Laboratory Services
Forensic Biology

These are management system documents utilized by ATF Laboratories. They are provided for informational purposes only. Sensitive or copyrighted information has been redacted. The documents are used in ATF Laboratories and not published with the intent of setting a policy or analysis standard for other laboratories. The inclusion of product names does not imply endorsement by ATF Laboratories.

These documents are current as of March 26, 2024. ATF management system documents are reviewed annually and revised as needed. For specific requests, submit a Freedom of Information Act (FOIA) request. Instructions on how to file a FOIA request are found at: www.atf.gov/resource-center/freedom-information-act-foia.

INDEX

ATF-LS-FB01 Standard Approach to Forensic Biology Examination

ATF-LS-FB01.1 Appendix A - Standard Approach to Forensic Biology Examinations

ATF-LS-FB02 Alternate Light Source

ATF-LS-FB05 Phenolphthalein Test for Blood

ATF-LS-FB08 General QIAamp Micro DNA Extraction

ATF-LS-FB09 General QIAamp Investigator DNA Extraction

ATF-LS-FB12 30kD Microcon Concentration

ATF-LS-FB14 Identifiler

ATF-LS-FB16 Genemapper ID

ATF-LS-FB17 Identifiler STR Interpretation Guidelines

ATF-LS-FB17 Appendix A - Overall Combined PHR Data

ATF-LS-FB17.1 Appendix D - Three Person Mixture Interpretation Guide

ATF-LS-FB18 Forensic Biology Report Wording

ATF-LS-FB19 Combined DNA Index System (CODIS)

ATF-LS-FB20 Forensic Biology Case Note Glossary

ATF-LS-FB21 DNA Collection

ATF-LS-FB22 Staff DNA Index

ATF-LS-FB23 DNA Extraction using the QIAamp® Investigator Kit on the QIAcube® and QIAcube® Connect Robotic Workstation

Laboratory Services
Forensic Biology

ATF-LS-FB26 Outsourced Casework Samples

ATF-LS-FB28 Quality Control of Reagents

ATF-LS-FB29 Quality Control of Instruments and Equipment Maintenance

ATF-LS-FB30 GlobalFiler Amplification

ATF-LS-FB31 Applied Biosystems 3130 Genetic Analyzer - GlobalFiler™

ATF-LS-FB32 Applied Biosystems GeneMapper® ID-X Software

ATF-LS-FB33 Interpretation Guidelines - GlobalFiler/STRmix v2.4/3130

ATF-LS-FB34 Reporting the Results of Forensic Biology Analysis using STRmix™

- ATF-LS-FB34.1 Reporting of GlobalFiler / STRmix Results Supplemental
- ATF-LS-FB34.2 Report Appendix for Likelihood Ratios

ATF-LS-FB36 Quantifiler HP

ATF-LS-FB37 Rinse and Swab Collection from Fired Cartridge Cases

ATF-LS-FB38 Applied Biosystems™ 3500xL Genetic Analyzer - GlobalFiler™

ATF-LS-FB39 Interpretation Guidelines - GlobalFiler_3500_STRmix2.6

- ATF-LS-FB39.1 Work Instructions for Interpretation of GlobalFiler™ / 3500 / STRmix™ v2.6

ATF-LS-FB39.2 Guidance on Analyzing DNA Profiles with Pull-up

ATF-LS-FB40 BlueStar Presumptive Test for Blood

1. Scope

This protocol describes the general approach to Forensic Biology examinations. The protocol is not meant to encompass all possible circumstances that may be encountered in forensic casework. An analyst may deviate from this protocol as circumstances necessitate based on his/her training and experience. Significant deviations must be approved by the DNA Technical Leader.

2. References

2.1. Ensuring high standards, in: DNA Technology in Forensic Science, National Academy Press, Washington, D.C., 1992, pp. 97-110.
2.2. Ensuring high standards of laboratory performance, in: The Evaluation of Forensic DNA Evidence, National Academy Press, Washington, D.C., 1996, pp. 75-88.
2.3. DNA Advisory Board, Quality assurance standards for forensic DNA testing laboratories, 2000.
2.4. R. Saferstein, Examining evidence and removing biological stains, in: Forensic Science Handbook, Vol. I, second ed., Prentice Hall, Upper Saddle River, 2002, pp. 527-530.
2.5. N. Vandenberg, R.A. van Oorschot, The use of Polilight® in the detection of seminal fluid, saliva, and bloodstains and comparison with conventional chemical-based screening tests, J. Forensic Sci. 51 (2006) 361-370.
2.6. J.M. Butler, Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers, second ed., Elsevier Academic Press, Burlington, 2005.

3. Equipment

3.1. See equipment for appropriate protocols.

4. Safety/Quality Assurance

4.1. See Safety section for appropriate protocols.

5. Procedure

5.1. General Contamination Prevention Practices
5.1.1. At a minimum, a disposable lab coat, disposable gloves, and eye protection (as necessary) will be worn while examining evidence or performing procedures in the DNA laboratory. A face mask may be worn at the analyst's discretion.
5.1.1.1. All visitors must wear a disposable lab coat, face mask, and disposable gloves, unless those individuals choose to provide a DNA sample for the CODIS Staff Index.

5.1.2. Prior to and/or after examination of evidence, DNA extraction, PCR setup, and post-amplification procedures, examination work areas (hoods, countertops, etc.) and tools (tweezers, scissors, etc.) will be cleaned with a 10% bleach solution followed by 70% ethanol. Centrifuges, pipettors, Alternate Light Sources, and other equipment will be cleaned with 70% ethanol prior to and/or after use. Additionally, all evidence will be examined on a new sheet of bench paper.
NOTE: If examination of evidence takes place in the laboratory of a different section (Explosives, Latent Prints, etc.), these cleaning methods may not be feasible. In such cases, the cleaning methods/evidence handling practices of that section would apply.

5.1.3. All tube racks will be cleaned in a 10% bleach bath followed by rinsing with water after use. The 10% bleach bath will be made every two weeks.

5.1.4. At a minimum, all of the surfaces, hoods, pipettors, centrifuges, and other equipment in the DNA laboratories will be cleaned on a quarterly basis with the appropriate cleaning solutions (10% bleach solution followed by 70% ethanol or 70% ethanol only). The quarterly cleanings shall be documented in the appropriate log.

5.1.5. UV Crosslinkers will be used on the following items, at a minimum:

5.1.5.1. Tube racks, canisters – prior to use and after each cleaning

5.1.5.2. Non PCR-ready microcentrifuge tubes, 0.2 mL microcentrifuge tubes, and DNA IQ™ Spin Baskets

5.1.5.3. Aliquots of nuclease-free water and TE^{-4} prepared in 50 mL conical tubes (TE^{-4} purchased pre-mixed does not need to be cross-linked)

5.1.6. Contamination will be monitored by the evaluation of the control samples routinely analyzed during the course of casework analysis (i.e. extraction reagent blanks, positive controls, amplification negative controls, and quantitation plate blanks).

5.1.6.1. Contamination noted in negative controls (reagent blanks and amplification blanks), positive controls, or evidence samples that is observed during analysis and corrected by setting up a new plate for the CE does not require entry into the contamination log, a *Correction*, or *Corrective Action.* If the contamination occurred prior to CE analysis (e.g. DNA extraction, amplification, etc.), the event will be documented in the contamination log. If the contamination event appears to be indicative of a pattern or systemic problem, a *Correction* or *Corrective Action* may be initiated.

5.1.7. A contamination log will be maintained containing contamination incidents and the related documentation.

5.1.8. At a minimum, the contamination log book shall contain the following:

5.1.8.1. Case number

5.1.8.2. Analyst

5.1.8.3. Date

5.1.8.4. Sample contaminated (e.g. reagent blank, positive control, etc.)
5.1.8.5. Brief description of the incident including when the contamination was detected, investigation as to the source of the contamination, and the resolution.
5.1.8.6. Contaminating DNA profile
5.1.8.7. Documents such as case worksheets, notes, and electropherograms considered relevant to the investigation and resolution of the contamination event.

5.1.9. Staff Hits: If a DNA profile developed from evidence matches a member of the laboratory staff:
5.1.9.1. The contamination event will be documented in the contamination log (see above).
5.1.9.2. If the staff contamination is a single event and does not appear to be indicative of a pattern or systemic problem, a *Correction* may be documented.
5.1.9.3. If the staff contamination appears to be indicative of a pattern or a systemic problem, then a *Corrective Action* shall be initiated.

5.2. Sample Contamination Prevention Practices
5.2.1. To avoid sample mix-up or contamination, work with only one item at a time.
5.2.2. Handle evidence samples believed to contain low levels of biological material before samples believed to contain higher levels.
5.2.3. In general, evidence samples will be processed prior to the examination of reference samples. Evidence samples and reference samples will undergo evidence processing, extraction, quantification setup, and PCR setup in separate designated areas.
5.2.4. Pulse spin all microcentrifuge tubes before opening to remove liquid from the inside of the cap. Use care when opening microcentrifuge tubes to prevent splashing.
5.2.5. Only relevant paperwork will be carried into and out of the post-amplification laboratory. Do not return this paperwork to the pre-amplification laboratories.
5.2.6. Hands shall be washed prior to leaving the post-amplification laboratory.
5.2.7. To prevent sample switching:
5.2.7.1. Immediately prior to making a sample transfer, verify the labels on the sample tube and the new tube.
5.2.7.2. After making a sample transfer, it is recommended to move the sample tube(s) to a different location within the rack, or a different rack, to prevent using the same tube twice.

5.3. General Evidence Examination Procedure
5.3.1. Items will first be visually examined to determine whether a Forensic Biology exam is appropriate. In those instances where a Forensic Biology exam is not appropriate, the reason(s) will be recorded in the analyst's notes.

5.3.2. An Alternate Light Source (ALS) may be used by the analyst to assist in conducting the visual exam. It may be useful when examining clothing for biological stains or to help locate areas of the clothing to sample when attempting to determine the wearer. Refer to the Alternate Light Source protocol for proper use of the ALS.

5.3.3. If hairs are observed on an item, or if a Trace Evidence exam has been requested, the analyst will take precautions to assure that no hairs or other trace evidence is lost during the Forensic Biology exam. A Trace Evidence examiner shall be consulted before proceeding with DNA analysis on hair roots.

5.3.4. After a visual examination, applicable serology examinations may be conducted at the analyst's discretion based on the circumstances (sample size, probative value, etc.).

5.3.5. The phenolphthalein test can be used as a presumptive test for blood. Refer to the Phenolphthalein Test for Blood protocol for detailed instructions on performing this procedure.

5.3.6. The analyst may swab, take cuttings, or use another approved method to collect biological evidence from items for PCR testing. The size of cuttings and/or number of swabs used will be determined by the analyst based on his/her training and experience. The analyst should try, if feasible, to leave a portion of the stain untested in the event that re-testing is required. If this is not feasible, the analyst will obtain permission from the prosecuting attorney or investigating agent to consume the stain. The approximate size/amount of stain cut for DNA and/or remaining should be indicated in the analyst's notes.

5.3.7. DNA extraction and purification of evidence samples will be performed at a separate time/space from reference samples. Refer to the appropriate extraction protocol for detailed instructions on performing this procedure. In general, semen-containing samples will not be analyzed by the ATF Laboratory and should be forwarded to a laboratory that routinely analyzes this sample type.

5.3.8. Multiple swabs/cuttings from the same stain/item may be extracted in separate tubes and then combined into a single sample using a Microcon filter.

5.3.9. The DNA concentration of the extracts will be estimated using a validated quantification kit and the Applied Biosystems™ 7500 Real-Time PCR instrument. Refer to the Quantifiler® protocol for detailed instructions on performing this procedure.

5.3.10. If necessary, samples may be concentrated using Microcon® filters. Refer to the 30 kD Microcon® DNA Concentration/Purification protocol for detailed instructions on performing this procedure.

5.3.11. Using the quantification data, samples will be amplified using the GlobalFiler™ PCR Amplification Kit and a validated thermal cycler. The target concentration of DNA template for this amplification will generally be 0.5 ng. It may be necessary for the analyst to amplify a lesser or greater amount of DNA depending on the

sample type/situation. The analyst will use his/her training and experience to make the determination on when this should be done. Refer to the GlobalFiler™ protocol for detailed instructions on performing this procedure.

5.3.12. DNA typing will be performed on all amplified samples using a validated Applied Biosystems™ Genetic Analyzer. Samples may be injected multiple times at the analyst's discretion. Refer to the Applied Biosystems™ Genetic Analyzer protocol for detailed instructions on performing this procedure.

5.3.13. Evidence samples and reference samples from the same case may not be loaded on the same Genetic Analyzer plate.

5.4. Disposition of Evidence

5.4.1. When a case has been completed, the associated evidence (exhibits and DNA extract tubes) will be properly sealed and returned to the evidence custodian. If the entire extract has been consumed during analysis, the extract tube may be discarded. The remaining extract tubes shall be packaged individually (e.g. small zip-lock bags). All extracts associated with the case (multiple submissions, if desired) will be sealed in an outer envelope labeled at a minimum with the case number, exhibit number(s), analyst's initials, and date. The extracts will be retained by the ATF Laboratory at approximately -20°C. The disposition of the extract tubes (i.e. retained or discarded) will be recorded in the closing inventory. Amplified product shall be considered work product and discarded after the case report has undergone a technical review.

5.4.2. Prior to returning the original evidence, the outer packaging of all exhibits submitted to the laboratory for DNA analysis that may reasonably still contain biological material will be labeled so as to clearly identify the exhibit as potentially containing biological evidence and therefore should be stored in a temperature controlled environment.

5.5. Reinterpretation of Legacy Data

5.5.1. Legacy data, as defined by the current version of the Quality Assurance Standards for Forensic DNA Testing Laboratories, shall only be reinterpreted by a qualified analyst.

5.5.2. Technical reviews of legacy data shall only be performed by a qualified analyst.

5.5.3. An analyst may be determined to be qualified by one of the following manners:

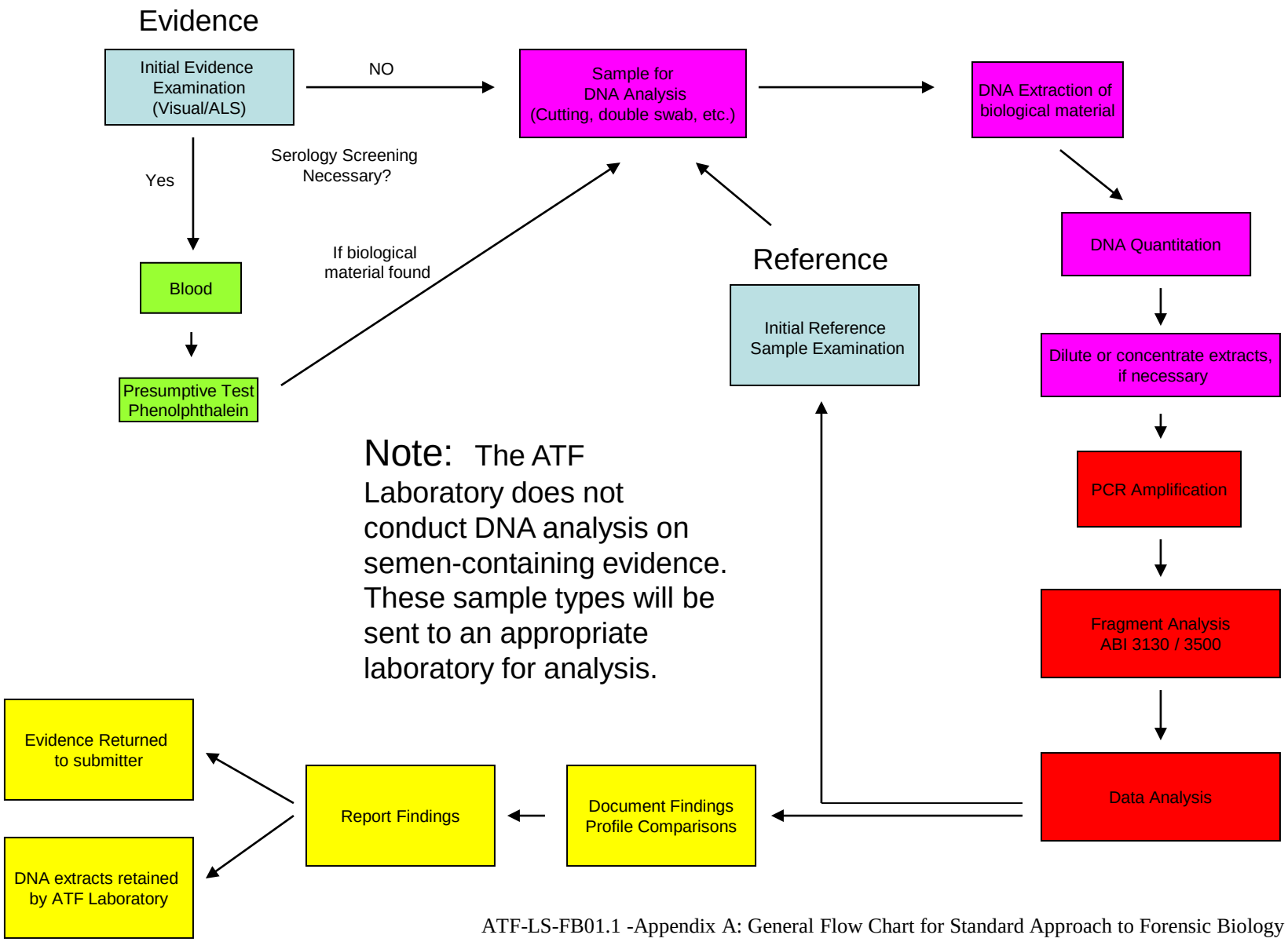
5.5.3.1. Previously qualified analyst in the laboratory for interpretation of the specific legacy data: the analyst will undergo a refresher training covering the technical skills and knowledge for the reinterpretation of the legacy data on an annual basis.

5.5.3.2. An analyst not previously qualified in the laboratory for the interpretation of the specific legacy data: the analyst shall successfully complete the training required to demonstrate the ability to interpret data, reach conclusions, and generate reports for the legacy data. The analyst must successfully complete a competency set, including practical components

of reinterpretation, to the extent of the analyst's participation in casework analyses.

5.5.3.3. Documentation of the refresher training for previously qualified analysts or the successful completion of the training, including the competency set, for analysts that were not previously qualified shall be maintained by the laboratory.

ATF-LS-FB01.1 - APPENDIX A: GENERAL FLOW CHART FOR STANDARD APPROACH TO FORENSIC BIOLOGY EXAMINATIONS



1. Scope

This protocol applies to the visualization of various body fluids, which will fluoresce in the presence of certain wavelengths of light. An Alternate Light Source (ALS) — here to include a laser — utilizes specific wavelengths of light to help locate body fluid stains on clothing or other items of evidence. Semen, saliva, vaginal fluid, and perspiration all fluoresce when using an ALS while blood may appear darker. The ALS can be used to supplement the visual examination of evidence exhibits. Stains located using an ALS can be marked for further serology/DNA testing. Stained areas located with the aid of the ALS are not identified as biological material based on the ALS alone. If no stained areas are observed on an exhibit based on visual and ALS-aided examination, it does not mean that no biological material is on the exhibit, it only indicates that no biological material was observed.

Alternate Light Sources can also be used to visualize friction ridge detail or smudging after Latent Print processing. Section 4 Safety/Quality Assurance measures and Section 5 Procedure apply to the visualization of friction ridge detail or smudging, with the exception of 4.6 and 5.4, which only apply to the visualization of body fluids.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

2.1. Foster + Freeman Crime-lite 82S User Guide, September 2016.
2.2. SPEX Forensics HandScope® Xenon Operation Manual, March 2006.
2.3. TracER Operator's Manual, August 2008.
2.4. Federal Bureau of Investigation Laboratory Serology Protocol Manual, Procedure for the Application of an Alternate Light Source to Aid in the Detection of Biological Fluids, July 2006.
2.5. R. Saferstein, The identification of semen and other body fluids, in: Forensic Science Handbook, Vol. II, second ed., Prentice Hall, Upper Saddle River, 2005, pp. 330-331.
2.6. E. Springer, J. Almog, A. Frank, Z. Ziv, P. Bergman, W.G. Qiang, Detection of dry body fluids by inherent short wavelength UV luminescence: preliminary results, Forensic Sci. Int. 66 (1994) 89-94.

3. Equipment

3.1. Disposable Gloves
3.2. Colored ALS protective goggles
3.3. Lab coat
3.4. Approved ALS or Laser:

3.4.1. Coherent TracER Laser
3.4.2. Foster + Freeman Crime-Lite® 82S
3.4.3. SPEX Forensics HandScope® Xenon HSX-5000
3.5. Known positive controls (semen, saliva, or sweat) and negative control
3.6. 10% bleach solution
3.7. 70% ethanol
3.8. Bench paper

4. Safety/Quality Assurance

4.1. Wear appropriate ALS protective goggles while using the ALS.
4.2. Do not stare directly into the light as this can cause damage to the eyes.
4.3. Do not block the exhaust fan on the rear panel as light may overheat.
4.4. A lab coat must be worn at all times while performing this procedure.
4.5. Disposable gloves shall be worn when handling reagents and evidence.
4.6. View a positive control (semen, saliva, or sweat) and a negative control (unstained cloth) before examining evidence with the light. **The ALS model used, along with the results of the controls, shall be documented in the casework notes.**
4.7. When practical, only one item of evidence shall be open at a time.
4.8. Clean the ALS with a wipe dampened with 70% ethanol. Do not use bleach to clean the ALS.
4.9. The laboratory bench surface shall be cleaned before and after use with 10% bleach solution followed by 70% ethanol. Fresh bench paper shall then be placed on the surface prior to examination.
4.10. Evidence and controls shall be evaluated in a darkened room.
4.11. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and should be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. Plug the ALS into the Power Supply or Battery Pack.
5.2. Select the appropriate wavelength and goggles depending on the ALS to be used:
5.2.1. Coherent TracER LASER: 460-577 nm, orange protective goggles
5.2.2. HandScope® Xenon HSX-5000: 450 nm, orange protective goggles
5.2.3. Foster + Freeman Crime-Lite® 82S: 445-510 nm, orange protective goggles
5.3. Adjust the collimator, if necessary, to focus the light to a spot-size suitable for the evidence.
5.4. Evaluate the ALS with a positive control (semen, saliva, or sweat) and a negative control

5.4.1. The positive control should fluoresce, the negative control should display no fluorescence.

5.4.2. If control results are abnormal, do not proceed with the exam.

5.5. Examine evidence using the ALS; observed stains may be marked. Serology testing may be performed on these stains if necessary.

1. Scope

This protocol is to be used when examining an exhibit of evidence for the presence of blood and is only a presumptive test. A presumptive test is one that is highly sensitive but not completely specific. The phenolphthalein test (also known as the Kastle-Meyer test) is a presumptive test for the presence of hemoglobin, a component of the red blood cells in blood. The phenolphthalein test is based on the peroxidase activity of a substance, in this case heme or heme derivatives, which catalyzes the reaction with peroxide which in turn oxidizes phenolphthalin to phenolphthalein. A positive reaction is observed when the colorless phenolphthalin solution turns pink as it is oxidized to phenolphthalein. The intensity of the pink color is directly proportional to the amount of hemoglobin and, therefore, blood that is present. Other substances such as plant peroxidases and chemical oxidants may also cause a positive reaction. Therefore, a positive phenolphthalein test only indicates the presence of blood but does not confirm the presence of blood.

2. References

2.1. EVIDENT Phenolphthalein Presumptive Blood Testing Kit.
2.2. R.E. Gaensslen, Identification of blood, in: Sourcebook in Forensic Serology, Immunology and Biochemistry, U.S. Department of Justice, Washington, D.C., 1983, pp. 73-133.
2.3. H.C. Lee, Identification and grouping of bloodstains, in: R. Saferstein, Forensic Science Handbook, Prentice Hall, Englewood Cliffs, 1982, pp. 267-337.

3. Equipment

3.1. Disposable gloves
3.2. Lab coat
3.3. Eye protection
3.4. Sterile swabs
3.5. Sterile water
3.6. Presumptive blood test kit (alcohol, phenolphthalein reagent, hydrogen peroxide – E.g. Evident Crime Scene Products: catalog #3658)
3.7. Known positive control (dried blood)
3.8. Known negative control
3.9. Scissors
3.10. Forceps
3.11. 70% ethanol or alcohol wipes
3.12. 10% bleach solution
3.13. Bench paper

4. Safety/Quality Assurance

4.1. Any utensils used to cut or manipulate swabs or other types of evidence must be cleaned between uses with 10% bleach solution followed by 70% ethanol or alcohol wipes.
4.2. Disposable gloves shall be worn when handling kit reagents and evidence.
4.3. Record the lot number of the phenolphthalein testing kit in the case notes. Do not use the reagents after the expiration date. Discard the phenolphthalein reagent if the colorless solution becomes pink.
4.4. At a minimum, each day the reagents are to be used, a negative control and positive control must be tested and documented in the analyst's notes. If either of the controls fail, a different lot of reagents shall be used.
4.5. This test is only a presumptive test and does not confirm the presence of blood. It is not human specific.
4.6. Lab coat and eye protection must be worn at all times while performing this procedure.
4.7. When practical, only one item of evidence shall be open at a time.
4.8. The laboratory bench surface shall be cleaned before and after use with 10% bleach solution or other sanitizing agent and may be followed by 70% ethanol. Fresh bench paper shall then be placed on the surface prior to examination.
4.9. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. Moisten a sterile swab with sterile water and lightly rub the suspected stain concentrating the sample on the swab tip. Alternatively, a cutting, filter paper rubbing or other appropriate method of sampling may be taken from the stain to be used for testing at the analyst's discretion.
5.2. Add one (1) drop of the alcohol reagent on the substrate surface.
5.3. Add one (1) drop of the phenolphthalein reagent. A pink color change at this point indicates the presence of other oxidative agents and the test is deemed inconclusive.
5.4. Add one (1) drop of the hydrogen peroxide reagent.
5.5. An immediate color change to vivid pink indicates a positive reaction. No color change indicates a negative reaction. Interpretation of results must be made immediately (within 5 seconds). A pink color change may occur after 30 seconds, which is a normal catalytic reaction that may occur without the presence of blood.
    5.5.1. Positive Result: an immediate color change to vivid pink (within 5 seconds)
    5.5.2. Negative Result: no color change within 5 seconds
    5.5.3. Inconclusive: color change to pink after the addition of the phenolphthalein reagent but before the addition of the hydrogen peroxide, color change to pink after 5 seconds, or any other anomalous result

5.6. Record results by noting the color change or lack of color change and the conclusion of the test.

5.7. A cutting or swabbing of an unstained area adjacent to the stained area may be tested as a substrate control at the analyst's discretion.

1. Scope

This protocol can be used to extract and purify DNA from reference samples and evidence samples containing biological material. DNA can be extracted from dried biological stains or liquid biological material efficiently using the QIAamp DNA Micro Kit and extraction protocol described below. The method is composed of four basic steps. First, the biological material is digested and the cells lysed in a buffer containing a detergent and a protease. Next, the lysate is passed through a membrane that binds the DNA. Contaminants are then washed off the membrane through two washing steps. Finally, the purified DNA is eluted off the membrane in a small volume of an appropriate buffer.

The DNA extract should be free of contaminants, but further purification methods may be used to remove inhibitory substances that remain, if necessary.

This method is recommended for use on biological samples where high levels of DNA are expected such as neat bloodstains or reference samples. Analysts should refer to ATF-LS-FB09 (General QIAamp Investigator DNA Extraction) or ATF-LS-FB35 (QIAamp Micro Double AL DNA Extraction) for extraction of low-level evidence and hair samples in order to maximize recovery of DNA.

2. References

2.1. Qiagen QIAamp® DNA Micro Handbook, Aug. 2003.

2.2. I. van Niekerek, C. Snyman, J. van Niekerk, A. Greyling, Efficient purification of genomic DNA from forensic samples using QIAamp® Kits, Qiagen News 1 (2005) 10-12.

2.3. V. Castella, N. Dimo-Simonin, C. Brandt-Casadevall, P. Mangin, Forensic evaluation of the QIAshredder/QIAamp DNA extraction procedure, Forensic Sci. Int. 156 (2006) 70-73.

2.4. D.J. Johnson, L. Martin, K.A. Roberts, STR-typing of human DNA from human fecal matter using the Qiagen QIAamp Stool Mini Kit, J. Forensic Sci. 50 (2005) 802-808.

2.5. N. Vandenberg, R.A. van Oorschot, Extraction of human nuclear DNA from feces samples using the QIAamp DNA Stool Mini Kit, J. Forensic Sci. 47 (2002) 993-995.

2.6. J.M. Butler, Sample collection, DNA extraction, and DNA quantitation, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 42-50.

2.7. P. Gill, A.J. Jeffreys, D.J. Werrett, Forensic application of DNA ‘Fingerprints’, Nature 318 (1985) 577-579.

2.8. R. Higuchi, C.H. von Beroldingen, G.F. Sensabaugh, H.A. Erlich, DNA typing from single hairs, Nature 332 (1988) 543–546.

2.9. C.T. Comey, B.W. Koons, K.W. Presely, J.B. Smerick, C.A. Sobieralski, D.M. Stanley, F.S. Baechtel, DNA extraction strategies for amplified fragment length polymorphism analysis, J. Forensic Sci. 39 (1994) 1254-1269.

2.10. S.A. Greenspoon, M.A. Scarpetta, M.L. Drayton, S.A. Turek, QIAamp spin columns as a method of DNA isolation for forensic casework, J. Forensic Sci. 43 (1998) 1024-1030.
2.11. K. Sinclair, V.M. McKehnie, DNA extraction from stamps and envelope flaps using QIAamp and QIAshredder, J. Forensic Sci. 45 (2000) 229-230.
2.12. Qiagen, Developmental validation of the QIAamp DNA Investigator Kit, July 2015, available at: https://www.qiagen.com/us/resources/resourcedetail?id=1d9de4e8-cbd4-4be7-aec2-dd9f79f038fb&lang=en&autoSuggest=true.

3. Equipment

3.1. Disposable gloves
3.2. Eye protection
3.3. Lab coat
3.4. Sterile swabs
3.5. Sterile water
3.6. Disposable scalpels or razor blades
3.7. Scissors
3.8. Forceps
3.9. 70% ethanol or alcohol wipes
3.10. 10% bleach solution
3.11. QIAamp® DNA Micro Kit (Qiagen Catalog # 56304) containing the following:
  3.11.1. QIAamp® MinElute™ columns
  3.11.2. Collection Tubes
  3.11.3. Buffer ATL
  3.11.4. Buffer AL
  3.11.5. Buffer AW1
  3.11.6. Buffer AW2
  3.11.7. Buffer AE (not used)
  3.11.8. Carrier RNA
  3.11.9. Proteinase K (typically not used)
3.12. Invitrogen Proteinase K
3.13. Dithiothreitol (DTT)
3.14. Pipettes
3.15. Disposable aerosol-resistant pipettor tips
3.16. Microcentrifuge tubes
3.17. NAO™ Baskets/QIAGEN® Investigator Lyse and Spin tubes
  OPTIONAL: DNA IQ™ Spin Baskets
3.18. Benchtop hood
3.19. TE^{-4} (10mM Tris-HCl, 0.1mM EDTA, pH 8.0)
3.20. Bench paper
3.21. Thermomixer

3.22. Centrifuge
3.23. Vortexer

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling kit reagents and evidence.
4.2. Perform extraction steps, where possible, in a hood to reduce the risk of contamination. Clean surfaces with 10% bleach solution before and after use. After exiting the hood, turn on the UV light (automatically set for 15 minutes of exposure).
4.3. When possible, reference samples will be processed in a separate dedicated area after the analysis of evidence samples has been completed.
4.4. Use aerosol-resistant pipettor tips when transferring liquids containing DNA.
4.5. Change pipettor tips after transferring any liquids potentially containing DNA.
4.6. Record the lot number of each reagent used in notes. Do not use the reagents after the expiration date.
4.7. Initiate at least one reagent blank as the final sample of the set of extractions.
4.8. Lab coat and eye protection must be worn at all times while performing this procedure.
4.9. Only tubes associated with one sample shall be open at a time.
4.10. Exercise caution when opening tubes.
4.11. The laboratory bench surface shall be cleaned before and after use with 10% bleach solution or other sanitizing agent and may be followed by 70% ethanol. Fresh bench paper shall then be placed on the surface prior to examination.
4.12. Final DNA extract tubes shall be labeled, at a minimum, with case number, item number, and analyst's initials.
4.13. Samples thought to contain lower levels of DNA will be handled before those thought to contain large amounts of DNA.
4.14. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Sample Preparation

5.1. Any utensils used to cut or manipulate the swabs or other types of evidence must be cleaned between uses with 10% bleach solution followed by 70% ethanol or alcohol wipe. Alternatively, single-use disposable razor blades may be used.
5.2. To maximize lysate recovery, cuttings of swab tips or evidence substrate should be placed in a clean NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket with tube. Alternatively, a clean microcentrifuge tube may be used.
5.2.1. Cuttings from biological stains/substrate (clothing, carpet, etc.) should be approximately 5 mm x 5 mm in size. Some specimens may require cuttings of differing sizes depending on the type, condition and concentration of the biological

material. Larger sized cuttings may be cut into smaller pieces before placing them into the tube.

5.3. For liquid samples, place a portion of the liquid (1-100 µL) directly into a clean microcentrifuge tube.

5.4. For reference samples, cut a small portion of blood card (up to 5 mm^2), swab tip, or other reference material and place in a clean microcentrifuge tube (or in a clean NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket with tube if maximum recovery of lysate is a priority).

6. Procedure

6.1. Check the reagent logs or the reagent bottles to ensure that Buffer AW1, Buffer AW2, and carrier RNA have been appropriately prepared. Prior to use, Buffers ATL, AW1, and AW2 must contain no precipitates. If precipitates have formed, gently heat the bottles prior to dispensing the reagents.

6.2. A Reagent Blank shall be initiated as the last sample in the set of samples. The Reagent Blank shall contain all the liquid reagents contained in the evidentiary samples except for the biological material. The Reagent Blank shall be handled in a manner that is identical to the evidentiary samples and use the most sensitive volumes and steps used with the evidentiary samples. For example, if carrier RNA is used with only a few of the evidentiary samples being extracted in a set, carrier RNA will be added to the Reagent Blank, as well.

6.2.1. If the analyst determines that two or more evidentiary extracts may be combined during the concentration step, the same number of reagent blanks shall be initiated at the DNA extraction step. For example, if the analyst determines that the extracts of three sets of swabs from a firearm may be combined and concentrated later in the analysis, then the analyst shall initiate three reagent blanks at the DNA extraction step.

6.2.2. If an NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket is used, the tips of two swabs should be placed in the Reagent Blank to facilitate the recovery of the entire volume of sample.

Cell Lysis

6.3. Add **300-400 µL of Buffer ATL** and **20 µL of ProK** (20 mg/mL) to the sample tube.

6.3.1. The volume of ATL buffer may be increased to ensure that the sample is completely immersed in the lysis solution. If the volume of Buffer ATL is increased, the volume of ProK should be increased proportionally at a 20:1 ratio (e.g. 500 µL of ATL and 25 µL of ProK).

6.4. Vortex for approximately 10 seconds.

6.5. Place the samples in a thermomixer and incubate at 56° C with shaking at 900 rpm for at least 1 hour.

NOTE: Samples may be incubated overnight; however, incubation times greater than 18 hours have been shown to decrease DNA yield.

6.6. If NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket is used for improved lysate recovery, centrifuge samples for 1 - 3 minutes at 18,000 x g. Baskets and substrate may be discarded at this point. Continue at step 6.8.

6.7. Briefly centrifuge the samples to remove condensation from inside the lid.

6.8. Add **300-400 µL of Buffer AL** (use a volume equal to the volume of Buffer ATL used in step 6.3).

6.9. **1 µL of carrier RNA** (1 µg/µL) can be added at this point if, due to the concentration or condition of the biological material, it is determined by the analyst that carrier RNA may aid in the recovery of the DNA.

6.10. Vortex for approximately 10 seconds.

6.11. Place the tubes in a thermomixer and incubate at 70° C with shaking at 900 rpm for at least 10 minutes.

NOTE: Any white precipitate that formed when the Buffer AL was added should disappear during this incubation step.

6.12. Briefly centrifuge the samples to remove condensation from the inside of the lid.

6.13. If still present, the solid substrate can be removed and discarded.

NOTE: If maximum recovery of lysate is a priority, and NAO™ Baskets/QIAGEN® Investigator Lyse and Spin baskets are unavailable, the solid substrate can be placed in a DNA IQ™ Spin Basket for centrifugation.

6.13.1. If DNA IQ™ Spin Baskets were used, centrifuge tubes for 2 minutes at full speed.

6.13.2. Discard basket and substrate after centrifugation.

Bind DNA

6.14. Carefully transfer up to 700 µL of the supernatant from the microcentrifuge tube to a QIAamp MinElute™ column without wetting the rim of the column.

6.15. Centrifuge the column at 6000 x g for 1 minute. Place the MinElute ™ column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

6.16. If any supernatant remains, repeat steps 6.14 and 6.15.

Wash DNA

6.17. Carefully open the QIAamp MinElute™ column and add **500 µL of Buffer AW1** without wetting the rim. Close the lid and centrifuge at 6000 x g for 1 minute.

6.18. Place the QIAamp MinElute™ column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

6.19. Carefully open the QIAamp MinElute™ column and add **500 µL of Buffer AW2** without wetting the rim. Close the lid and centrifuge at 6000 x g for 1 minute.

6.20. Place the QIAamp MinElute™ column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

6.21. Centrifuge at full speed for 3 minutes to dry the membrane completely.
6.22. Place the QIAamp MinElute™ column in a clean microcentrifuge tube and discard the collection tube containing the flow-through.

Elute Purified DNA

6.23. Carefully apply **20-50 µL of TE^{-4}** to the center of the membrane.
6.24. Incubate at room temperature for 0-5 minutes.
6.25. Centrifuge at full speed for 1 minute.
OPTIONAL: Repeat steps 6.23 through 6.25 to increase yield.
6.26. Remove the QIAamp MinElute™ column and cap the tube. The QIAamp MinElute™ column can be discarded. The DNA extract may be concentrated at this point or may proceed to quantitation directly.

1. Scope

This protocol is to be used to extract and purify DNA from reference samples and samples of evidence containing biological material. DNA can be extracted from dried biological stains or liquid biological material efficiently using the QIAamp DNA Investigator Kit extraction protocol described below. The method is composed of four basic steps. First, the biological material is digested and the cells lysed in a buffer containing a detergent and a protease. Next, the lysate is passed through a membrane that binds the DNA. Contaminants are then washed off the membrane through three washing steps. Finally, the purified DNA is eluted off the membrane in a small volume of an appropriate buffer.

The DNA extract should be free of contaminants but further purification methods may be used to remove inhibitory substances that remain, if necessary.

This method is appropriate for use on most dried stains, liquid samples, and other biological materials encountered in forensic casework.

2. References

2.1. Qiagen QIAamp® DNA Investigator Handbook, June 2012.

2.2. I. van Niekerek, C. Snyman, J. van Niekerk, A. Greyling, Efficient purification of genomic DNA from forensic samples using QIAamp® Kits, Qiagen News 1 (2005) 10-12.

2.3. V. Castella, N. Dimo-Simonin, C. Brandt-Casadevall, P. Mangin, Forensic evalutation of the QIAshredder/QIAamp DNA extraction procedure, Forensic Sci. Int. 156 (2006) 70-73.

2.4. D.J. Johnson, L. Martin, K.A. Roberts, STR-typing of human DNA from human fecal matter using the Qiagen QIAamp Stool Mini Kit, J. Forensic Sci. 50 (2005) 802-808.

2.5. N. Vandenberg, R.A. van Oorschot, Extraction of human nuclear DNA from feces samples using the QIAamp DNA Stool Mini Kit, J. Forensic Sci. 47 (2002) 993-995.

2.6. J.M. Butler, Sample collection, DNA extraction, and DNA quantitation, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 42-50.

2.7. P. Gill, A.J. Jeffreys, D.J. Werrett, Forensic application of DNA 'Fingerprints', Nature 318 (1985) 577-579.

2.8. R. Higuchi, C.H. von Beroldingen, G.F. Sensabaugh, H.A. Erlich, DNA typing from single hairs, Nature 332 (1988) 543–546.

2.9. C.T. Comey, B.W. Koons, K.W. Presely, J.B. Smerick, C.A. Sobieralski, D.M. Stanley, F.S. Baechtel, DNA extraction strategies for amplified fragment length polymorphism analysis, J. Forensic Sci. 39 (1994) 1254-1269.

2.10. S.A. Greenspoon, M.A. Scarpetta, M.L. Drayton, S.A. Turek, QIAamp spin columns as a method of DNA isolation for forensic casework, J. Forensic Sci. 43 (1998) 1024-1030.

2.11. K. Sinclair, V.M. McKehnie, DNA extraction from stamps and envelope flaps using QIAamp and QIAshredder, J. Forensic Sci. 45 (2000) 229-230.
2.12 Qiagen, Developmental validation of the QIAamp DNA Investigator Kit, July 2015, available at: https://www.qiagen.com/us/resources/resourcedetail?id=1d9de4e8-cbd4-4be7-aec2-dd9f79f038fb&lang=en&autoSuggest=true.

3. Equipment

3.1. Disposable gloves
3.2. Eye protection
3.3. Lab coat
3.4. Sterile swabs
3.5. Sterile water
3.6. Disposable scalpels or razor blades
3.7. Scissors
3.8. Forceps
3.9. 70% ethanol or alcohol wipes
3.10. 10% bleach solution
3.11. QIAamp DNA Investigator Kit (Qiagen Catalog # 56504) containing the following:
  3.11.1. QIAamp MinElute™ columns
  3.11.2. Collection Tubes
  3.11.3. Buffer ATL
  3.11.4. Buffer AL
  3.11.5. Buffer AW1
  3.11.6. Buffer AW2
  3.11.7. Buffer AE
  3.11.8. Carrier RNA
  3.11.9. Proteinase K (typically not used)
3.12. Invitrogen ProK
3.13. 96-100% Ethanol
3.14. Dithiothreitol (DTT)
3.15. Pipettes
3.16. Disposable aerosol-resistant pipette tips
3.17. Microcentrifuge tubes
3.18. NAO™ Baskets/QIAGEN® Investigator Lyse and Spin baskets and tubes
OPTIONAL: DNA IQ™ Spin Baskets
3.19. Benchtop hood
3.20. TE^{-4} (10 mM Tris-HCl , 0.1 mM EDTA, pH 8.0)
3.21. Bench paper
3.22. Thermomixer
3.23. Centrifuge
3.24. Vortexer

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling kit reagents and evidence.

4.2. Perform extraction steps, where possible, in a hood to reduce the risk of contamination. Clean surfaces with 10% bleach solution prior to use. After exiting the hood, turn on the UV light (automatically set for 15 minutes of exposure).

4.3. Where possible, reference samples will be processed in a separate designated area after the analysis of evidence samples has been completed.

4.4. Use aerosol-resistant pipette tips when transferring liquids containing DNA.

4.5. Change pipette tips after transferring any liquids potentially containing DNA.

4.6. Record the lot number of each reagent used in notes. Do not use the reagents after the expiration date.

4.7. Initiate a reagent blank as the final sample of the set of extractions.

4.8. Lab coat and eye protection must be worn at all times while performing this procedure.

4.9. When practical, only tubes associated with one sample shall be open at a time.

4.10. Exercise caution when opening tubes.

4.11. The laboratory bench surface shall be cleaned before use with 10% bleach solution or other sanitizing agent and may be followed by 70% ethanol. Fresh bench paper shall then be placed on the surface prior to examination.

4.12. Final DNA extract tubes shall be labeled, at a minimum, with case number, item number, and analyst initials.

4.13. Samples thought to contain lower levels of DNA will be handled before those thought to contain large amounts of DNA.

4.14. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Sample Preparation

5.1. Any utensils used to cut or manipulate the swabs or other types of evidence must be cleaned between uses with 10% bleach solution and followed by 70% ethanol or alcohol wipe. Alternatively, single use disposable razor blades may be used.

5.2. Cuttings of swab tips or evidence substrate should be placed in a clean NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket with tube if recovery of lysate is a priority. Alternatively, a clean microcentrifuge tube may be used.

5.2.1. Cuttings from biological stains/substrate (clothing, carpet, etc.) should be approx. 5mm x 5mm in size. Some specimens may require cuttings of differing sizes depending on the type, condition and concentration of the biological material. Larger sized cuttings may be cut into smaller pieces before placing them into the tube.

5.3. For liquid samples, place a portion of the liquid (1-100 µL) directly into a clean microcentrifuge tube.

5.4. Hair Roots

5.4.1. Prior to processing any hairs for DNA, consult with a trace evidence examiner.

5.4.2. When appropriate, gently rinse hair with 70% ethanol followed by sterile water prior to cutting.

5.4.3. Cut a 0.5-1 cm piece of the root end of the hair and put it in a microcentrifuge tube containing the extraction reagents.

5.4.4. If hair is not cleaned with ethanol and water, generate a control portion of hair by cutting another 0.5-1 cm piece of hair (non-root) and process it alongside the sample.

5.5. For reference samples, cut a small portion of blood card (up to 5 mm2), swab tip, or other reference material and place in a clean microcentrifuge tube (or in a clean NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket with tube if recovery of lysate is a priority).

6. Procedure

6.1. Check the reagent logs or the reagent bottles to ensure that Buffer AW1, Buffer AW2, and carrier RNA have been appropriately prepared. Prior to use, Buffers ATL, AW1, and AW2 must contain no precipitates. If precipitates have formed, gently heat the bottles prior to dispensing the reagents.

6.2. A Reagent Blank shall be initiated as the last sample in the set of samples. The Reagent Blank shall contain all the liquid reagents contained in the evidentiary samples except for the biological material. The Reagent Blank shall be handled in a manner that is identical to the evidentiary samples and use the most sensitive volumes and steps used with the evidentiary samples. For example, if carrier RNA is used with only a few of the evidentiary samples being extracted in a set, carrier RNA will be added to the Reagent Blank as well.

6.2.1. If the analyst determines that two or more evidentiary extracts may be combined during the concentration step, the same number of Reagent Blanks shall be initiated at the DNA extraction step. For example, if the analyst determines that it is possible that the extracts from three sets of swabs from a firearm may be combined and concentrated later in the analysis, then the analyst shall initiate three Reagent Blanks at the DNA extraction step.

6.2.2. If an NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket is used, the tips of two swabs should be placed in the Reagent Blank to facilitate the recovery of the entire volume of sample.

Cell Lysis

6.3. Add **400 µL of Buffer ATL** and **20 µL of ProK** (20 mg/mL) to the sample.

6.3.1. The volume of ATL may be increased to ensure that the sample is completely immersed in the lysis solution.
NOTE: A 20:1 ratio of ATL:Pro K, and a 2:2:1 ratio of ATL:AL:EtOH must be maintained. For example: 500 µL ATL : 25 µL Pro K : 500 µL AL : 250 µL EtOH.

6.4. For **hair samples**, also add **20 µL of 1M DTT**.

6.5. Vortex for approximately 10 seconds.

6.6. Place samples in a thermomixer and incubate at 56° C with shaking at 900 rpm.

6.6.1. **Evidence samples** should be incubated for at least 3 hours.

6.6.2. **Reference samples** should be incubated at least 1 hour.

NOTE: Samples may be incubated overnight; however, incubation times greater than 18 hours have been shown to decrease DNA yield.

6.7. If NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket is used for improved lysate recovery, centrifuge samples for 1 minute at 10000 x g. Baskets and substrate may be discarded at this point. Continue at step 6.9.

6.7.1. Centrifuge time can be increased up to 3 minutes at full speed if residual lysate is observed in the baskets.

6.8. Briefly centrifuge the samples to remove condensation from inside the lid.

6.9. Add **400 µL Buffer AL** (or appropriate volume of Buffer AL equal to the original volume of Buffer ATL).

6.10. **1 µL of carrier RNA** (1 µg/µL) can be added at this point if, due to the concentration or condition of the biological material, it is determined by the analyst that carrier RNA may aid in the recovery of the DNA.

6.11. Vortex for approximately 15 seconds.

6.12. Place the tubes in a thermomixer and incubate at 70° C with shaking at 900 rpm for at least 10 minutes.
NOTE: Any white precipitate that formed when the Buffer AL was added should disappear during this incubation step.

6.13. Briefly centrifuge the samples to remove condensation from inside the lid.

6.14. If still present, the solid substrate can be removed and discarded.
OPTIONAL: If lysate recovery is a priority, and NAO™ Baskets/QIAGEN® Investigator Lyse and Spin baskets are unavailable, the solid substrate can at this point be placed in a DNA IQ™ Spin Basket. Centrifuge for 2 minutes at full speed, then discard the basket and substrate.

6.15. Add **200 µl 96-100% non-denatured EtOH** (or appropriate volume equal to ½ of the original volume of Buffer ATL).

6.16. Vortex for approximately 15 seconds.

6.17. Centrifuge the tubes for 2 minutes at full speed.

Bind DNA

6.18. Transfer up to 700 µL of supernatant from the lysate tube to a QIAamp MinElute™ column without wetting the rim of the column.

6.19. Centrifuge the column at 6000 x g for 1 minute. Place the MinElute™ column in a clean 2 mL collection tube and discard the collection tube containing the flow through.

6.20. Add any remaining supernatant from the lysate tube to the MinElute™ column without wetting the rim of the column.

6.21. Centrifuge the column at 6000 x g for 1 minute. Place the MinElute™ column in a clean 2 mL collection tube and discard the collection tube containing the flow through.

Wash DNA

6.22. Carefully open the QIAamp MinElute™ column and add **500 µL of Buffer AW1** without wetting the rim. Close the lid and centrifuge at 6000 x g for 1 minute.

6.23. Place the QIAamp MinElute™ column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

6.24. Carefully open the QIAamp MinElute™ column and add **700 µL of Buffer AW2** without wetting the rim. Close the lid and centrifuge at 6000 x g for 1 minute.

6.25. Place the QIAamp MinElute™ column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

6.26. Carefully open the QIAamp MinElute™ column and add **700 µL of ethanol** (96-100%) without wetting the rim. Close the lid and centrifuge at 6000 x g for 1 minute.

6.27. Place the QIAamp MinElute™ column in a clean 2 mL collection tube and discard the collection tube containing the flow-through.

6.28. Centrifuge at full speed for 3 minutes to dry the membrane completely.

6.29. Place the QIAamp MinElute™ column in a clean microcentrifuge tube and discard the collection tube containing the flow-through.

6.30. Carefully open the lid of the QIAamp MinElute™ column and incubate at room temperature (15-25º C) for 10 min.

Elute Purified DNA

6.31. Carefully apply **20 – 50 µL of TE^{-4}** to the center of the membrane.

6.32. Incubate at room temperature for 1 – 5 minutes.

6.33. Centrifuge at full speed for 1 minute.
OPTIONAL: Repeat steps 6.31 through 6.33 to increase yield.

6.34. Remove the QIAamp MinElute™ column and close the lid of the tube. The QIAamp MinElute™ column can be discarded. The DNA extract may be concentrated at this point or may proceed to quantification directly.

1. Scope

This protocol is used to concentrate and/or further purify DNA extracts. DNA typing will not be successful if there is insufficient starting material during the amplification process. Additionally, the presence of certain substances called inhibitors (e.g. fabric dyes) may prevent amplification from occurring. Microcon® filters offer a means of concentrating DNA and removing the inhibitors from a DNA extract. During centrifugation, the filter captures larger molecules (DNA), and allows the liquid and smaller molecules (inhibitors) to pass through. In the final step, the DNA is eluted from the filter in a volume of liquid determined by the analyst. This method may be used to concentrate DNA extracts down to as little as 10 µL, if desired. While the Microcon® can be used to further purify a DNA extract, it may not remove all inhibitory substances. A small percentage of the DNA in the original extract may be lost during the Microcon® filtration. It is important to weigh the benefit of concentrating and/or further purifying the DNA with the possibility of losing a portion of the DNA.

2. References

2.1. Millipore Microcon® Centrifugal Filter Devices User Guide, June 2005.
2.2. Internal validation studies.
2.3. C.T. Comey, B.W. Koons, K.W. Presely, J.B. Smerick, C.A. Sobieralski, D.M. Stanley, F.S. Baechtel, DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis, J. Forensic Sci. 39 (1994) 5.
2.4. A.M. Krowczynska, K. Donoghue, L. Hughes, Recovery of DNA, RNA, and protein from gels with Microconcentrators, Biotechniques, 1995, 18(4): pp. 698-703.

3. Equipment

3.1. Disposable gloves
3.2. Eye protection
3.3. Lab coat
3.4. 30 kD Microcon® (sample reservoir and retentate/filtrate vials)
3.5. TE^{-4} (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
3.6. Carrier RNA (cRNA, 1 µg/µl, Qiagen)
3.7. 10% bleach solution
3.8. 70% EtOH
3.9. Vortexer
3.10. Pipettes
3.11. Benchtop hood
3.12. Disposable aerosol-resistant pipette tips
3.13. Centrifuge

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn during this procedure.

4.2. Perform concentration steps, where possible, in a hood to reduce the risk of contamination. Clean surfaces with 10% bleach solution prior to use. After exiting the hood, turn on the UV light.

4.3. Use aerosol-resistant pipette tips when transferring liquids containing DNA.

4.4. Change pipette tips after transferring any liquids potentially containing DNA.

4.5. Record the lot number of each reagent used in notes. Do not use the reagents after the expiration date.

4.6. The reagent blank must be concentrated with associated DNA extracts.

4.7. Lab coat and eye protection must be worn at all times while performing this procedure.

4.8. Where practical, only tubes associated with one sample shall be open at a time.

4.9. Final DNA extract tubes shall be labeled, at a minimum, with case number, item number, and analyst's initials.

4.10. In general, samples thought to contain significantly lower levels of DNA will be handled before those thought to contain large amounts of DNA.

4.11. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. In benchtop hood, appropriately label the assembled filters (sample reservoir and retentate/filtrate vial) and extra vials. The clear side of the sample reservoir should be facing upwards.

5.2. Add 1 µl of cRNA (1 µg/µl) to each DNA extract tube being concentrated.

NOTE: If combining samples, cRNA should only be added to one of the extracts being combined for concentration.

5.3. Vortex and briefly centrifuge samples to remove extract from the inside of lid.

5.4. Transfer the DNA extract into the sample reservoir without touching the membrane with the pipette tip.

NOTE: **Multiple extracts can be combined at this step if desired. Up to 500 µl of volume can be added to the sample reservoir at a time.**

NOTE: If combining multiple extracts, an equal number of reagent blanks that were extracted concurrently with the evidentiary samples shall be combined and concentrated to the same level as the evidentiary sample(s) with the lowest volume. The reagent blanks to be combined should include any reagent blanks with a detectable DNA concentration as determined

during quantitation. If no reagent blanks exhibit a detectable DNA concentration, then the reagent blanks will be selected in order 1 through X (X being the number of evidentiary samples combined). If no DNA extracts are combined and more than one reagent blank was initiated, all reagent blanks that demonstrated a detectable DNA concentration, as determined during quantification, shall be processed to completion. If no reagent blanks demonstrated a detectable DNA concentration as determined during quantification, the first reagent blank of the set shall be processed to completion. The unused reagent blanks shall be retained, unless all of the associated evidentiary DNA extracts are consumed in analysis.

5.5. Close the filter assembly and place in the centrifuge. Spin at 14,000 x g for approximately 4-6 minutes. Spin time will vary based on starting volume. Do not excessively dry the membrane.

5.6. Inspect bottom of sample reservoir. If excess liquid is present, filter assembly may be spun for additional time.

5.7. Add 100 µl of TE^{-4} to the reservoir for each sample and spin at 14,000 x g for approximately 4-6 minutes. Spin time may vary. Do not excessively dry the membrane.

Optional: Additional washes with TE^{-4} buffer may be performed to further purify DNA extract. Pipette desired wash amount (up to 300 µL) into the sample reservoir and repeat step 5.5. If performing repeated washes, additional vials may be required.

5.8. Remove sample reservoir from vial containing filtrate and invert sample reservoir (white side up) into a new labeled vial.

Optional: Pipette additional TE^{-4} buffer (1-40 µL) into bottom of sample reservoir prior to inversion. Reservoirs may be gently agitated using low setting on vortexer after inversion. Discard vial containing filtrate.

5.9. Spin at 1,000 x g for 3-5 minutes to collect the concentrated DNA extract in the new vial.

Optional: After the recovery spin, additional TE^{-4} buffer may be added to DNA extract to achieve desired volume.

5.10. DNA extracts can be stored at 4ºC for short periods and should be stored at -20ºC for longer term storage.

NOTE: If the DNA extract(s) is/are stored at -20°C for a longer period of time, the extract may be transferred to a properly labeled 1.5 ml or 2 ml microcentrifuge tube.

1. Scope

This protocol is to be used with the Applied Biosystems (AB) AmpFlSTR™ Identifiler™ PCR Amplification Kit to amplify DNA extracted from known reference samples or biological material found on items of evidence at the following loci: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA, and the sex-determining locus Amelogenin. The AB AmpFlSTR™ Identifiler™ PCR Amplification Kit utilizes the polymerase chain reaction (PCR) to make copies of specific regions of extracted DNA samples. These regions are called short tandem repeats (STR) and, taken together, allow the analyst to characterize the source of the DNA. Identifiler allows for the simultaneous amplification of 15 tetranucleotide repeat regions (loci) and the gender marker Amelogenin in one reaction tube. Amplification is performed in a thermal cycler.

The Identifiler kit contains all reagents needed for amplification which includes: primer sets specific for the various loci, PCR reaction buffer, AmpliTaq® Gold DNA polymerase, and the positive control DNA (9947A). Allelic ladders are also included in the kit; however, they are not needed during amplification. The primer sets contained within each kit consist of both unlabeled primers and those that are labeled with one of four distinctive fluorescent dyes. The incorporation of fluorescent dyes during the amplification process allows for the subsequent detection, characterization, and sizing of the fragments on the Applied Biosystems 3130 Genetic Analyzer. The use of multicolor dyes permits the analysis of loci with overlapping size ranges.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

2.1. Applied Biosystems AmpFlSTR™ Identifiler™ PCR Amplification Kit User's Manual, 2005.
2.2. Prince George's County (MD) Police Forensic Services Division, Serology/DNA Laboratory Short Tandem Repeat (STR) Analysis Protocol, 2006.
2.3. P.J. Collins, L.K. Hennessy, C.S. Leibelt, R.K. Roby, D.J. Reeder, P.A. Foxall, Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: The AmpFlSTR™ Identifiler™ PCR Amplification Kit, J. Forensic Sci. 49 (2004) 1265-1277.
2.4. C. Leibelt, B. Budowle, P. Collins, Y. Daoudi, T. Moretti, G. Nunn, D. Reeder, R. Roby, Identification of a D8S1179 primer binding site mutation and the validation of a primer designed to recover null alleles, Forensic Sci. Int. 133 (2003) 220-227.
2.5. J.M. Butler, Commonly Used Short Tandem Repeat Markers and Commercial Kits, Biology of STRs, and Forensic Issues in Forensic DNA Typing, in: Biology, Technology, and Genetics of STR Markers, second ed., Elsevier Academic Press, 2005, pp 85-180.
2.6. A. Edwards, A. Civitello, H. Hammond, C.T. Caskey, DNA typing and genetic mapping with trimeric and tetrameric tandem repeats, Am. J. Hum. Genet. 49 (1991) 746–756.

2.7. H.A. Hammond, L. Jin, Y. Zhong, C.T. Caskey, R. Chakraborty, Evaluation of 13 short tandem repeat loci for use in personal identification applications, Am. J. Hum. Genet. 55 (1994) 175–189.
2.8. B. Budowle, T.R. Moretti, K.M. Keys, B.W. Koons, J.B. Smerick, Validation studies of the CTT STR multiplex system, J. Forensic Sci. 42 (1997) 701–707.
2.9. C.J. Fregeau, R.M. Fourney, DNA typing with fluorescently tagged short tandem repeats: A sensitive and accurate approach to human identification, BioTechniques 15 (1993) 100–119.
2.10. P.S. Walsh, N.J. Fildes, R. Reynolds, Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA, Nucleic Acids Res. 24 (1996) 2807-2812.
2.11. R.K. Saiki, T.L. Bugawan, G.T. Horn, K.B. Mullis, H.A. Erlich, Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes, Nature 324 (1986) 163-166.
2.12. R.K. Saiki, D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, H.A. Erlich, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, Science 239 (1988) 487-491.

3. Equipment

3.1. Disposable gloves
3.2. Lab coat
3.3. Eye protection
3.4. Benchtop hood
3.5. TE^{-4} (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
3.6. AB Identifiler PCR Amplification Kit
  3.6.1. AmpFlSTR™ Identifiler™ Primer Set
  3.6.2. AmpFlSTR™ PCR Reaction Mix
  3.6.3. AmpliTaq Gold® DNA Polymerase
  3.6.4. AmpFlSTR™ Control DNA 9947A
3.7. Thermal cycler
3.8. 0.2mL thin-walled PCR tubes
3.9. Microcentrifuge tubes
3.10. 10% bleach solution
3.11. 70% ethanol (EtOH)
3.12. Tube racks
3.13. Pipette
3.14. Disposable pipette tips
3.15. Vortexer
3.16. Centrifuge

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling reagents and DNA extracts.
4.2. Change gloves frequently.

4.3. Lab coat and eye protection must be worn at all times while performing this procedure.
4.4. PCR setup for evidence samples shall be performed separately from PCR setup for known samples. PCR setup for known samples shall be performed in a dedicated area.
4.5. Samples thought to contain lower levels of DNA will be handled before those thought to contain large amounts of DNA.
4.6. Sample setup must be performed in a PCR setup hood. Clean surfaces with 10% bleach solution followed by 70% EtOH prior to and after use. After exiting hood, turn on UV light.
4.7. Exercise caution when opening tubes.
4.8. Use aerosol-resistant pipette tips when transferring liquids containing DNA.
4.9. Change pipette tips after transferring any liquids potentially containing DNA.
4.10. Only tubes associated with one sample shall be open at a time.
4.11. The reagent blank must be amplified at a sensitivity level equal to or exceeding the sensitivity of its associated DNA extracts.
4.12. Tubes containing DNA extract shall not be opened in benchtop hood prior to aliquoting master mix into 0.2 mL tubes.
4.13. A positive amplification control (9947A) must be initiated.
4.14. Add 10 uL TE^{-4} to the negative control tube last, after all DNA samples have been added to the other tubes. This tube functions as a negative control for the PCR setup.
4.15. Record the lot number of each reagent used. Do not use the reagents after the expiration date.
4.16. Store the DNA amplification reagents in a refrigerator or freezer separate from the DNA extracts and evidence.
4.17. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. Turn on the thermal cycler.
5.2. If DNA extracts have been frozen, allow them to thaw.
5.3. Dilutions can be prepared at any time prior to adding DNA extracts and/or dilutions to 0.2 mL PCR tubes. If preparing dilutions in PCR setup hood, do not prepare them until after master mix has been aliquotted to 0.2 mL PCR tubes. Determine the amount of DNA extract and TE^{-4} required for each sample and control based on quantification results. Optimal target amount of template DNA per sample and positive control is approximately 0.8 ng.

NOTE: If necessary, lesser or greater quantities of DNA may be used to obtain the desired result.

5.4. Determine total number of sample and control tubes.
5.5. In the PCR setup hood, appropriately label 0.2 mL PCR tubes and 1.5 mL master mix tube(s). Place the 0.2 mL PCR tubes in a clean tray dedicated for PCR setup.

5.6. Vortex and briefly spin the amplification reagents (Identifiler primers, PCR Reaction mix, and AmpliTaq® Gold DNA polymerase).

5.7. Add appropriate volumes of reagents to 1.5 mL tube(s) to create the PCR master mix. The following volumes are 'per sample' and include excess to allow for volume lost in pipetting. Multiply the volumes by the number of samples and controls to be amplified.

PCR Reaction Mix: 10.5 µL
Identifiler™ Primer Set: 5.5 µL
AmpliTaq® Gold DNA Polymerase: 0.5 µL

5.8. Vortex and briefly spin master mix.

5.9. Aliquot 15 µL master mix to each sample and control 0.2 mL tube.

5.10. Vortex and briefly spin DNA extracts and dilutions (if necessary).

5.11. Add appropriate volume of DNA extracts or dilutions, and reagent blank(s) to 0.2 mL PCR tubes. Add TE^{-4} as necessary to bring the final total volume of the PCR reaction to 25 µL.

5.12. Vortex and briefly spin 9947A positive control DNA.

5.13. Add appropriate volume of 9947A to positive control 0.2 mL tube.

5.14. Add 10 µL TE^{-4} to negative control 0.2 mL tube.

5.15. Clean the benchtop hood with 10% bleach followed by 70% EtOH. Turn on the UV light when leaving the benchtop hood.

5.16. Briefly spin tray on plate centrifuge.

5.17. Place tubes in thermal cycler.

5.18. Ensure that all 0.2 mL tube caps are tightly sealed.

5.19. Close thermal cycler cover.

5.20. Select Identifiler protocol (*ID-28*), verify that thermal cycling conditions are as follows, and press start.

HOLD	95° C	11 min	Initial Incubation Step
CYCLE (28 cycles)	94° C	1 min	Denature
	59° C	1 min	Anneal
	72° C	1 min	Extend
HOLD	60° C	60 min	Final Extension
HOLD	4° C	∞	Final Step

5.21. When the amplification is complete, samples can be stored in the refrigerator or freezer. Amplicons can be discarded after the case has been completed and the report has undergone technical and administrative reviews.

1. Scope

This protocol is used to analyze the raw data generated by the Applied Biosystems™ (AB) 3130 Genetic Analyzer. The data collected by the AB™ 3130 Genetic Analyzer for each sample is displayed graphically as an electropherogram. These samples include questioned and known specimens, positive controls, negative controls, and allelic ladders. The intensity, color, and scan number of each peak detected for the sample are recorded in the electropherogram. The intensity of the peak corresponds to the amount of amplified DNA present; the scan number corresponds to the size of the fragment. AB™ GeneMapper® ID is an automated genotyping software program which converts the information contained in the electropherograms ("raw data") to a more readily usable format ("analyzed data").

The size of each fragment of amplified DNA is determined by comparison to the internal size standard, GS500. GS500 fragments, which contain a range of sizing fragments between 35 and 500 base pairs, are labeled with the LIZ dye and are displayed as orange peaks. The 75 – 450 base pair range is required for sizing. GeneMapper® ID generates allelic designations from the sized data. This is accomplished by comparison to the allelic ladder. The GeneMapper® ID software recognizes the first allele of the ladder and creates approximately one base pair bins (+/- 0.5 base pair) around each allele in the ladder. Sample peaks are labeled by comparison of their size to the size of the ladder bins. The ladders are composed of the more common alleles in the general population. They also contain virtual bins for some less common alleles. If a sample peak falls into a bin or a virtual bin, an allelic designation will be assigned to the peak. However, if the peak is not represented by either a bin or a virtual bin created by the ladder, the GeneMapper® ID software will designate this peak as "OL" for "off-ladder" allele.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

2.1. Applied Biosystems™ GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide, 2004.
2.2. Prince George's County (MD) Police, Forensic Services Division, Serology / DNA Laboratory Short Tandem Repeat (STR) Analysis Protocol, 2006.
2.3. J.M. Butler, DNA Separation Methods, DNA Detection Methods, Instrumentation For STR Typing, and STR Genotyping Issues in: Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers, second ed., Elsevier Academic Press, 2005, pp 313-388.
2.4. S.B. Klein, J.M. Wallin, M.R. Buoncristiani, Addressing the Ambient Temperature Variation Effects on Sizing Precision of AmpFlSTR® Profiler Plus™ Alleles Detected on the AB Prism® 310 Genetic Analyzer, Forensic Sci. Comm. 5 (2003) 1.

3. Equipment

3.1. Computer with GeneMapper® ID software.

4. Safety/Quality Assurance

4.1. The positive control (9947A) for each amplification set-up must type correctly in order to use the associated samples.
4.2. Negative controls (negative amplification controls, reagent blanks, and formamide blanks) must be run and analyzed along with the associated samples. Refer to the "STR Interpretation Guidelines – Identifiler™" protocol for a discussion of negative controls.
4.3. At least one positive control and one formamide blank must be included in each GeneMapper® ID project.
4.4. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience. Deviations shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. Turn on the computer station and login.
5.2. If not performing data analysis on a computer directly attached to a 3130 Genetic Analyzer, then connect the selected computer to the network and copy the run folder of interest to the selected computer.
5.3. Launch the GeneMapper® ID software and login.
5.4. Select *File > Add Samples to Project* from the menu. Find the run folder of interest, highlight that folder (or the sample files in the folder) and click *Add to List*. When all samples have been selected for the project, click *Add*.
5.5. In the tree pane of the GeneMapper® ID window, highlight the samples. Select *View > Raw Data* from the menu. Viewing the raw data may be useful in determining the start and stop points for the analysis range, the overall quality of the injection and electrophoresis, and whether or not artifacts are present.
5.6. Select *View > Samples* from the menu. Under the *Samples* tab, make sure that the correct *Sample Type* ("sample, negative control, positive control, or allelic ladder"), *Analysis Method, Panel* ("Identifiler_v1"), and *Size Standard* ("CE_G5_HID_GS500") are selected. See the Appendix for further information.
5.7. Select *Analysis > Analyze* from the menu or click on the green triangle icon. A "Save Project" window will pop up. Type in the name of the project. The project name shall include at a minimum the date the project was created, the analyst's initials, and the instrument name. Be sure to save your project frequently when working with it.
5.8. After analyzing the data, look at the "Sizing Quality (SQ)" column at the far right hand side of the GeneMapper® ID window. If the icon in this column is yellow or red for

any samples, verify that all of the GS500 peaks for that sample fall within the range of analysis.

In the tree pane, select the sample that has a yellow/red "SQ" icon.
Select *View>Raw Data* from the menu.
Place the pointer at the 75 bp peak and 450 bp peak, and note the number listed at each "Data Point".
Select *View>Samples* from the menu.
Select *Analysis>Analysis Method Editor* from the menu (or use the bell curve icon).
Make sure that the "Start Point" number is less than the "Data Point" number for the 75 bp peak.
Make sure that the "Stop Point" number is greater than the "Data Point" number for the 450 bp peak.

5.8.1. If necessary, adjust the range of analysis to encompass all of these peaks, 75 bp to 450 bp.

In the "Analysis Method Editor", make the "Start Point" number lower than the "Data Point" number for the 75 bp peak.

5.8.2. Alternatively, a different "Analysis Method" can be chosen that encompasses the necessary range.

NOTE: All samples in a project shall be analyzed with the same method. To choose a different analysis method:

Select *View>Samples* from the menu.
For the first sample, choose a method in the "Analysis Method" drop-down menu.
Click on the column heading "Analysis Method" to highlight the whole column.
Type *Ctrl-D* to copy down the Analysis Method to all samples.
Select *Analysis > Analyze* from the menu or click on the green triangle icon to re-analyze the samples.

5.9. Select *Analysis>Display Plots* from the menu. Choose the appropriate plot from the "Plot Setting" drop-down menu. See the appendix for the plot requirements. Also reference Chapter 8 of the "Applied Biosystems™ GeneMapper® ID Software Version 3.1 Human Identification Analysis User Guide" for more detailed information on plot windows. Verify that all of the peaks in the GS500 size standard have been correctly assigned for each sample.

5.10. The 250 base pair peak in the GS500 standard, which is not automatically assigned a size, can be used to gauge whether or not the sample(s) will type correctly based on any given allelic ladder. The actual size of the 250 base pair peak depends on the electrophoresis conditions at the time of when the sample(s) ran on the 3130 Genetic Analyzer. If the 250 base pair peak for a sample is greater than one base pair away (+/- 0.5 base pairs) from the same peak in the allelic ladder, it will probably not type correctly. Removing ladders (changing Sample Type from "Allelic Ladder" to

"Sample") and/or creating multiple GeneMapper® ID projects for one run may be necessary in order for all of the samples to type correctly.

5.11. Each project created from one run must contain, at a minimum, one allelic ladder, a correctly typed positive control, and a negative control in addition to the samples of interest. It may be necessary to re-inject samples along with an allelic ladder, positive control, and a negative control if these criteria cannot be met.

5.12. Ensure that the peaks of the allelic ladder are labeled with the correct allele designations. For guidance, see "STR Interpretation Guidelines - Identifiler™" protocol.

5.13. While in the "Samples Plot" window, view each sample and examine the peak labels. It may be necessary to edit peak labels to account for things such as stutter, spectral pull-up, or spikes. For guidance, see "STR Interpretation Guidelines – Identifiler™" protocol.

5.13.1. To edit allele calls, peak labels must be displayed. In the "Samples Plot" window, select *View>Labels>Vertical Labels* to display peak labels. To edit a peak label, select the peak and then right-click the mouse.

If the peak is already labeled, the options are:

- "Delete the allele call"
- "Rename the allele"
- "View the History"

If the peak is not labeled, the options are:

- "Add an allele call"
- "View the History"

Choose the appropriate option by clicking on it.

When renaming or adding an allele call, choose the allele call or enter a custom name.

5.13.2. After making the necessary changes, enter an "Allele Edit Comment", and Click *Ok*. The following are common "Allele Edit Comments":

Stutter – used for stutter peaks.

Spike – used for spikes.

Artifact – used for non-specific artifact peaks.

Pull-up – used for pull-up peaks.

Minus A – used for minus A peaks.

NOTE: All allele edits (computer-generated or by hand) must be displayed on the electropherogram.

5.14. Print the analyzed data and place it in each associated case file. See the Appendix for specifics on printing plots. In the "Samples Plot" window, select *File>Print***.** The following data must be printed:

The electropherogram for each case sample.

The electropherograms for ALL allelic ladders, positive controls, and negative controls (reagent blanks, negative amplification controls, formamide blanks) associated with the case samples.

The GS500 Size Standard Plot for ALL of the associated samples and controls within the project.

The raw data for ALL negative amplification controls and reagent blanks.

NOTE: If multiple cases are in the same GeneMapper® ID project, the data for the associated allelic ladders, positive controls, negative controls, GS500 plots, and raw data will have to be printed FOR EACH CASE.

5.15. Next, print a table showing the list of samples within the project. In the GeneMapper® ID window, select the appropriate table from the "Table Setting" drop-down menu. Select *File>Print* from the menu. On the "Page Setup" tab in the "Print" screen, select the *Landscape* button. See Appendix 1 for specifics on printing the table.

5.16. Run information, including instrument settings and data collection settings, will be printed for each associated case.

Highlight a positive control sample in the project and select *View>Sample Info* from the menu.

Print the "Sample Info" (two pages).

Contents

1. Scope 3
2. References 3
3. Equipment 5
4. Safety / Quality Assurance 5
5. Procedure 5
5.1 General DNA Profile Interpretation Steps 5
5.2 Evaluation of the Controls 7
Verification of Internal Size Standard (GS-500) 7
Verification of the AmpFlSTR™ Identifiler™ Allelic Ladder 8
Evaluation of Negative Controls 9
Amplification Positive Control (9947A) 12
Single Injection/Capillary Events 13
5.3 Evaluation of Sample Profiles 14
Analytical Threshold (AT) 14
Stochastic Threshold (ST) 14
Off-Scale Peaks 14
Artifacts 15
Off-Ladder Alleles 18
Peak Height Imbalance 19
Composite Profiles 21
Interpretation/Statistical Calculation Documentation 21
Preliminary Evaluation of Sample DNA Profile 22
5.4 Single Contributor DNA Profile Interpretation 23
Comparison and Conclusions 25
Statistical Analysis of Assumed Single Contributor DNA Profiles 25
5.5 Multiple Contributor DNA Profile Interpretation 27
Statistical Analysis of Multiple Contributor DNA Profiles 32
For three person mixture interpretation, see Appendix D. 35
Unique Statistical Situations 35
Documentation 37

Appendix B..... 38

Appendix C..... 42

1. Scope

The interpretation guidelines are intended to be a general guide for the evaluation of the typing results for DNA amplified with the AmpFlSTR™ Identifiler™ PCR Amplification Kit and analyzed with the Applied Biosystems (AB) 3130 Genetic Analyzer and GeneMapper® ID v3.2 analysis software. The guidelines will ensure consistency in the analysis and evaluation of the typing results between DNA analysts. These guidelines cannot encompass the entire range of samples or circumstances that will be encountered in forensic casework. Exceptions may be made based on the analyst's training and experience and must be approved by the DNA Technical Leader. The guidelines were based on the manufacturer's recommendations, manufacturer's user's manuals, internal validation studies, interpretation guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDAM), and the scientific literature.

2. References

2.1. Applied Biosystems GeneMapper® *ID-X* Software Version 1.3 User Bulletin, revision A, October 2011.
2.2. AmpFlSTR® Identifiler™ PCR Amplification Kit User's Manual, 2005.
2.3. Internal validation studies
2.4. J.M. Butler, DNA Separation Methods, DNA Detection Methods and Instrumentation for STR Typing in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp. 313-372.
2.5. R. Saferstein, The PCR reaction, in: Forensic Science Handbook, Vol. 1, second ed., Prentice Hall, Upper Saddle River, NJ, 2002, pp 569-570.
2.6. J.M. Butler, Commonly used short tandem repeat markers and commercial kits, Biology of STRs, and Forensic issues, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005 pp 85-180.
2.7. A. Edwards, A. Civitello, H. Hammond, C.T. Caskey, DNA typing and genetic mapping with trimeric and tetrameric tandem repeats, Am. J. Hum. Genet. 49 (1991) 746–756.
2.8. H.A. Hammond, L. Jin, Y. Zhong, C.T. Caskey, R. Chakraborty, Evaluation of 13 short tandem repeat loci for use in personal identification applications, Am. J. Hum. Genet. 55 (1994) 175–189.
2.9. B. Budowle, T.R. Moretti, K.M. Keys, B.W. Koons, J.B. Smerick, Validation studies of the CTT STR multiplex system, J. Forensic Sci. 42 (1997) 701–707.
2.10. C.J. Fregeau, R.M. Fourney, DNA typing with fluorescently tagged short tandem repeats: a sensitive and accurate approach to human identification, BioTechniques 15 (1993) 100–119.
2.11. A. Urquhart, N.J. Oldroyd, C.P. Kimpton, P. Gill, Highly discriminating heptaplex short tandem repeat PCR system for forensic identification, BioTechniques 18 (1995) 116–121.
2.12. E.S. Mansfield, J.M. Robertson, M. Vainer, A.R. Isenberg, R.R. Frazier, K. Ferguson, S. Chow, D.W. Harris, D.L. Barker, P.D. Gill, B. Budowle, B.R.

McCord, Analysis of multiplexed short tandem repeat (STR) systems using capillary array electrophoresis, Electrophoresis 19 (1998) 101–107.

2.13. B. Budowle, J.B. Smerick, K.M. Keys, T.R. Moretti, United States population data on the multiplex short tandem repeat loci-HUMTHO1, TPOX, and CSF1PO and the variable number tandem repeat locus D1S80, J. Forensic Sci. 42 (1997) 846-849. Erratum, Journal of Forensic Sciences (1998) 43(1):253.

2.14. K. Lazaruk, P. Walsh, F. Oaks, D. Gilbert, B. Rosenblum, S. Menchen, D. Scheibler, H. Wenz, C. Holt, J. Wallin, Genotyping of forensic short tandem repeat (STR) systems based on sizing precision in a capillary electrophoresis instrument, Electrophoresis 19 (1998) 86-93.

2.15. C.A. Crouse, S. Rogers, E. Amiott, S. Gibson, A. Masibay, Analysis and interpretation of short tandem repeat microvariants and three-banded allele patterns using multiple allele detection systems, J. Forensic Sci. 44 (1999) 87-94.

2.16. C.A. Crouse, J. Schumm, Investigation of species specificity using nine PCR-based human STR systems, J. Forensic Sci. 40 (1995) 952-956.

2.17. A.M. Lins, C.J. Sprecher, C. Puers, J.W. Shumm, Multiplex sets for the amplification of polymorphic short tandem repeat loci - silver stain and fluorescent detection, BioTechniques 20 (1996) 882-889.

2.18. P.S. Walsh, N.J. Fildes, R. Reynolds, Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA, Nucleic Acids Res. 24 (1996) 2807-2812.

2.19. B. Leclair, C.J. Fregeau, K.L. Bowen, R.M. Fourney, Systematic analysis of stutter percentages and allele peak height and peak area ratios for heterozygous STR loci for forensic casework and database samples, J. Forensic Sci. 49 (2004) 968-980.

2.20. T.M. Clayton, J.P. Whitaker, R. Sparkes, P. Gill, Analysis and interpretation of mixed forensic stains using DNA STR profiling, Forensic Sci. Int. 91 (1998) 55-70.

2.21. P.M. Schneider, R. Fimmers, W. Keil, G. Molsberger, D. Patzelt, W. Pflug, T. Rothamel, H. Schmitter, H. Schneider, B. Brinkmann, The German Stain Commission: recommendations for the interpretation of mixed stains, Int. J. Legal Med. 123 (2009) 1-5.

2.22. J. Bright, J. Turkington, J. Buckleton, Examination of variability in mixed DNA profile parameters for the Identifiler multiplex, Forensic Sci. Int. Genet. 4 (2010) 111-114.

2.23. P. Gill, C.H. Brenner, J.S. Buckleton, A. Carracedo, M. Krawczak, W.R. Mayer, N. Morling, M. Prinz, P.M. Schneider, B.S. Weir, DNA Commission of the International Society of Forensic Genetics: recommendations on the interpretation of mixtures, Forensic Sci. Int. Genet. 160 (2006) 90-101.

2.24. Scientific Working Group on DNA Analysis Methods, Interpretation guidelines for autosomal STR typing by forensic DNA testing laboratories, 2010.

2.25. B. Budowle, A.M. Giusti, J.S. Waye, F.S. Baechtel, R.M. Fourney, D.E. Adams, L.A. Presley, H.A. Deadman, K.L. Monson, Fixed-bin analysis for statistical evaluation of continuous distributions of allelic data from VNTR loci for use in forensic comparisons, Am. J. Hum. Genet. 48 (1991) 841-855.

2.26. B. Budowle, A.J. Onorato, T.F. Callaghan, A.D. Manna, A.M. Gross, R.A. Guerrieri, J.C. Luttman, D.L. McClure, Mixture interpretation: defining the relevant features for guidelines for the assessment of mixed DNA profiles in forensic casework, J. Forensic Sci. 54 (2009) 810-821.

3. Equipment

3.1. Computer with GeneMapper® ID analysis software

4. Safety / Quality Assurance

4.1. Safety: Not applicable

4.2. Quality Assurance: See Procedure Section

5. Procedure

5.1 General DNA Profile Interpretation Steps

5.1.1 Evaluation of the standards, allelic ladders and controls

Internal Size Standard for each sample

Allelic Ladder(s)

Negative controls (extraction reagent blank, amplification reagent blank, formamide blank)

Positive amplification control

5.1.2 Evaluation of evidence sample DNA profiles

Designate allelic peaks, mark artifacts if present.

Determine if each sample contains sufficient data for interpretation and/or comparison.

For those profiles containing sufficient data for interpretation and/or comparison, determine if each sample profile is from a single contributor or multiple contributors.

If the sample is determined to originate from a single contributor, follow the steps listed under the "Single Contributor DNA Profile Interpretation" listed below.

If the sample is determined to originate from multiple contributors, follow the steps listed under the "Multiple Contributor DNA Profile Interpretation" listed below.

5.1.3 Analysis of known DNA sample profiles

After the interpretation of the evidentiary samples, the results of the known samples can then be reviewed and interpreted. The interpretation of each evidentiary DNA profile will be documented prior to the review of any known samples associated with the case,

where possible. This may not be possible in all circumstances, for example, if additional evidence is submitted after the evidentiary and known samples have been analyzed.

5.1.4 Comparing known DNA profiles to the Evidentiary DNA profiles

After the interpretation and documentation of the evidentiary profiles, the known DNA profiles can then be compared to the evidentiary profiles. In addition to comparing the evidentiary samples to known DNA profiles, comparisons should be made to the other evidentiary samples associated with the case, if present. Two conclusions can be drawn from this comparison:

- Excluded: the individual associated with the known DNA sample cannot be a contributor to the evidentiary sample.
- Not Excluded: the individual associated with the known DNA sample can be a contributor to the evidentiary sample.

5.1.5 Statistical Analysis

If it is determined that an individual associated with a known DNA profile cannot be excluded as a possible contributor to the evidence profile, a statistical calculation will be performed to lend weight to the meaning of inclusion except in cases as noted in the "Statistical Calculation" section below.

5.1.6 CODIS

All profiles suitable for comparison purposes will be compared to the Staff Index, Profiles Generated Index, and LDIS database prior to being reported as a way of detecting possible contamination. See CODIS Manual for instructions on entering and searching profiles in CODIS.

5.1.7 Report

The results of the DNA analysis are then detailed in a laboratory report.

5.2 Evaluation of the Controls

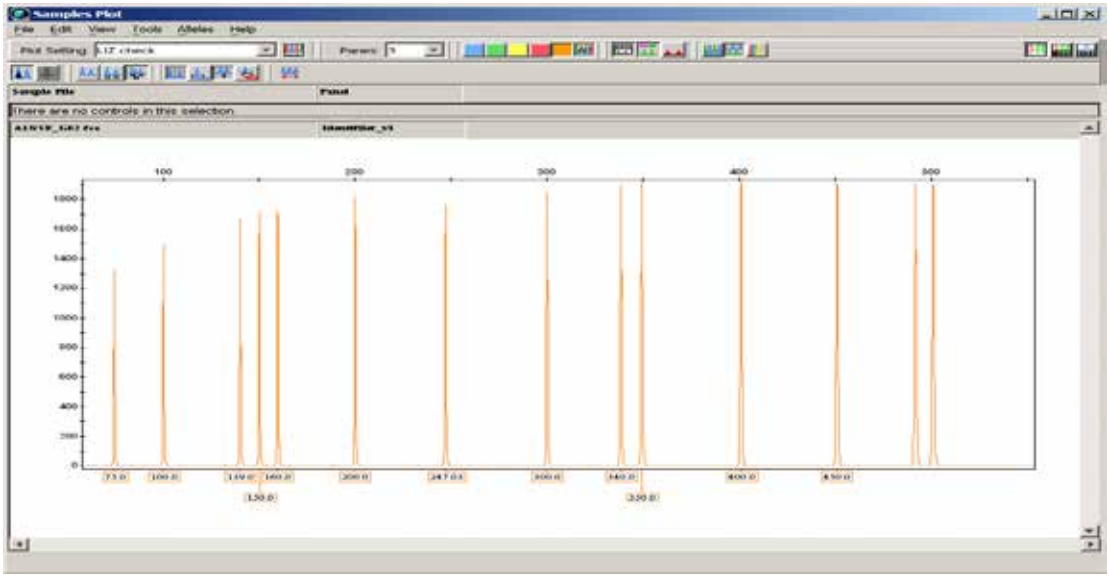
Verification of Internal Size Standard (GS-500)
The Internal Size Standard (GS-500, LIZ dye labeled) consists of twelve peaks of known base pair size ranging from 35 base pairs to 500 base pairs.

All Internal Size Standard peaks ranging from 75 to 450 base pairs must be present, have the expected peak morphology, have good peak balance across the range of sizing, and be labeled correctly with peak heights greater than 50 RFU for the associated profile to be acceptable.

Extraneous peaks observed in the Internal Size Standard can be disregarded as long as they are not incorrectly labeled as peaks of the Internal Size Standard.

	Internal Size Standard (GS-500) peaks necessary for the analysis of AB AmpFlSTR™ Identifiler™ amplified products
Base Pair Sizes (bp)	75, 100, 139, 150, 160, 200, 250*, 300, 340, 350, 400, 450

** The electrophoresis of the 250 base pair peak is not automatically assigned since fluctuations in the laboratory's temperature will result in variations in the electrophoretic mobility of the peak. It can be used as an additional control. The calculated size of the 250 peak should not vary by more than one base pair across the run. If the variation is greater than 1 base pair, the run may need to be broken up into separate projects. If an individual sample or ladder is the only sample that falls outside the acceptable range and it can be reasonably concluded that it was an individual capillary event, the sample can be disregarded and the rest of the run analyzed.*



Typical Internal Size Standard (GS-500)

Verification of the AmpFlSTR™ Identifiler™ Allelic Ladder

The presence of all alleles of the allelic ladder must be verified (See *Characterization of the AmpFlSTR™ Identifiler™ Loci* table below).

Allelic ladders missing one or more alleles or containing allele peaks that are marked off-ladder are not valid.

Sample profiles must be associated with one or more valid allelic ladders to be considered for interpretation.

The Sample Type of allelic ladders that are found not to be valid may be changed from "Allelic Ladder" to "Sample." If this is done, the project must be re-analyzed.

Locus Designation	Chromosome Location	Alleles Included in Identifiler Allelic Ladder	Dye Label	Control DNA 9947A
D8S1179	8	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	6-FAM	13[a]
D21S11	21q11.2-q21	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38		30[b]
D7S820	7q11.21-22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 11
CSF1PO	5q33.3-34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 12
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	VIC	14, 15
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3		8, 9.3
D13S317	13q22-31	8, 9, 10, 11, 12, 13, 14, 15		11[c]
D16S539	16q24-qter	5, 8, 9, 10, 11, 12,13, 14, 15		11, 12
D2S1338	2q35-37.1	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28		19, 23

Locus Designation	Chromosome Location	Alleles Included in Identifiler Allelic Ladder	Dye Label	Control DNA 9947A
D19S433	19q12-13.1	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	NED	14, 15
vWA	12p12-pter	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24		17, 18
TPOX	2p23-2per	6, 7, 8, 9, 10, 11, 12, 13		8[d]
D18S51	18q21.3	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27		15, 19
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	PET	X
D5S818	5q21-31	7, 8, 9, 10, 11, 12, 13, 14, 15, 16		11[e]
FGA	4q28	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2		23, 24

Characterization of the AmpFlSTR® Identifiler™ Loci. AmpFlSTR® Identifiler™ PCR Amplification Kit User's Manual, 2005. a. For CODIS purposes, profile reported as 13, 13. b. For CODIS purposes, profile reported as 30,30. c. For CODIS purposes, profile reported as 11,11. d. For CODIS purposes, profile reported as 8,8. e. For CODIS purposes, profile reported as 11,11.

Evaluation of Negative Controls

Reagent Blank

The reagent blank is initiated at the extraction step and is processed in parallel with the associated evidentiary sample(s). It consists of all the reagents used during the extraction. The reagent blank is used to detect DNA contamination from the reagents, disposables, or environment that may affect the evidentiary samples. Contamination detected in the reagent blank may be systemic in nature or a randomly occurring event. If contamination is detected, an effort should be made to identify the source to prevent future contamination events.

An acceptable reagent blank will contain no peaks above the analytical threshold (50 RFU).

A reagent blank containing a peak above the analytical threshold (50 RFU) between 100 base pairs and 362 base pairs, which is believed to be an artifact such as a spike or spectral pull-up from the internal size standard, shall be re-injected. If, upon re-injection, no peaks above the analytical threshold are present, the reagent blank is acceptable.

Artifacts outside the 100 to 362 base pair window are not significant and do not necessitate the re-injection of the control.

If at least one non-artifact peak above the analytical threshold or a recognizable pattern of peaks below the threshold is present in the reagent blank, all samples associated with the reagent blank must be re-analyzed, if possible.

Based on the results of the other controls (e.g. amplification blank, positive control, etc.) associated with the evidentiary samples, the re-analysis may initially consist of re-injecting all samples, re-amplification of all samples, or re-extraction of all associated samples, if possible.

If it is not possible to re-extract a sample associated with a contaminated reagent blank and it is reasonable to assume that the integrity of the DNA analysis was not affected, the results shall be reported as described in the Report Wording protocol. The issues shall be noted in the case file.

For evidentiary samples associated with a contaminated reagent blank where it is reasonable to assume that the integrity of the DNA analysis may have been affected, the results shall be reported as described in the Report Wording protocol (ATF-LS-FB18). In addition, a note shall be made in the report briefly describing the issue and stating the results should be interpreted with caution.

If a reagent blank associated with a set of samples to be re-amplified cannot be re-amplified due to limited volume, and it was determined to be valid from a previous analysis, the samples associated with the reagent blank can still be analyzed as long as the sensitivity of the re-amplified samples does not exceed the original sensitivity of the reagent blank.

A printout of the raw data demonstrating the presence of the primer peaks will be included with the case material along with a printout of the electropherogram with a maximum Y-axis of 200 RFU.

Amplification Negative Control

The amplification negative control is initiated at the amplification step and consists of all the reagents used in the amplification, except TE^{-4} is used in the place of the template DNA.

An acceptable amplification negative control shall contain no peaks above the analytical threshold (50 RFU).

An amplification negative control containing a peak above the analytical threshold between 100 base pairs and 362 base pairs which is believed to be an artifact, such as a spike or spectral pull-up from the internal size standard, shall be re-injected. If, upon re-injection, no peaks above the analytical threshold are present, the amplification negative control is acceptable.

Artifacts outside the 100 to 362 base pair window are not significant and do not necessitate the re-injection of the control.

If at least one non-artifact peak above the analytical threshold or a recognizable pattern of peaks below the threshold is present in the amplification negative control, all samples associated with the amplification negative control must be re-analyzed.

> The re-analysis may consist of re-injection or re-amplification, if possible.
> If it is not possible to re-amplify a sample associated with a contaminated amplification negative control and it is reasonable to assume that the integrity of the DNA analysis was not affected, the results shall be reported as described in the Report Wording protocol. The issues shall be noted in the case file.
> For evidentiary samples associated with a contaminated amplification negative control, where it is reasonable to assume that the integrity of the DNA analysis may have been affected, the results shall be reported as described in the Report Wording protocol. In addition, a note shall be made in the report briefly describing the issue and stating the results should be interpreted with caution.
> A printout of the raw data demonstrating the presence of the primer peaks will be included with the case material along with a printout of the electropherogram with a maximum Y-axis of 200 RFU.

Formamide Blank

A formamide blank consists of the formamide solution with ILS, but without amplified DNA product.

At least one formamide blank will be analyzed with each set of samples run on the Genetic Analyzer.

An acceptable formamide blank shall contain no peaks above the analytical threshold (50 RFU).

> It is typical for the formamide blank to contain spectral pull-up from the ILS due to the lack of primers and amplified product during the electrokinetic injection which results in a significant increase of ILS fragments entering the capillary. The pull-up peaks detected will mimic the ILS pattern and can be marked as such. These peaks do not preclude the interpretation of the associated samples nor does their presence require the samples to be re-injected.

Formamide blanks containing a peak above the analytical threshold between 100 base pairs and 362 base pairs which is believed to be an artifact other than that mentioned in the preceding paragraph, such as a spike, shall be re-injected. If, upon re-injection, no peaks above the analytical threshold are present (other than possible ILS pull-up), the formamide blank is acceptable.

> Artifacts outside the 100 to 362 base pair window are not significant and do not necessitate the re-analysis of the control.

If at least one non-artifact peak above the analytical threshold or a recognizable pattern of peaks below the threshold is present in the formamide blank, all samples associated with the formamide blank, must be re-analyzed.

The re-analysis may consist of re-injection from the same plate or setting up a new plate for the Genetic Analyzer.

Amplification Positive Control (9947A)

The amplification positive control is initiated at the amplification step and is processed in parallel with the evidentiary samples. It consists of all the reagents used in the amplification reaction and template DNA of a known profile (9947A). The amplification positive control monitors the amplification reaction and ensures the reliability of the results for the associated evidentiary samples.

The typing results for the positive control should match the expected profile (see below).

Locus	Genotype	Locus	Genotype
D8S1179	13,13	D2S1338	19,23
D21S11	30,30	D19S433	14,15
D7S820	10,11	vWA	17,18
CSF1PO	10,12	TPOX	8,8
D3S1358	14,15	D18S51	15,19
TH01	8,9.3	Amelogenin	X,X
D13S317	11,11	D5S818	11,11
D16S539	11,12	FGA	23,24

Expected typing results for Control DNA 9947A

If the allelic peak heights are greater than the relevant thresholds (analytical and stochastic, 50 and 200 RFU, respectively), match the expected profile and meet the minimum expected peak height ratio (See Appendix A), the positive control is valid.

On rare occasions, the heterozygote peak height balance may fall outside expectations. The positive control is still valid in this situation. If multiple loci demonstrate greater than expected imbalance, this may be an indication of an issue with amplification or capillary electrophoresis.

If the typing results do not match the expected profile (incorrect profile, additional alleles due to possible contamination or partial/no results), the positive control is not valid and all evidentiary samples associated with the positive control shall be re-analyzed. The re-analysis may consist of re-injection or re-amplification.

If it is not possible to re-analyze a sample associated with a failed positive control, where it is reasonable to assume that the integrity of the DNA analysis was not affected, the results shall be reported as described in the Report Wording protocol (*ATF-LS-FB18*). The issues shall be noted in the case file.

For evidentiary samples associated with a failed positive control where it is reasonable to assume that the integrity of the DNA analysis may have been affected, the results shall be reported as described in the Report Wording protocol. In addition, a note shall be made in the report briefly describing the issue and stating the results should be interpreted with caution.

Single Injection/Capillary Events

In general, if a single capillary or single injection event occurs that results in a poor injection as evidenced by the ILS peaks, the sample or samples specifically affected may be re-injected alone and the entire run does not have to be re-injected and analyzed. This is true for all samples except for the positive control. Each run must have a valid positive control.

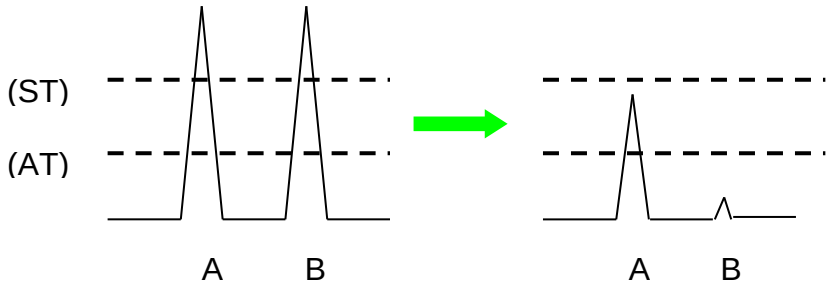
5.3 Evaluation of Sample Profiles

Analytical Threshold (AT)
The Analytical Threshold is the relative fluorescent unit (RFU) value that, when exceeded by peaks that display the expected peak morphology, allows those peaks to be considered "real" products of amplification.

The Analytical Threshold is set at 50 RFU.

Stochastic Threshold (ST)
Due to the inherent nature of PCR amplification of low levels of DNA, the results may contain dramatic peak height imbalance and allele drop-out. The Stochastic Threshold is the RFU value that, when exceeded by a single allelic peak, the DNA analyst can be confident that the sister peak of a heterozygous pair would be detected (i.e. would be above the Analytical Threshold). The Stochastic Threshold listed below refers to a single source sample. The use of the Stochastic Threshold must be modified to account for possible allele sharing during the interpretation of mixed DNA profiles due to the possible additive effects of allele sharing.

The Stochastic Threshold is set at 200 RFU.



Off-Scale Peaks
An Off-Scale Peak is a peak which exceeds the linear dynamic range of the instrument. Off-Scale Peaks may result in raised baselines and/or excessive "pull-up" in one or more colors.

Analyzed data from Off-Scale Peaks should not be used for quantitative comparisons since the true quantity of signal is not known. For example, this can result in disproportionably high stutter calculations.

Typically, Off-Scale Peaks will be associated with other artifacts such as –A peaks, increased n-4 peaks, n-8 peaks, and n+4 peaks which cause difficulties during interpretation.

Sample profiles containing Off-Scale data may be re-analyzed at the discretion of the analyst.

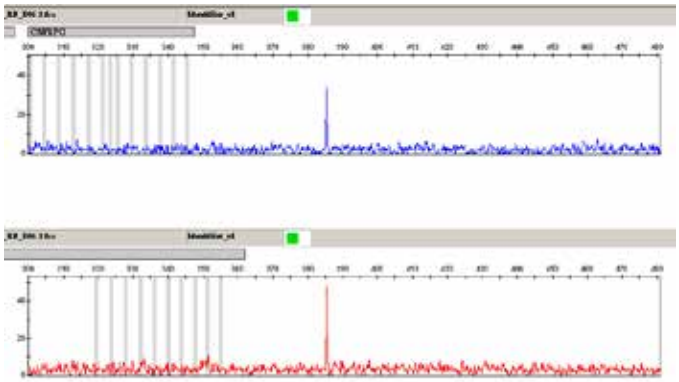
Re-analysis may consist of adding less amplified product to the AB 3130 set-up tray, diluting the amplified product in formamide prior to adding it to the 3130 set-up tray, or re-amplifying the sample. Re-amplification is preferred and may be done at the analyst's discretion.

Artifacts

Spikes

A spike is defined as a non-specific, non-reproducible, and non-allelic peak, which is typically observed in one or more colors and is an artifact of the electrophoresis and signal detection. A spike does not typically display the same peak morphology as an allelic peak with respect to peak width, starting slope and ending slope.

If a spike is present in the analytical range (100 – 362 base pairs), the spike may be confirmed to be an artifact by re-injection if it interferes with interpretation. Upon re-injection, the same spike should not be present.



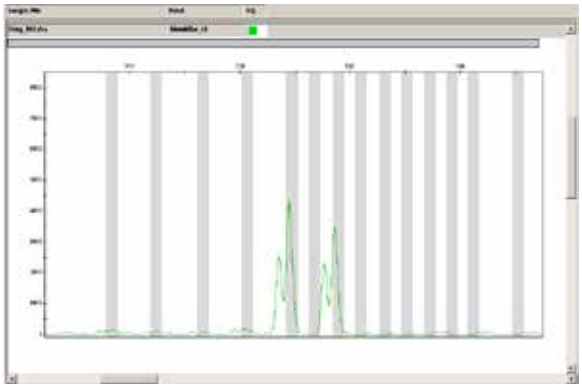
Example of a spike exhibiting the typical characteristics (narrow peak width, detected in multiple channels, similar peak height in different channels).

Non-Template Nucleotide Addition

The Taq polymerase has the inherent property of non-template directed nucleotide addition. This nucleotide addition is reverse primer dependent. In the AmpFlSTR™ Identifiler™ reaction, the non-template nucleotide addition is favored to the extent that, under normal conditions, the majority of the amplified product contains the additional nucleotide.

In extreme conditions, a portion of the amplified product will not contain the additional nucleotide resulting in a fragment one base pair shorter than the expected allele. Since the nucleotide typically added is adenine (A), the peak representing amplified product in which the non-template directed nucleotide addition did not occur is oftentimes referred

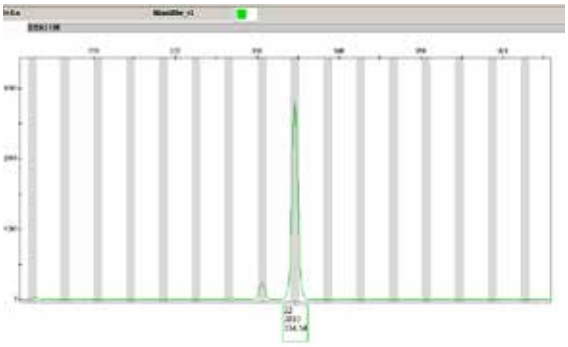
to as the –A peak. One extreme circumstance occasionally encountered is the addition of excess template DNA.



Example of –A peak

Stutter

Stutter peaks are an artifact of the Polymerase Chain Reaction when amplifying repeat regions of DNA. The primary peak is representative of the actual repeat number contained in the template DNA. The stutter peak is an artifact usually one repeat shorter in length than the primary peak. For tetrameric repeats, the stutter peak is n-4 base pairs where n is the base pair length of the primary peak. Occasionally n+4 peaks may be observed.



Example of a stutter peak (unlabeled) one repeat in length shorter than the primary peak (labeled as "22" allele).

Stutter peaks can be characterized both by their size and their peak height proportion when compared to the associated primary peak expressed as the percentage of the stutter peak height compared to the primary peak. The expected maximum stutter percentage for each locus will vary and can be experimentally defined. The table below lists the maximum stutter percentages expected for each locus.*

Locus	Maximum Expected Stutter %*	Locus	Maximum Expected Stutter %*
D8S1179	8.2	D2S1338	11.1
D21S11	9.4	D19S433	13.3
D7S820	8.2	vWA	12.6
CSF1PO	9.2	TPOX	4.8
D3S1358	10.7	D18S51	17.0
TH01	5.1	Amelogenin	---
D13S317	8.0	D5S818	6.8
D16S539	10.4	FGA	14.7

**AB Maximum Stutter Values are taken from the AmpFlSTR® Identifiler™ PCR Amplification Kit User's Manual, 2005. It is a compilation of data from the analysis of 1,187 samples*

Minor peaks cannot be absolutely identified as stutter peaks even though they meet both the size and stutter peak percentage criteria.

If there is no other indication of a mixture in a profile, the minor peaks meeting the stutter peak criteria can be attributed to stutter.

If evidence of a mixture is observed in a profile, minor peaks falling within the stutter peak criteria should be taken into consideration during the interpretation.

Under some circumstances, stutter peaks may exceed the maximum expected stutter percentages. These peaks may be called stutter at the analyst's discretion based on the specific circumstances.

Stutter peak calculations associated with off-scale allele peaks will not be reflective of the true percentage.

Stutter peaks between two alleles that differ by two repeats (e.g. 15 and 17 allelic peaks with a stutter peak at the 16 position) may exceed the maximum expected stutter percentages due to the n-4 contribution from the 17 allele and the n+4 contribution from the 15 allele.

Stutter peak percentages may increase at lower levels of input template DNA.

Matrix Failure (Spectral Pull-Up)

Matrix Failure, also known as spectral pull-up, results in peaks from one color "bleeding" into another color when the wavelength ranges emitted by the fluorescent tags overlap. The software is designed to use a matrix algorithm to separate the fluorescent signal given off by each of the fluorescent tags. This separation is not always complete resulting in minor peaks being detected in the adjacent spectral colors. These pull-up peak heights are typically less than 5% of the peak height of the source peak. The pull-up peak is usually sized within ±0.25 base pairs of the source peak.

If pull-up peaks are observed in a sample, the sample may be re-analyzed at the analyst's discretion based on the specific circumstances.

Raised Baseline

Raised baseline may be observed in samples containing excess template DNA or due to instrument failure. The raised baseline appears as non-specific elevation along the x-axis of the electropherogram.

If excess raised baseline is observed in a sample, the sample may be re-analyzed at the analyst's discretion.

Other Artifacts

Other peaks that do not represent genetic data from the template DNA may be observed in the sample electropherogram. These peaks or series of peaks may result from dissociated fluorescent dyes or other causes due to the instrument, capillary electrophoresis, or amplification kit. For example, a series of peaks is routinely observed between 90 and 100 base pairs in the VIC dye (green). If these artifacts appear within the analytical range (100 – 362 base pairs) and are excessive, the sample should be re-analyzed.

Off-Ladder Alleles

Most common alleles are represented by the allelic ladder or virtual bins set by the software. Occasionally, non-artifact peaks will be observed outside of the allele bins and will be labeled as "OL."

Off-ladder alleles may result from temperature fluctuations in the instrument's environment during the course of the electrophoresis. The change in temperature may cause a migration shift of allelic peaks that consequently fall outside the ±0.5 base pair bins even though the alleles are represented in the ladder.

Micro-variant and rare variant alleles may also be observed that fall between alleles within a locus or the range of alleles between two loci. Micro-variant and rare variant alleles shall be re-injected to confirm the sizing.

Off-ladder allelic peaks (OL) observed between alleles within a locus should be labeled as a variant of the smaller flanking allele.

If the OL peak is approximately one base pair longer than the smaller flanking allele (X), the OL peak will designated as X.1.

If the OL peak is approximately two base pairs longer than the smaller flanking allele, it will be designated as X.2.

If the OL peak is approximately three base pairs longer than the smaller flanking allele, it will be designated as X.3.

For example, an OL peak falling between the 8 and 9 alleles that is approximately two base pairs longer than the 8 allele would be designated as an 8.2.

An OL allelic peak that falls between the allele range of two loci first should be associated with one of the loci and then be labeled as described below.

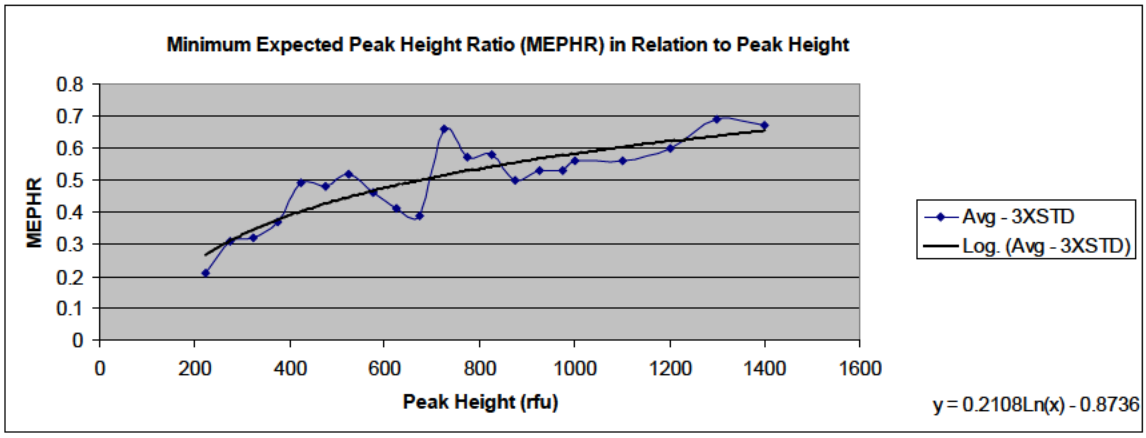
- To associate the OL peak with one of the flanking loci, the alleles present in the flanking loci along with the base pair sizes of the alleles need to be considered.
- If one of the flanking loci contains two alleles and the other locus contains one, the OL peak will be associated with the latter locus.
- If both loci contain one allele, the base pair size of the OL peak should be compared to the base pair sizes of the flanking allelic ladders. If the OL peak is a "perfect repeat" of one of the allelic ladders, it will be associated with that locus. If it is not a "perfect repeat" of either flanking allelic ladder, then both loci will be considered inconclusive and may only be used for exclusionary purposes.
- If the OL peak has been associated with a locus, it will then be designated as "<" or ">" the last allele of the associated allelic ladder.

Peak Height Imbalance

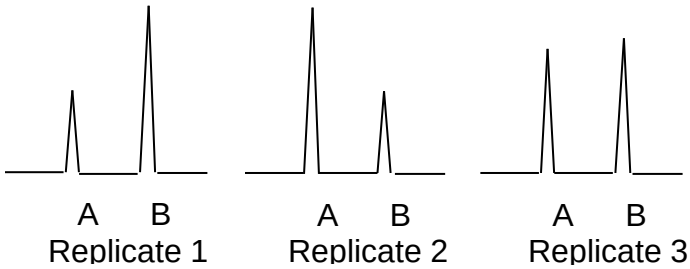
Expected Variation in Peak Height Balance

It is not unusual for PCR to produce imbalanced peak heights for heterozygous loci. As the amount of template DNA decreases, the imbalance may become more dramatic.

The following chart displays the minimum expected peak height balance within a locus for a single source sample. The ranges are based on the peak height of the allele with the greatest peak height. The balance is calculated as a percentage of the lesser peak height as compared to the greater peak height. The chart displays a smooth curve based on the average peak height balance minus three Standard Deviations. Therefore by definition, it would be expected that 99.7% of the heterozygous peaks examined would meet these expectations. This chart and the supporting data are contained in Appendix A. Alleles with peaks below 200 RFU would be have a minimum expected peak height ratio of <25% and could result in sister peaks below the analytical threshold. For alleles with peaks above 1,400 RFU, the minimum expected peak height ratio is 65%.



For heterozygous loci with alleles separated by relatively few repeats, the imbalance is not significantly affected by allele size. Hence, the imbalance will affect the larger allele as much as the smaller allele. Therefore, if a sample is amplified multiple times using the same template quantity, the imbalance may vary and may favor the larger allele one time and the smaller allele another time (see below).



For heterozygous loci with alleles separated by a larger number of repeats (such as the locus FGA), the imbalance may be greater than the expectations defined by the chart below and may favor the smaller allele. This imbalance will not be reproducible.

Peak Height Imbalance due to Primer Site Mutation

A sequence mutation in the primer region may have one of three effects. If the mutation causes significant destabilization, the allele will not be amplified and therefore not detected (null allele). If the mutation does not affect the stability of the primer binding, no effect will be observed. If, however, the mutation causes some destabilization of the primer binding but not to the extent to prevent amplification, the resulting peak balance at the locus may be outside the expected range. This form of imbalance is reproducible.

If the peak height imbalance falls outside the expected range at a single locus in an assumed single source profile, the sample can be re-amplified to attempt to reproduce the imbalance.

If the second amplification displays a similar peak imbalance, then the locus can be called.

If the second amplification produces peak balance within the expected range, the locus can be called.

If the second amplification does not demonstrate the presence of the second allele or if the samples cannot be re-amplified, the locus may be used for comparison purposes at the discretion of the analyst based on training and experience. Stochastic effects can cause imbalance outside of expectations, but so can a low level second contributor.

Expected Peak Height Balance in Mixed DNA Profiles

The above chart displays the Minimum Expected Peak Height Balance for single source samples. If a DNA profile is the result of DNA from multiple contributors, the additive effects of allele sharing must be considered (see Mixed Sample Interpretation section below for more details).

Composite Profiles

Combining data from multiple injections/amplifications resulting from a single DNA extract is called a composite profile. For example, if a sample is severely degraded, the initial amplification using 1ng of template DNA may have produced interpretable data at a few of the smaller molecular weight loci. However, when the same DNA extract was re-amplified using 2ng of template DNA, additional interpretable data was obtained from a few of the higher molecular weight loci. The smaller molecular weight loci in the second amplification are now not interpretable due to saturation. The data from the two amplifications can be combined into a single composite profile.

In some instances, it may be useful to re-inject or re-amplify a sample to obtain interpretable data for additional loci.

Composite profiles must be the product of data generated from a single DNA extract.

Data obtained from a second cutting from the same stained area cannot be combined with the data from the initial cutting of the stained area.

Interpretation/Statistical Calculation Documentation

An Interpretation/Statistical Calculation (ISC) worksheet shall be completed for each unique evidentiary DNA profile suitable for comparison.

The ISC documents the possible contributing genotypes to the observed profile, and calculates the random match probability.

This includes two or more DNA profiles that are consistent with each other but may be lacking data at one or more loci.

This ISC worksheet and accompanying information (if necessary) will document the interpretation of the DNA profile and results in a list of possible contributing genotypes and the Random Match Probability for the profile.

Major and minor components shall have separate ISC worksheets. If the minor component is not suitable for comparison purposes, it is not necessary to complete the ISC worksheet.

The ISC worksheet shall be completed prior to viewing the DNA profile from relevant reference DNA profiles.

- There will be some circumstances where this is not feasible (e.g. subsequent submissions, etc.).

Preliminary Evaluation of Sample DNA Profile

The entire DNA profile should be evaluated. Several conclusions can be drawn at this point.

Is the profile suitable for comparison purposes?

- If only a few alleles are detected across the entire profile and the peak heights are relatively low, the analyst may determine that the DNA profile is not suitable for comparison purposes. A specific number of alleles are not listed due to the wide variation in results that may be observed. For example, only two loci may demonstrate results with two alleles at each, all above the stochastic threshold, for a total of four alleles detected. This profile may be deemed interpretable. Another DNA profile may also have four alleles at four different loci, all below the stochastic threshold. This profile may be deemed uninterpretable.
- If the DNA profile is the result of four or more contributors and the peak heights are relatively low, the mixed DNA profile is not be suitable for comparison purposes.

Is the DNA profile consistent with resulting from a single contributor?

Is the DNA profile consistent with resulting from multiple contributors?

- Peaks below the analytical threshold may be used to aide in the determination of the number of contributors as it affects the subsequent interpretation at the analyst's discretion based on training and experience.
- The interpretation of an apparent single source profile with allelic peaks significantly above the stochastic threshold (e.g. 1000 RFU) will not be affected by one or more possible peaks below the analytical threshold.
- The interpretation of a low level apparent single or multiple source profile may be affected by the presence of possible allelic peaks just below the analytical threshold.

5.4 Single Contributor DNA Profile Interpretation

A DNA profile may be considered to be from a single source under the following conditions:

No more than two alleles are present at each locus (excluding stutter and other explainable artifacts).

- Tri-allele patterns are not common, but can be observed. If three alleles are observed at a single locus and all other loci display no more than two alleles, the possibility of a tri-allele pattern should be considered. However, it is also possible that the third allele is the product of a second low level contributor only detected at a single locus (especially at the low molecular weight loci).

Loci containing two allelic peaks meet the minimum expected peak height ratio.

Typically, peaks below the Analytical Threshold can be ignored. However, numerous peaks detected with the expected allelic peak morphology and falling within allelic bins just below the analytical threshold may be considered when interpreting a low level sample. While these peaks cannot be considered true allelic peaks, their presence may be an indication of additional contributors which could influence the interpretation of low levels samples.

A sufficient number of alleles and loci are detected from which a conclusion as to the number of contributors can be reliably drawn. For example, a partial DNA profile in which only three loci have one or two alleles above the analytical threshold is not sufficient to determine the number of contributors reliably. This determination will be made based on the analyst's training and experience.

- Information specific to the case and the item of evidence should be taken into account when making this determination. For example, a small blood stain collected from a sidewalk may be assumed to be from a single contributor. A swab from a doorknob in a public building will most likely contain DNA contributions from multiple individuals.

If a profile is determined to be from a single contributor, this assumption will be documented in the notes.

The possible contributing genotypes will be documented in the notes.

<u>Assumed Single Source – Two Alleles</u>

Loci containing two peaks above the stochastic threshold may be used for comparison and statistical purposes.

If the profile is of sufficient quality and the assumption has been made that it is the result of a single contributor, then loci containing two alleles with either one or both peaks between the analytical threshold and the stochastic threshold, may be used for comparison and statistical purposes.

This assumption will be noted in the report preceding any statistical analyses.

The two peaks detected at a locus for an assumed single source profile should fall within the minimum expected peak height balance range (See Appendix A).

If the peak height balance is outside the range, this could be an indication of a mixture or a primer binding site mutation. If this imbalance is observed at multiple loci, it is most likely the result of a mixture and the sample cannot be treated as a single source.

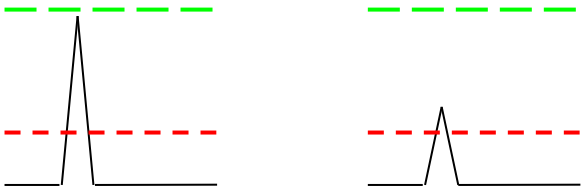
Assumed Single Source – One Allele

Loci containing one peak above the stochastic threshold may be used for comparison and statistical purposes.

If the profile is of sufficient quality and the assumption has been made that it is the result of a single contributor, loci containing one peak below the stochastic threshold but above the analytical threshold may be used for comparison and statistical purposes under the following conditions:

Due to the potential loss of allelic data due to drop-out of a sister allele to the allele detected, all genotypes associated with the detected allele must be considered a possible contributor to the DNA profile. For example, if the A allele is detected at locus X and the peak height is 120 RFU, all genotypes associated with the A allele must be considered a possible contributor to the DNA profile. If the alleles at locus X are A through E, then the possible contributing genotypes are AA, AB, AC, AD and AE.

The probability that drop-out occurred decreases as the peak height of the detected allele approaches the stochastic threshold. For example, the probability that the sister allele of an allele with a peak height of 190 RFU dropped-out is much less than the probability that the sister allele of an allele with a peak height of 53 RFU dropped-out. This should be taken into account when making the comparison of the evidentiary sample DNA profile to a known DNA profile. If multiple loci of an assumed single source profile demonstrate the presence of a single peak with a peak height of 195 RFU and the known DNA reference is heterozygous at each of these loci; the possibility of excluding the individual associated with the known DNA sample should be considered.



A
Probability of drop-out approaches zero

A
Probability of drop-out >>0

Comparison and Conclusions

Excluded

The individual associated with the known DNA sample cannot be a contributor to the evidentiary sample DNA profile.

If the reference sample profile contains a genotype not consistent with the possible genotypes documented as possible contributors for the evidentiary DNA profile, the individual associated with the reference sample is excluded as a possible contributor.

Not Excluded

The individual associated with the known DNA sample can be a contributor to the evidentiary sample.

If the reference sample genotypes are consistent with the genotypes observed at all loci present in the evidentiary sample, then the individual associated with the reference sample cannot be excluded as a possible contributor.

Profiles may also be deemed "not suitable for comparison or statistical purposes" due to limited results or the complexity of the mixture (e.g. four or more contributors).

If during the comparison process, the determination that a specific genotype which was previously determined not to meet expectations is changed, then the reason shall be sufficiently documented. This would only be expected to occur due to a rare event such as a primer site mutation or tri-allelic pattern.

If a Y allele peak is detected above the analytical threshold at the Amelogenin locus, even in the absence of the X allele, the presence of male DNA can be established.

Statistical Analysis of Assumed Single Contributor DNA Profiles

Values for p and q represent allele frequencies for the identified alleles at each locus and are obtained from reference population data. If the profile is assumed to be the result of a single contributor, loci with alleles that meet the interpretation expectations as described above may be used for statistical purposes.

A Random Match Probability shall be calculated using the appropriate software and the formulae listed below to estimate the rarity of the profile in the relevant populations.

Heterozygous locus:

$$\mathbf{2pq}$$ (*NRCII formula 4.1b*)

Homozygous locus, allelic peak above the stochastic threshold:

$$\mathbf{p^2 + p(1\text{-}p)\theta}$$

where $\theta = 0.01$ for most populations. A θ value of 0.03 may be used for small isolated populations (*NRCII formula 4.4a).*

Homozygous locus, allelic peak below the stochastic threshold:

$2p - p^2$ (*NRCII recommendation 4.1)*

- This formula is the sum of all genotype frequencies associated with the single allele detected and is equal to $a^2 + 2ab + 2ac + 2ad + 2ae$.

The Random Match Probability for the entire profile is the product of the Random Match Probabilities calculated for each locus.
See Appendix B for examples.

5.5 Multiple Contributor DNA Profile Interpretation

The general flow to mixture interpretation is listed below (Clayton, et al., 1998):

Identify the presence of a mixture
Designate the allele peaks
Identify the minimum number of contributors
Estimate the relative ratio of the individual contributors to the mixture
Consider all possible genotype combinations
Compare reference samples
Statistical analysis (if applicable)

Identify the presence of a mixture

A DNA profile may be considered to be from two or more sources under the following conditions:

Three or more alleles are present at one or more loci (excluding stutter and other explainable artifacts). Tri-allele patterns are not common but can be observed. If three alleles are observed at a single locus and all other loci display no more than two alleles, the possibility of a tri-allele pattern should be considered. However, it is also possible that the third allele is the product of a second low level contributor only detected at a single locus.

The peak balance at one or more loci fall significantly outside the expected range. A primer binding site mutation may result in a null allele or significant heterozygous peak imbalance. While this should be considered if only a single locus does not meet the expected peak height balance, primer mutations would not be expected to occur at multiple loci and the imbalance is reproducible.

Stutter peaks significantly exceed the expected range.

Designate allele peaks

Differentiate between allelic peaks and artifact peaks. See Artifacts section for description of potential artifact peaks.

Identify the minimum number of contributors

The minimum number of contributors to a mixed DNA profile can be estimated using the locus/loci demonstrating the largest number of alleles.

For example, if the maximum number of alleles at one locus is four, it can be inferred that the minimum number of contributors is two. It is possible in this case that more than two individuals contributed to the biological material, and no more than four alleles were detected at a locus due to homozygosity or allele sharing, but the minimum number of contributors is two. In addition, more than two individuals could have contributed to the biological material, but due to the ratio of contributions from each individual, only the genetic information from two of the individuals is being detected.

Largest # of alleles at a locus	Minimum # of contributors
2	1
3	2
4	2
5	3
6	3
7	4
8	4

Estimate the relative ratio of the individual contributors to the mixture

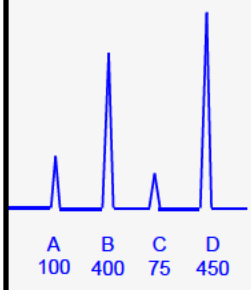
The ratio can be estimated by examining loci with the maximum number of alleles expected for the assumed number of contributors. For example, loci with four alleles would be used for a two person mixture.

The Mixture Ratio (M_r) can be calculated using the following formula:

$$M_r = \frac{PH(a) + PH(b)}{PH(a) + PH(b) + PH(c) + PH(d)}$$

Where PH(a) = Peak Height of Allele A, etc.

Example:



M_r Contributor 1 $\frac{100 + 75}{100 + 400 + 75 + 450} = 0.17$

M_r Contributor 2 $\frac{400 + 450}{100 + 400 + 75 + 450} = 0.83$

Ratio of Contributor 1 : Contributor 2 = 0.17 / 0.83 = 1:4.8

The major and minor components of a mixture may be distinguished if the average estimated ratio is at least 4:1 (M_r = 80%).

- In these cases, the major component can be treated as a separate profile.
- For a single source major component, the major component can be treated as described in the Single Contributor DNA Profile Interpretation section.
- For a multiple contributor major component, the major component can be treated as an indistinguishable mixture for those alleles associated with the major component.

Because the mixture ratio may vary from locus to locus across the profile outside of any influences such as differing states of degradation, the calculated mixture ratio is considered an estimate. In general, a range of the mixture ratio is ± 1 (e.g. the range for a 3:1 ratio becomes 2:1 to 4:1) for high RFU DNA profiles. This range may increase as the amount of template DNA decreases or the amount of degradation increases. An increase in the mixture ratio range may also be observed in mixtures with a large difference between the individual contributions (e.g. 15:1). It may be beneficial to look at the range of the mixture ratio across the entire profile to estimate the range to be used during interpretation.

- For example, if the mixture ratio range across the profile varies from 3:1 to 7:1 and the genotype combination being considered would result in a 15:1 ratio, the combination should be excluded. If the genotype combination would result in an 8:1 mixture ratio, then it may be more conservative to include it as a possibility.
- It may also be necessary to take into account other factors, such as stutter, when making determinations based on mixture ratio.
    - § For example, if a minor component allele is in the stutter position of the major component allele, a portion of the minor component's peak height could be attributed to stutter. This would falsely increase its peak height and lower the mixture ratio.

The M_r may change across the entire profile due to differing states of degradation of the DNA contributions in the original sample. For example, if fresh blood was deposited on the handle of a knife on top of older, more degraded biological material, the M_r of the smaller loci may be significantly different than those of the larger loci where the effects of the degradation are more apparent.

or

If the average mixture ratio for the stronger component is less than 80% (i.e. 4:1), the mixture must be treated like an indistinguishable mixture.

Consider all possible genotype combinations

Each locus should be evaluated individually at this step. All possible genotype combinations should be examined to determine if they meet the interpretation expectations. During this step, the analyst may use the assumptions from *Steps 3 and 4* (the number of contributors and the estimated mixture ratio, respectively) to derive the peak height balance expectations.

The possibility of allele drop-out must be taken into consideration during the interpretation step if an allele or alleles fall below the stochastic threshold.

It is important to note that just because all alleles detected exceed the stochastic threshold does not implicitly mean that there is no potential allele drop-out associated with one of the alleles. The additive effects of a shared allele may cause the peak height of one allele of a heterozygous pair to exceed the stochastic threshold even though the sister allele was not detected. If this is the case, then the possibility of allele drop-out must be a consideration during the interpretation step.

See the table below for the genotype combinations for a two person mixture given the number of alleles detected.

Two Person Mixture Genotype Combinations*								
Four alleles (a,b,c,d)		**Three alleles (a,b,c)**		**Two alleles (a,b)**		**One allele (a)**	
a,b	c,d	a,a	b,c	a,a	a,b	a,a	a,a
a,c	b,d	b,b	a,c	a,b	a,b	a,a	a,x
a,d	b,c	c,c	a,b	a,a	b,b	a,x	a,x
		a,b	a,c	a,b	b,b		
		b,c	a,c	a,x	b,b		
		a,b	b,c	a,x	a,b		
		a,x	b,c	b,x	a,a		
		b,x	a,c	b,x	a,b		
		c,x	a,b	a,x	b,x		

**Modified from Clayton, et al., 1998 x = drop-out of sister allele of heterozygous pair*

Due to inhibition, degradation or other effects on the detection of the alleles, it is possible that at least a portion of the contributor's genotypes is not detected at all loci. As described on page 29, differing states of degradation between the two components of a mixture may cause the apparent contribution of one of the components to decrease as the loci increase in molecular weight. If it can be reasonably assumed that this has occurred and it has progressed to the point at which the component's genotype is completely undetected, then this observation will be documented in the analyst's notes and no genotype can be excluded as a possible contributor to the results at this locus.

If the minor contributor's contribution to a mixed DNA profile results in peaks similar in height to the stutter peaks, the stutter peaks must be considered potential true alleles even if the analysis software has filtered the peaks out. Assumptions about the mixture (number of contributors, mixture ratio, etc.) can be used to logically discount the incorporation of peaks in the stutter position as true alleles in the interpretation.

> For example, in an assumed two person mixture where four alleles are displayed at a locus, it is not necessary to consider peaks in the stutter position as possible true alleles.

The genotype combinations that do or do not meet expectations and the assumptions used to make the determinations must be documented in the analyst's notes.

These conclusions must be drawn prior to any comparison to reference sample profiles where possible. Exceptions to this include the submission of additional evidence subsequent to the analysis of the original evidentiary and reference samples.

The genotype combinations associated with three person mixtures can be much more complex, especially if four or fewer alleles are detected. In these cases, it is important to ensure that at least a portion of the genetic information from each of the three contributors is represented and the potential for allele drop-out associated with each of the alleles is examined.

It may not always be possible to draw reliable conclusions for mixtures containing four or more individuals due to their complexity.

For examples of interpretation, see Appendix C.

Compare reference samples

If a reference sample is available, the reference sample profile can be compared to the genotypes documented as possible contributors to the mixed DNA profile from the evidence sample.

Excluded

The individual associated with the known DNA sample cannot be a contributor to the evidentiary sample.

If the reference sample profile contains a genotype not consistent with the possible genotypes documented as possible contributors for the evidentiary DNA profile, the individual associated with the reference sample is excluded as a possible contributor.

Not Excluded

The individual associated with the known DNA sample can be a contributor to the evidentiary sample.

If the reference sample genotypes are included as possible contributing genotypes (previously determined) at all loci present in the evidentiary sample, then the individual

associated with the reference sample cannot be excluded as a possible contributor to the mixed DNA profile and the appropriate statistical calculation can be performed.

In lower level mixtures, it is sometimes necessary to consider the possibility of allele drop-out to explain the inclusion of a genotype as a potential contributor to the mixture when only one of the known profile's heterozygous pair of alleles is present. The probability that drop-out occurred decreases as the peak height of the detected allele approaches the stochastic threshold. For example, the probability that the sister allele of an allele with a peak height of 190 RFU dropped-out is much less than the probability that the sister allele of an allele with a peak height of 53 RFU. This should be taken into account when making the comparison of the evidentiary sample DNA profile to a known DNA profile. If the occurrence of allele drop-out is necessary to accommodate the inclusion of an individual associated with a known DNA profile at multiple loci where the detected allelic peak approaches the stochastic threshold, the possibility of excluding the individual associated with the known DNA sample should be considered.

Profiles deemed "not suitable for comparison or statistical purposes" due to limited results or the complexity of the mixture (e.g. four or more contributors) will not be compared to reference samples.

If during the comparison process, the determination that a specific genotype which was previously determined not to meet expectations is changed, then the reason shall be sufficiently documented. This would only be expected to occur in complex mixtures or due to a rare event such as a primer site mutation or tri-allelic pattern.

If a Y allele peak is detected above the analytical threshold at the Amelogenin locus, even in the absence of the X allele, the presence of male DNA can be established.

If a mixed DNA profile has been obtained from an intimate sample, the known contributor may be subtracted out by comparing the mixed DNA profile to the known DNA profile. Examples of intimate samples include but are not limited to vaginal swabs and breast swabs.

In these cases, depending on the remaining alleles, the remaining profile may be treated as a single source profile or a minor component of a distinguishable mixture if the potential for allele masking exists.

Statistical Analysis of Multiple Contributor DNA Profiles

The assumed number of contributors used during the interpretation and statistical calculation steps shall be noted in the laboratory report preceding the results of the statistical analysis.

Distinguishable Components of a Mixed DNA Profile

Major Component of a Distinguishable Mixed DNA Profile
A Random Match Probability shall be calculated using the appropriate software and the formulae as described in the Statistical Analysis of an Assumed Single Contributor DNA Profile section.

Minor Component of a Distinguishable Mixed DNA Profile
The statistical analysis of the minor component of a distinguishable mixture shall be performed in a similar manner as the statistical analysis of an indistinguishable mixed DNA profile.

All alleles detected shall be considered in the statistical analysis of the minor component, not just the minor alleles. Other alleles associated with the minor component may be masked by the major component alleles.

Genotypes excluded during the interpretation step may be excluded in the statistical analysis.

See Appendix C for examples of statistical analyses of mixed DNA profiles.

Indistinguishable Components of a Mixed DNA profile
The statistical analysis of a mixed DNA profile with indistinguishable components shall be conducted using the Random Match Probability.

The Random Match Probability at a specific locus is the sum of all the genotype frequencies documented as possible contributors to the mixed DNA profile.

The frequency of each genotype shall be calculated using the appropriate formulae listed below. Values for p and q represent allele frequencies for the identified alleles at each locus and are obtained from reference population data.

Heterozygous genotype:

2pq (*NRCII formula 4.1b*)

Single allelic peak, no zygotic ambiguity (i.e. no potential allele drop-out associated with the allele) as determined during the interpretation step:

$\mathbf{p^2 + p(1-p)\theta}$ (*NRCII formula 4.4a)*

where $\theta = 0.01$ for most populations. A θ value of 0.03 may be used for small isolated populations.

Single allelic peak, zygotic ambiguity exists (i.e. potential allele drop-out associate with the allele) as determined during the interpretation step:

$\mathbf{2p - p^2}$ (*NRCII formula 4.1)*

This formula is the sum of all genotype frequencies associated with the single allele detected and is equal to $a^2 + 2ab + 2ac + 2ad + 2ae$.

Due to the conservative nature of the calculations used, it is possible to obtain a Random Match Probability greater than 1. In these cases, the Random Match Probability assigned to the locus will be 1.

The Random Match Probability for a two-person mixture at loci displaying one, two, or three alleles where there is no indication of potential allele drop-out and no genotype combinations can be excluded based on peak height ratio or mixture ratio expectations is identical to the Combined Probability of Inclusion (CPI).

Random Match Probability is the square of the sum of the allele frequencies of the alleles detected.

Given the following results for Locus X, where A, B, and C are the alleles detected and a, b, and c are the corresponding allele frequencies:

$$\text{Random Match Probability} = (a + b + c)^2$$

The Random Match Probability for a two-person mixture at a given locus displaying four alleles is similar to that described immediately above, except the homozygous frequencies may be subtracted out.

$$\text{Random Match Probability} = (a + b + c + d)^2 - a^2 - b^2 - c^2 - d^2$$

Random Match Probability calculations for three person mixtures are more complex.

At lower levels, it is more conservative to treat each allele as if the potential exists for allele drop-out associated with each allele.

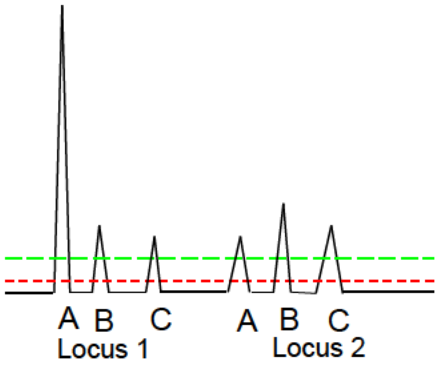
Allele drop-out does not need to be considered at loci displaying six alleles in an assumed three person mixture.

Loci at which possible complete drop-out of a component's genetic information has occurred shall be given a value of "1" (i.e. no genotype is excluded as a possible contributor). This determination will be made during the interpretation step.

The Random Match Probability for the entire profile is the product of the Random Match Probabilities calculated for each locus.

If the Mixture Ratio (M_r) of the components of the mixture vary significantly across the profile in a defined pattern (e.g. one component is more degraded than the other), then the analyst may choose one the following ways to interpret the mixture:

- A Random Match Probability for the distinguishable major component may be calculated at the loci of similar molecular weight which exceed the 4:1 average mixture ratio.
- The entire profile can be considered an indistinguishable mixture and the statistical calculation performed accordingly.
- A combination of the two methods list above may be employed (see example below). In this example, the major contributor at Locus 1 is an AA. At Locus 2, the mixture is indistinguishable, but the major contributor must still be one of the possible contributing genotypes at this locus (AA, AB, AC, BB, BC, or CC).



- The most discriminating profile shall be reported.

See Appendix C for examples of statistical analyses of mixed DNA profiles.

For three person mixture interpretation, see Appendix D.

Unique Statistical Situations

Use of Non-Standard Populations

In some instances, it may be desirable to use a population database that more accurately reflects the region of interest or person(s) of interest. In these cases, the analyst may

select an appropriate population database from a peer-reviewed journal to perform the statistical calculations in addition to the standard population databases described above.

Statistical Calculations for Familial Relationships

Statistical calculations for familial relationships shall be performed by an external qualified expert.

Body Identification and Criminal Paternity Interpretation

The ATF Laboratory may be requested to aid in the identification of a body or criminal paternity through DNA analysis. In these instances, the following guidelines should be followed.

The reference profiles shall be evaluated by the criteria described above. These profiles shall be provided to an external qualified expert to perform comparisons, draw conclusions and, if appropriate, perform the statistical analysis.

Staff Contamination

During the evaluation and comparison of the evidence, reference, and control DNA profiles, it may be determined that contamination from a "staff" member was detected. "Staff" includes laboratory personnel and some visitors, other ATF personnel, and other members of law enforcement.

If the contamination occurred in one of the control samples (reagent blank, amplification positive control, or amplification negative control), the contamination shall be treated in the manner previously described.

If a "staff" profile is detected on an item of evidence, the report shall make note of it, however the staff member name shall not appear in the report. If the DNA profile is a mixture and a portion of the mixture is consistent with a staff member, the remaining portion may be reported following the normal process described above. For example, an ATF agent may be detected on a weapon recovered during an arrest or a member of the laboratory staff may be detected on an exhibit that had been analyzed in the past. See ATF-LS-FB01 "Standard Approach to Forensic Biology Examination" for information on handling a "staff" contamination event.

If there is information that the evidence was not handled properly and the integrity of the DNA results may be affected, a cautionary statement will be added to the note describing the staff contamination event in the report. If the circumstances are sufficiently significant, the evidentiary profile(s) shall be determined to be not suitable for comparison purposes.

Comparison to Reference DNA Profiles Generated by Another Laboratory

In some instances, it may be necessary to compare an evidentiary DNA profile to a reference DNA profile generated by another laboratory. For example, the individual is deceased and there is no reasonable method to obtain a direct sample.

In these cases, a laboratory report containing the allele table for the reference DNA profile that has undergone a technical and administrative review or the allele table from the bench notes that has undergone a technical and administrative review can be used for comparison purposes. This information will be maintained in the case notes and a note shall be made in the report describing the source of the information.

Multiple DNA Profiles from the Same Exhibit

A statistical calculation to lend weight to a comparison where an individual cannot be excluded must be performed for each DNA profile, even when multiple consistent profiles that differ slightly due to allele drop-out, etc. are obtained from the same exhibit of evidence.

The statistical weight does not have to be calculated for items taken directly from an individual that are consistent with that individual (intimate samples such as fingernail scrapings, vaginal swabs, etc.).

If multiple DNA profiles are obtained from the same exhibit, but each has a different statistical weight, the group of profiles may be reported together using the lowest statistical weight to describe the entire group. See Report Wording Guidelines.

Documentation

Data Interpretation Sheet

A Data Interpretation Sheet shall be completed for each sample analyzed.

The Data Interpretation Sheet includes exhibit number, description of evidence, assumptions made during interpretation, brief summary of interpretation of profile (e.g. number of contributors in a mixture, completeness of profile, etc.), and the result of comparison, if applicable.

Allele Summary Table

An Allele Summary Table listing the alleles detected in the DNA profile following the guidelines listed below shall be included in the case notes for reference DNA profiles (this includes secondary references). It is not necessary to complete an ISC worksheet for reference samples. The allele table can then be used to compare the reference DNA

profiles to the list of documented possible contributing genotypes for the evidentiary samples.

- Each peak above the analytical threshold at a locus shall be listed in the table.
- Alleles at heterozygous loci shall be listed in ascending order (e.g. 15,20).
- The allele detected at a homozygous locus will be listed as a genotype (e.g. 15,15).
- If a single peak is detected at a locus and the allele's peak height is less than 200 RFU, the results for the locus shall be designated as "A,---" where A is the single allele.

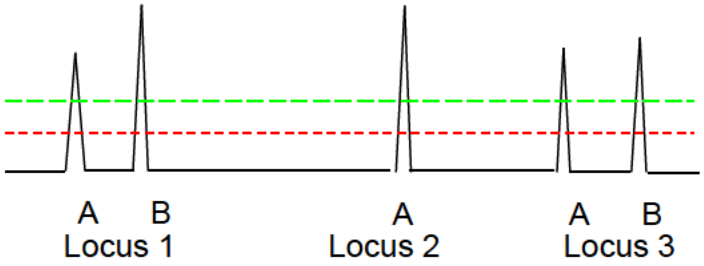
Appendix A

Appendix A is a separate Excel workbook that can be found in the Forensic Biology Worksheets folder on Qualtrax (ATF-LS-FB17 Appendix A).

Appendix B
Examples of Interpretation and Statistical Analyses of Assumed Single Contributor DNA Profiles

Alleles denoted as A and/or B
Allele frequencies denoted as a and/or b
Analytical Threshold: ----------
Stochastic Threshold: – – – – – –

Example B1



No allelic drop-out possible at any of the three loci

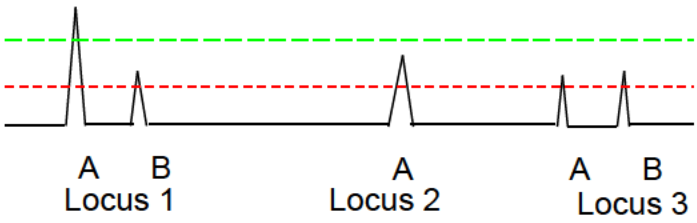
Genotypes included:

Locus 1: A,B
Locus 2: A,A
Locus 3: A,B

Statistical analysis:

Locus 1: 2ab
Locus 2: $a^2 + a(1-a)\theta$
Locus 3: 2ab

Example B2



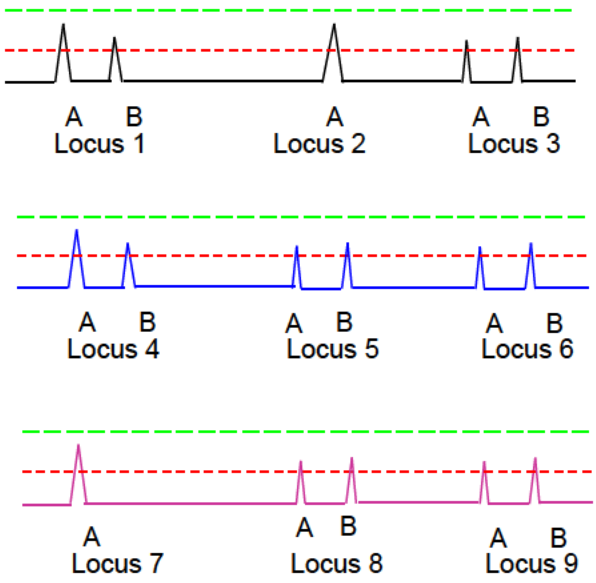
Allelic drop-out possible at Locus 2 only. Under the assumption that this is a single source profile, allele drop-out is not expected at loci displaying two alleles.
Genotypes included:

Locus 1: A,B
Locus 2: A,X (where X = any allele associated with allele A including A,A)
Locus 3: A,B

Statistical analysis:

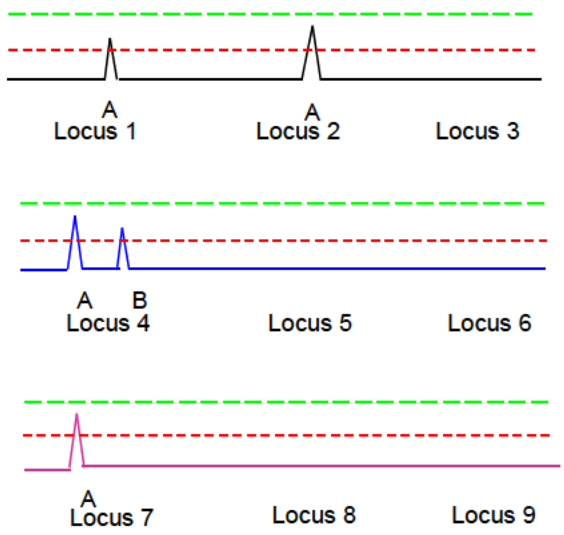
Locus 1: 2ab
Locus 2: $2a - a^2$
Locus 3: 2ab

Example B3



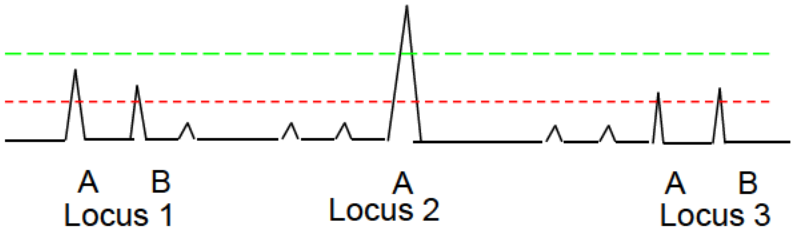
This DNA profile can be assumed to be a single source profile and can be used for matching and statistical purposes as described in Examples 1 and 2 even though none of the loci contain an allele exceeding the stochastic threshold. Sufficient data is present to reasonably make the assumption of a single contributor.

Example B4



While allelic peaks are detected in this DNA profile, there is not enough information to reasonably assume the number of contributors. If the number of contributors cannot be reasonable assumed, then the analyst cannot determine if all components are at least partially represented at a locus and therefore, each locus would be given a value of one (no genotype excluded). This profile may or may not be considered suitable for comparison purposes based on the context of the sample (e.g. drop of blood compared to a swabbing of gun grips).

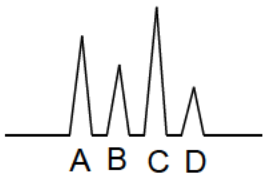
Example B5



In this case, the maximum number of alleles detected at a locus is two. However, a pattern of peaks below the analytical threshold can be seen. Assuming these peaks cannot be attributed to a form of artifact, have the expected allelic peak morphology, and fall within an allelic bin, the possibility of a mixed DNA profile should be considered during the interpretation. If the peaks detected are significantly greater than the stochastic threshold (e.g. 1000 RFU), the possible minor contribution below the analytical threshold does not need to be considered. If however, the detected alleles are closer to the stochastic threshold or even analytical threshold, the presence of the peaks below the analytical threshold may affect the interpretation of the DNA profile.

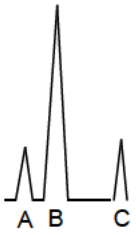
Appendix C
Examples of Interpretation and Statistical Analyses of Assumed Multiple Contributor DNA Profiles

Example C1



Assume the overall DNA profile is consistent with a two person mixture and the peak heights for the alleles are as follows: allele A (900 RFU), allele B (600 RFU), allele C (1000 RFU) and allele D (300 RFU). Since four peaks are present, the locus is a mixture of two heterozygous genotypes. Based on these assumptions, the A,D (Peak Height Ratio of 33%) and C,D genotypes (Peak Height Ratio of 30%) do not meet the Minimum Expected Peak Height Ratio expectations and are therefore excluded as possible genotypes. Because four alleles were detected, the potential for allele drop-out does not exist. This leaves the A,C and B,D genotypes that could be possible contributors.

Example C2



Assume the overall DNA profile is consistent with a two person mixture at an average mixture ratio of 2:1 and the peak heights of the alleles are as follows: allele A (500 RFU), allele B (2000 RFU) and allele C (500 RFU).

Genotype Combinations Examined:

Three alleles (a,b,c)		Possible?
a,a	b,c	No
b,b	a,c	Yes
c,c	a,b	No
a,b	a,c	No
b,c	a,c	No
a,b	b,c	No
a,x	b,c	No
b,x	a,c	No
c,x	a,b	No

The following genotypes are excluded because the remaining genotype does not meet the Minimum Expected Peak Height Ratio (MEPHR) based on the listed assumptions: AA, AX, CC, and CX. In these cases, the remaining genotype (B,C or A,B) falls outside the MEPHR.

The genotype combination A,B with a B,C also does not fall within the expected parameters. The maximum expected peak height associated with a peak at 500 RFU is approximately 895 RFU. Even at a 1:1 ration where both genotypes have the 500 RFU to 895 RFU ratio, the combined RFU for the B allele does not equal 2000 RFU.

The 2:1 ratio allows for the combination of an A,C mixed with a B,B. The average mixture ratio is assumed to be 2:1, which provides a range of 1:1 and 3:1. Because three alleles were detected and all exceed the stochastic threshold, no potential for allele drop-out exists.

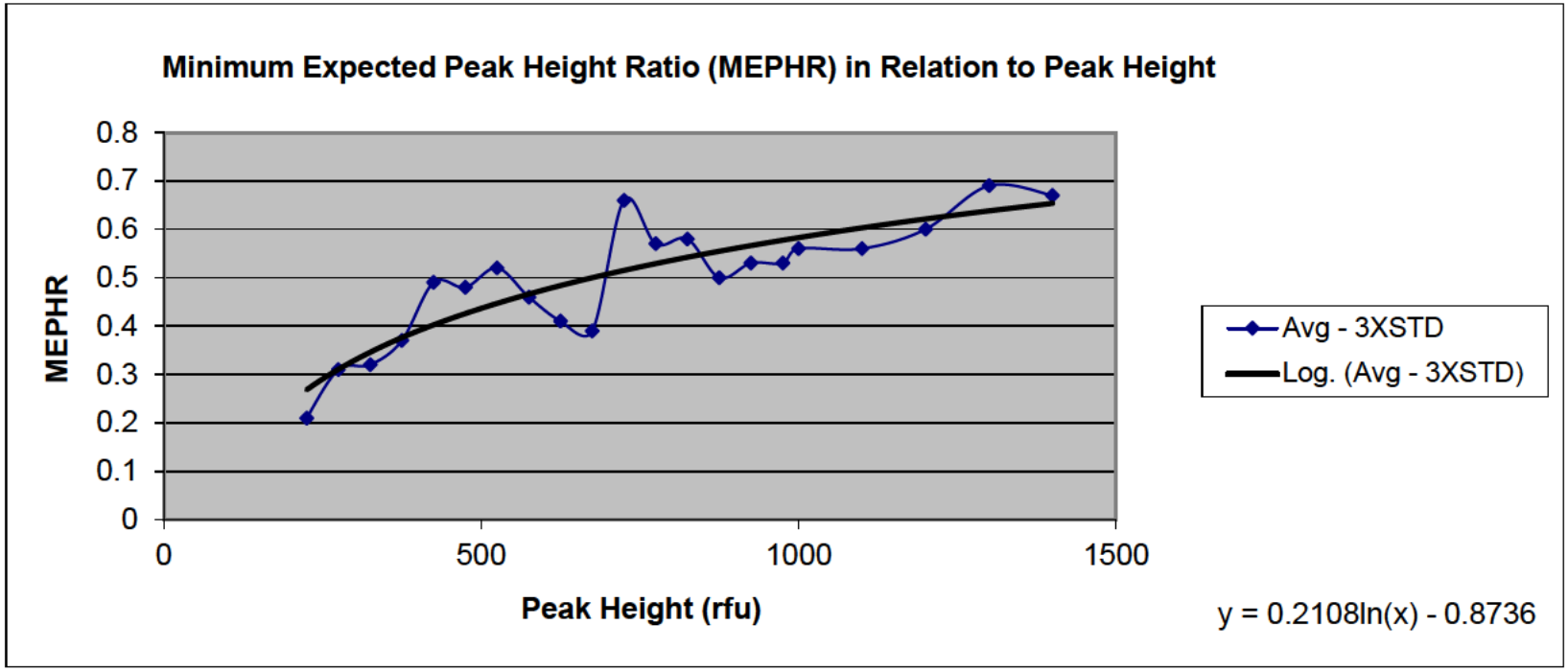
The statistical calculation is then the sum of the remaining genotype frequencies:

$$B,B = b^2 + b(1-b)\theta$$

$$A,C = 2ac$$

Appendix D

Appendix D is a separate PowerPoint document that can be found in the Forensic Biology Methods of Analysis (ATF-LS-FB17.1 Appendix D).



RFU of Peak	x =	5000
MEPHR	y =	65%
Minimum Expected Pk. Ht. of Sister Allele		3250

Peaks > 1400 rfu: MEPHR = 65%
Peaks <200 rfu: MEPH is < Analytical Threshold

ATF-LS-FB17.1 Appendix D – Three Person Mixture Interpretation Guide

3 Person Mixture Interpretation

- The following slides outline the interpretation of assumed three person mixtures.
- Mixtures with evidence of four or more contributors are not suitable for interpretation except in instances where the "minor" component(s) are degraded.
- In all cases, assuming a mixture is the result of the combination of three individuals must be done with caution. For example, approximately 70% of four person mixtures present as two or three person mixtures (based solely on number of alleles present).
- Sufficient data must be present to reasonably draw the conclusion that only three individuals contributed to the mixture. This conclusion is based on the number of alleles, peak height ratios, case information, origin of sample, sub-analytical threshold peaks, etc.
- The possible presence of first degree relatives contributing to the mixture can make the determination that there are three contributors to a mixture more difficult.

Documentation of interpretation

- Interpretation shall be documented on the electropherogram including the following information:
  - Single source or mixture
  - Number of assumed contributors if determined to be a mixture
    - At least two individuals, at least three individuals, etc.
  - Presence of male component
  - "major" component distinguishable, if applicable
  - Suitability for comparison purposes
    - Including "major" and "minor" components, if applicable
  - If profile is not suitable for comparison purposes, provide reason (e.g. lack of sufficient data, complexity of mixture)

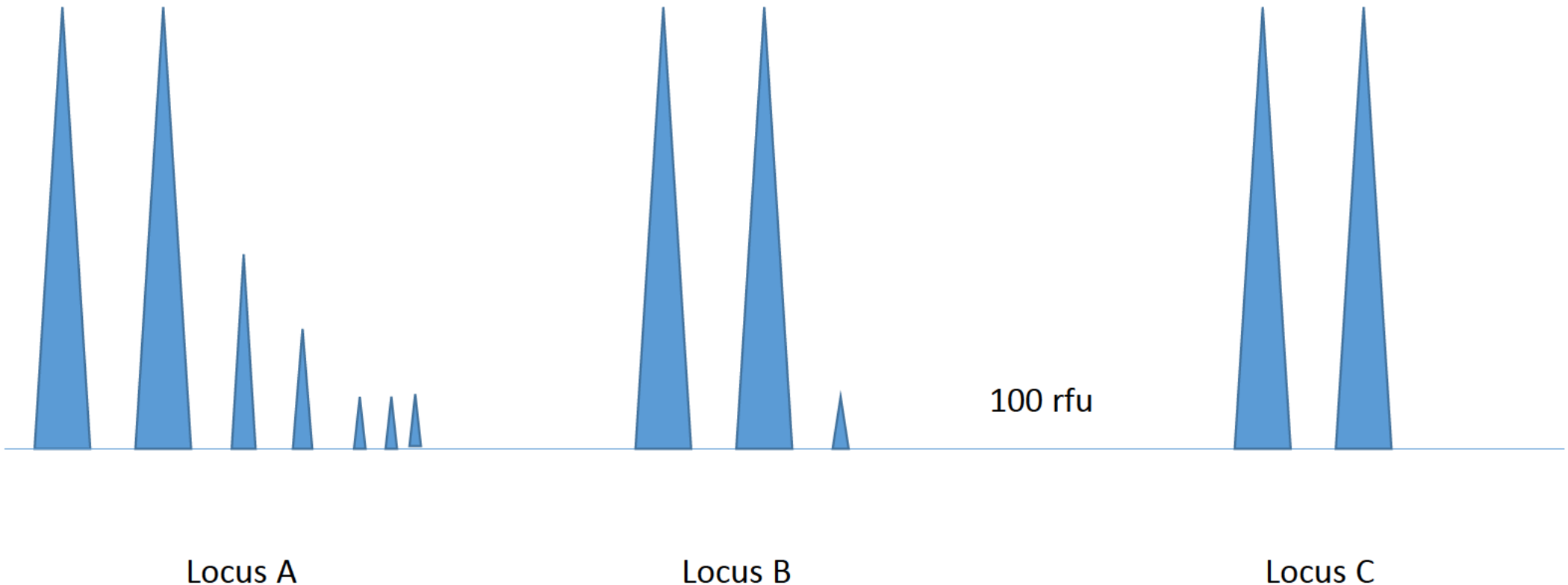
3 Person Mixture Interpretation

- Indistinguishable mixtures: unrestricted RMP calculations can be performed on loci where it can be reasonably assumed that no allele drop-out has occurred
  - Possible allele sharing, mixture ratio and degradation states should be considered when making this determination

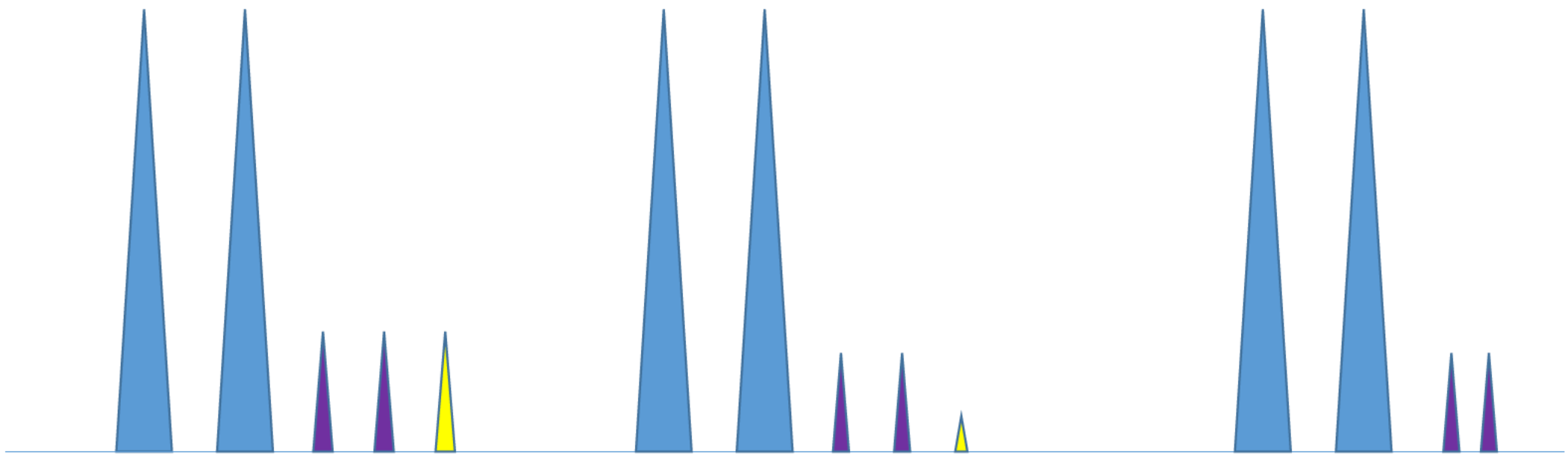
Greater sensitivity and degradation

- If it is apparent that the "minor" component is degraded, loci will be interpreted based on the number of remaining contributors. Due to the sensitivity of the smaller loci, evidence of the mixture may only be observed at these loci. One or more of the "minor" contributors may not be observed in the remaining loci and therefore, can be interpreted as mixtures based on the assumed number of remaining "minor" contributors due to degradation and decreased sensitivity at the larger loci.
- For example, evidence of a four person mixture may be observed at D8S1179, but there is only evidence of a three person mixture for the remaining loci. D8S1179 will be determined to be not suitable for comparison, but the remaining loci can be interpreted based on the guidelines detailed below. This should be done with caution.

Degraded "minor" component(s)



Degraded "minor" component(s)



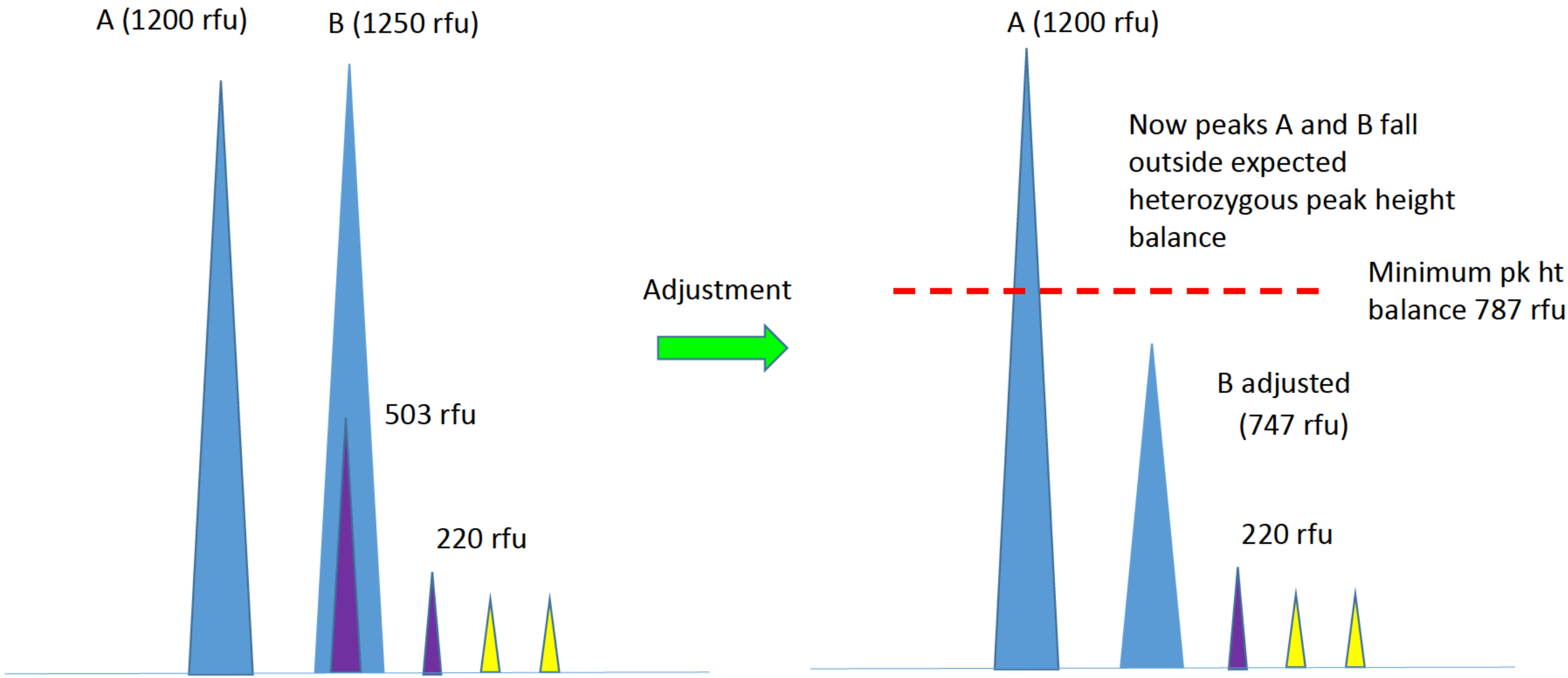
3 Person Mixture Interpretation

- Estimates for the overall mixture ratios for the three contributors can be estimated from loci with 5 and 6 alleles observed.
  - The mixture ratio for loci with 5 alleles observed can be estimated assuming the sister allele for one of the "minor" alleles is masked by the "major". Therefore, the highest peak height for a sister allele associated with the highest "minor" allele shall be calculated. This value shall be subtracted from the sum of the peak height(s) of the "major" component.

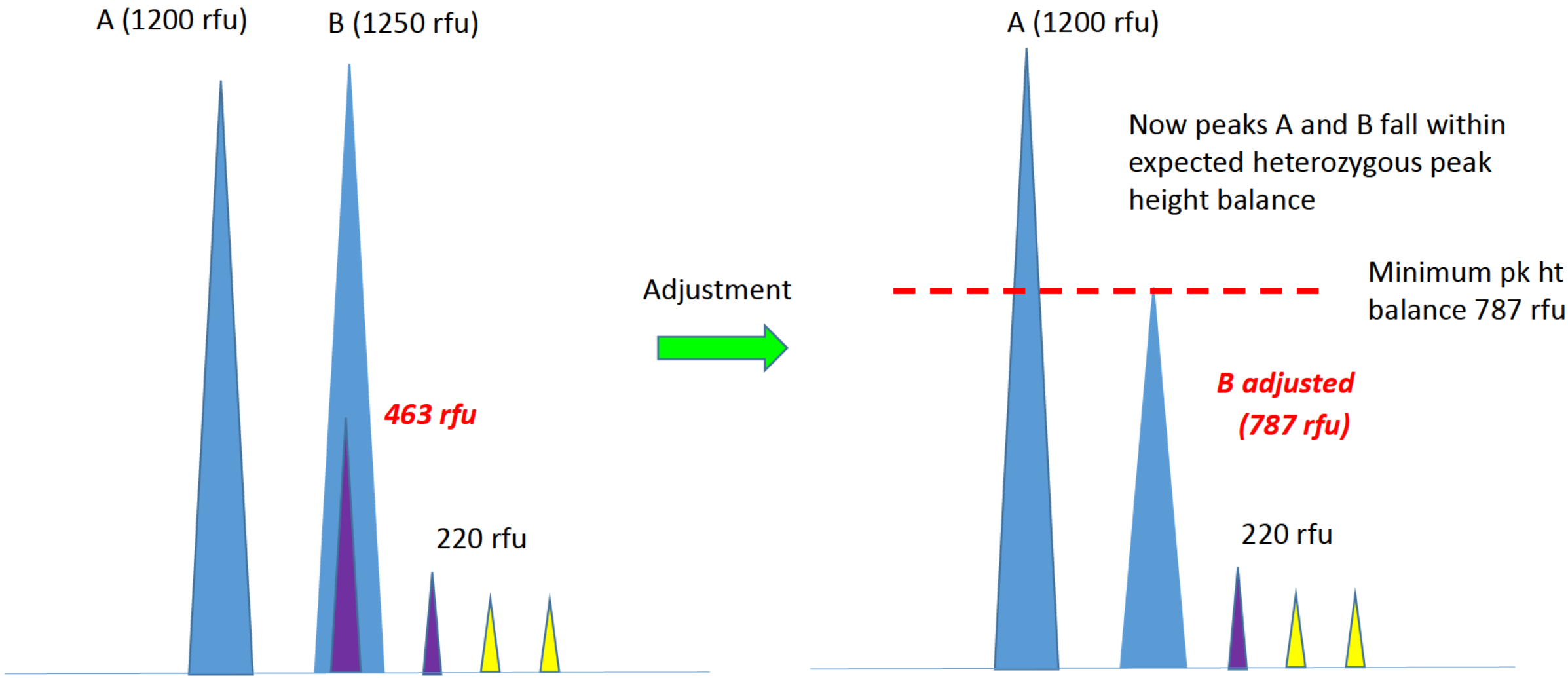
Adjusting “Major” component Total RFUs

- During the interpretation of three person mixtures and determining if a "major" component is distinguishable, some scenarios necessitate the adjustment of the total rfus of the “major” component.
- The total rfus of the "major" component is adjusted by subtracting out the possible contribution of overlapping “minor” alleles.
- Typically, the greatest possible contribution of the masked allele(s) will be subtracted.
- However, the amount subtracted cannot result in a pair of “major” component peaks that are outside of the expected heterozygous balance.
- In these cases, the maximum rfu contribution will be subtracted that still allows the “major” heterozygous pair to fall within the expected heterozygous balance. The subtraction will be taken from the highest of the two “major” peaks.

Adjusting “Major” component Total RFUs



Adjusting “Major” component Total RFUs

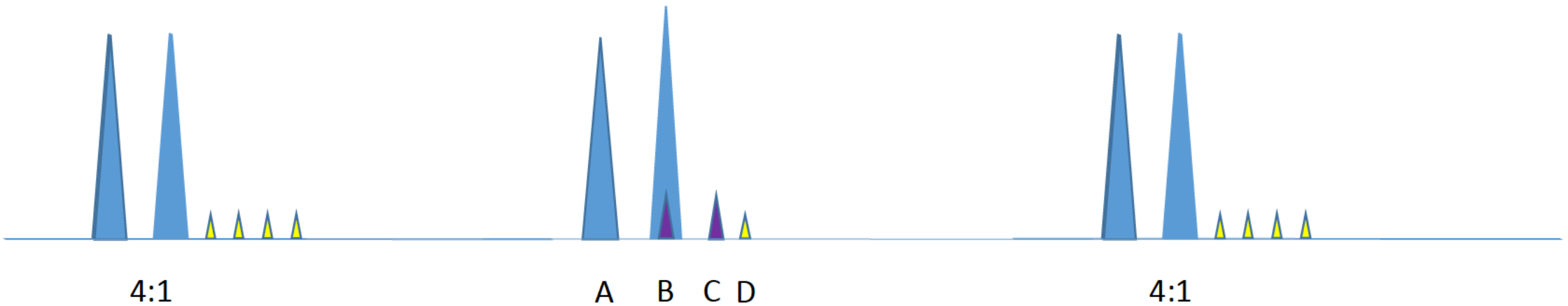


Determining if a "major" Component can be Distinguished

Determining if a “major” component can be distinguished

- As with all DNA profiles, it is critical to examine the entire profile during the interpretation. In some instances, the DNA profile observed provides sufficient data to clearly determine the presence of a "major" component. The "major" component may be single source or a mixture of two individuals. This can be determined if there are multiple loci with five or six alleles present across the size range being interpreted that demonstrate a mixture ratio of at least 4:1. The other loci within this size range can be used for comparison purposes if an assumption of an equal contribution from the tallest "minor" allele to the highest “major” allele still provides a 4:1 ratio.
- This should be done with caution and after considering possible degradation, allele sharing, locus to locus amplification efficiency variation and stutter contribution. For example, the D13 locus has demonstrated reduced amplification efficiency compared to the immediately adjacent loci and may demonstrate a different mixture ratio. In contrast, smaller loci such as D8 and D3 typically demonstrate greater amplification efficiency and thus greater sensitivity.
- Obviously, as the mixture ratio increases, the determination of the "major" component becomes more clear and loci with a smaller number of detected alleles can be used for comparison purposes.
- In situations where the mixture ratio is less clear due to degradation of one or more contributor’s DNA, inhibition, low levels of template DNA, or insufficient loci with five or six alleles, it is recommended to use the greatest possible peak height for the possible contribution of a shared sister allele (see below). However, this is not a reasonable assumption when the mixture ratio is more defined and the adjustment assuming the greatest possible contribution results in a mixture ratio significantly outside of the observed range.

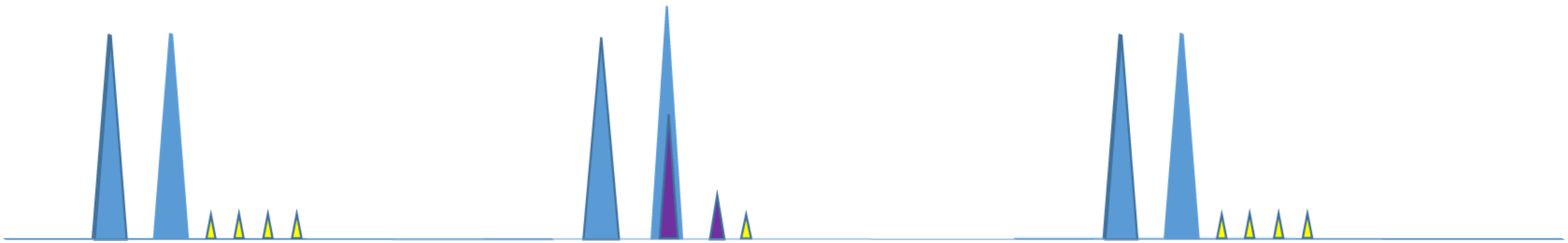
Determining if a “major” component can be distinguished



(A + (B-C)) > or = 4 * (2 *C)

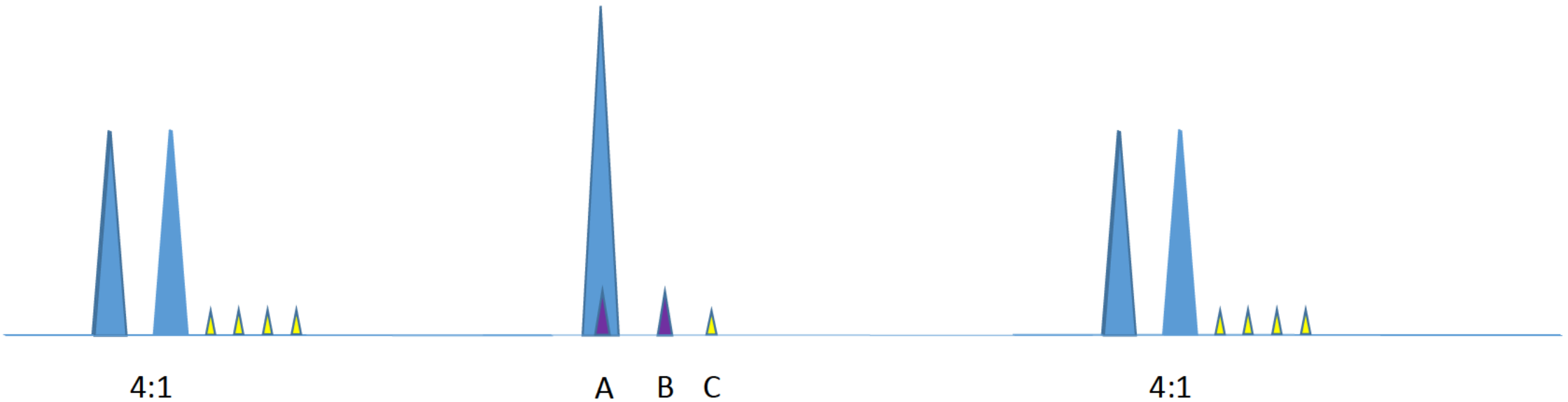
Assuming equal contribution of possible C sister allele to B

Determining if a “major” component can be distinguished



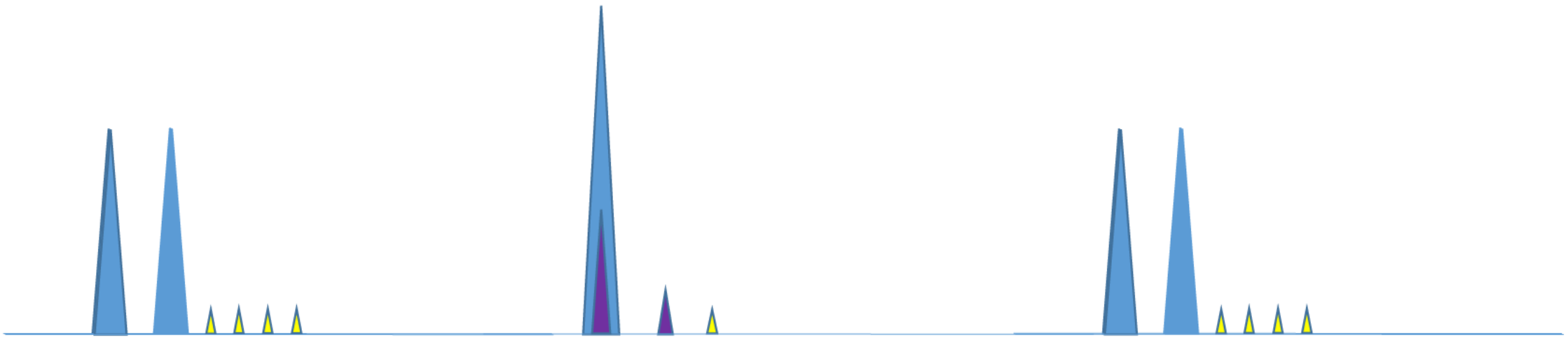
Assuming greatest possible contribution of possible C sister allele to B would result in the inability to determine a "major" component at this locus. ***This is not a reasonable assumption*** considering the entire profile.

Determining if a “major” component can be distinguished



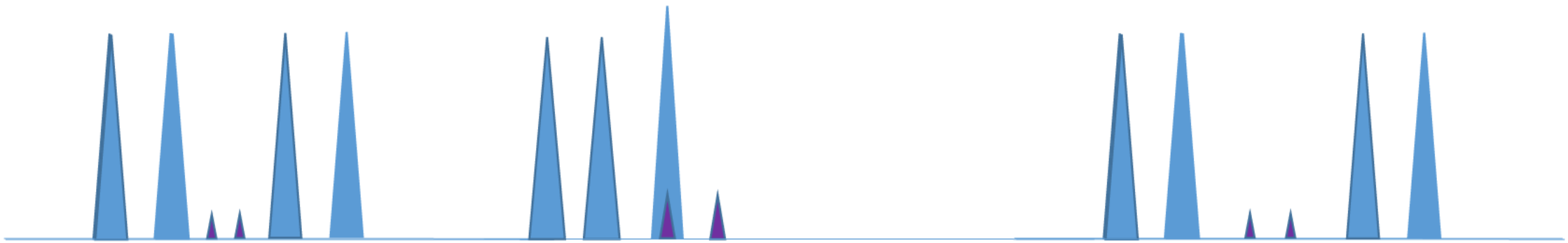
(A -B) > or = 4 * (2 *B)

Determining if a “major” component can be distinguished



Assuming greatest possible contribution of possible B sister allele to A would result in the inability to determine a "major" component at this locus. ***This is not a reasonable assumption*** considering the entire profile.

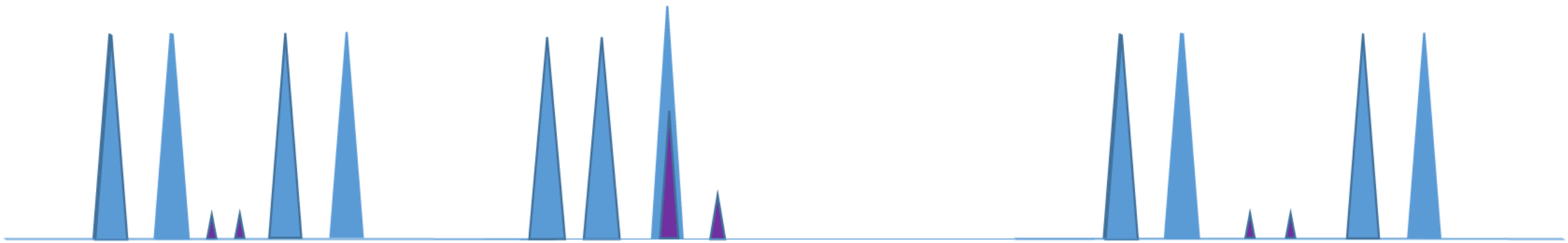
Determining if a two-person “major” component can be distinguished



(2 *C) - D > or = 4 * (2 *D)

Assuming equal contribution of possible D sister allele to C

Determining if a two-person “major” component can be distinguished

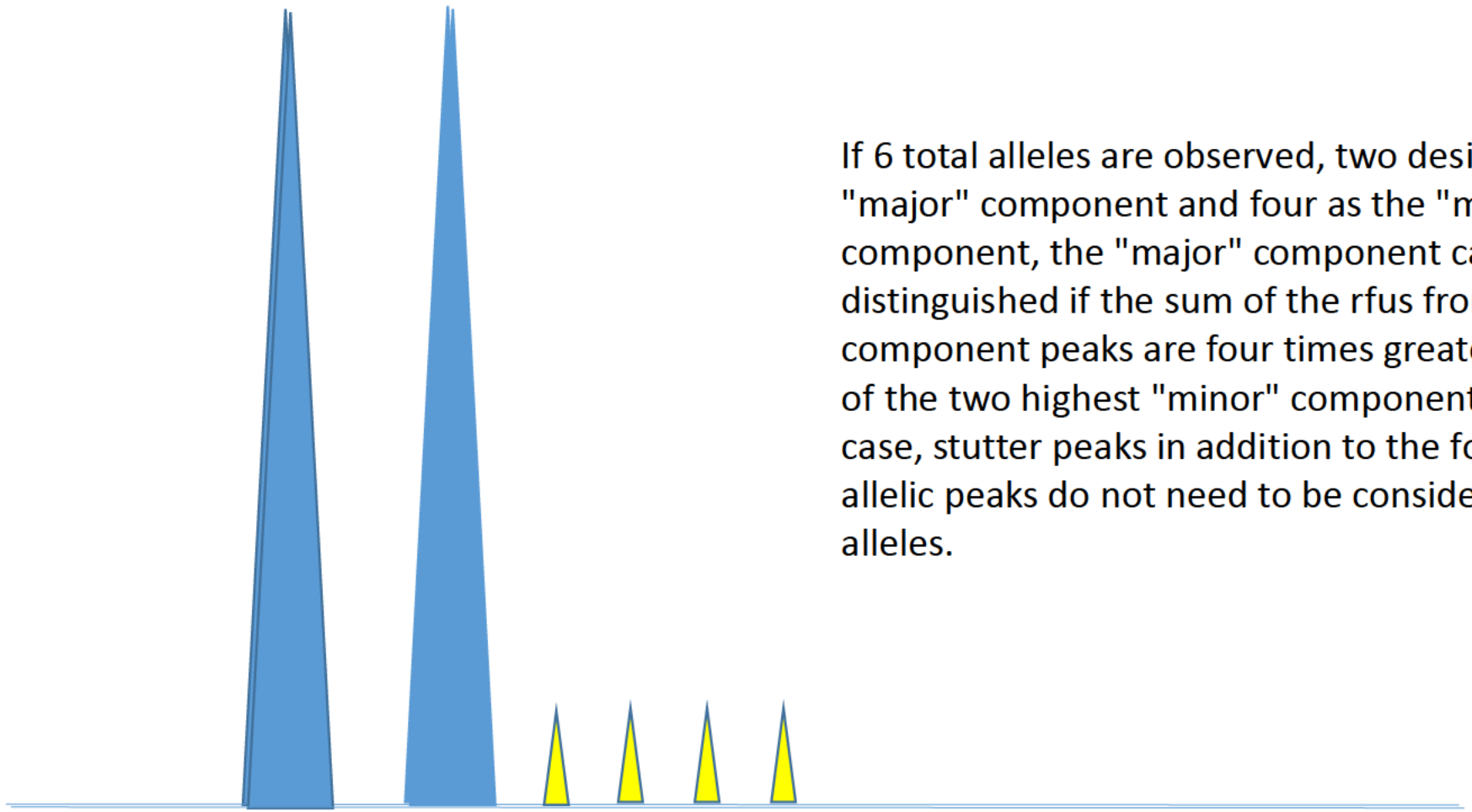


Assuming greatest possible contribution of possible D sister allele to C would result in the inability to determine a "major" component at this locus. ***This is not a reasonable assumption*** considering the entire profile.

Determining if a “major” component can be distinguished

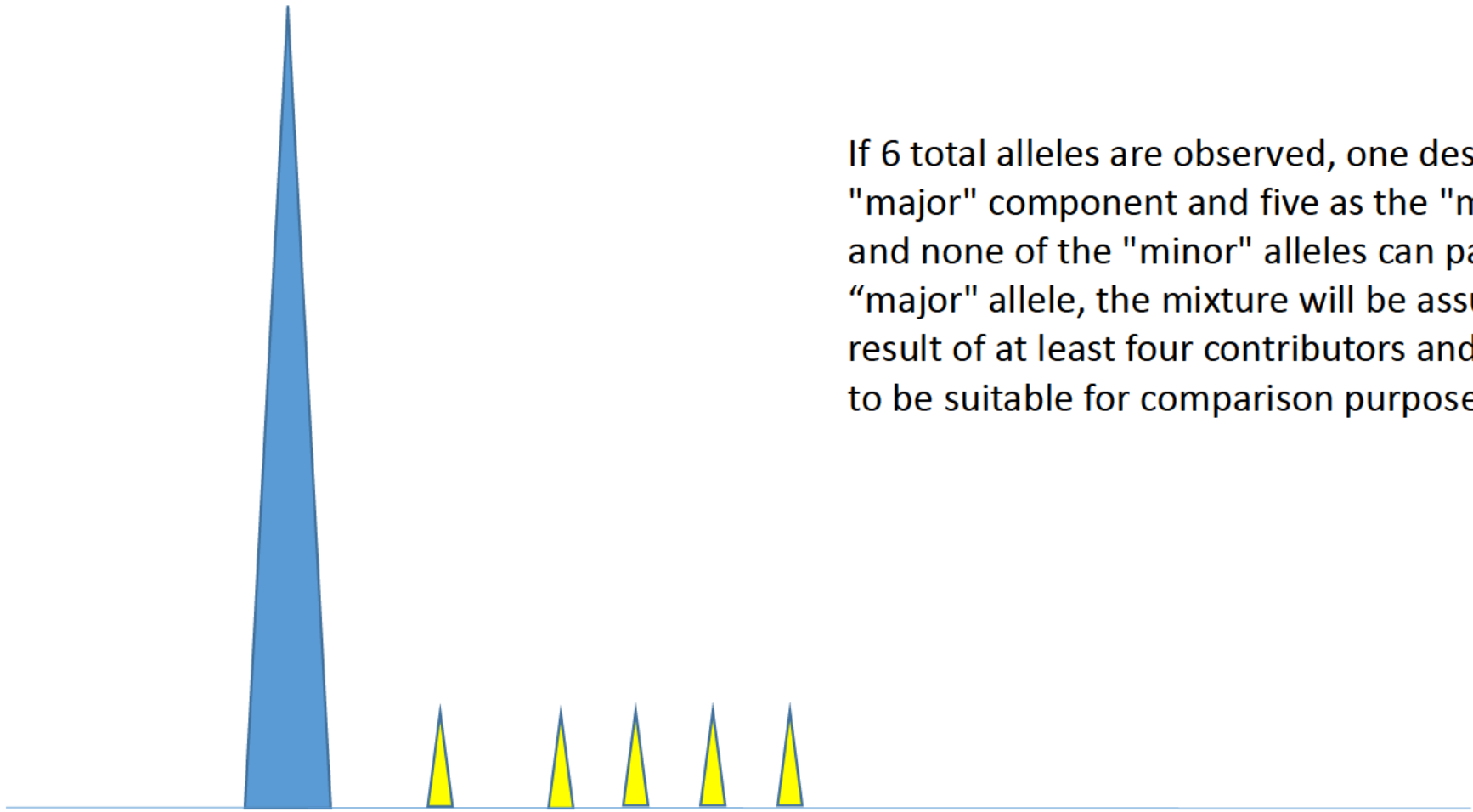
- In contrast to the mixed DNA profiles with clear "major" and "minor" components, there are some instances where the mixture ratio is difficult to determine or varies significantly across the entire profile. DNA profiles with limited DNA, degradation, inhibition or other issues may make it more difficult to determine if a "major" component can be distinguished.
- For these DNA profiles, the following provides guidance.

Six allele loci



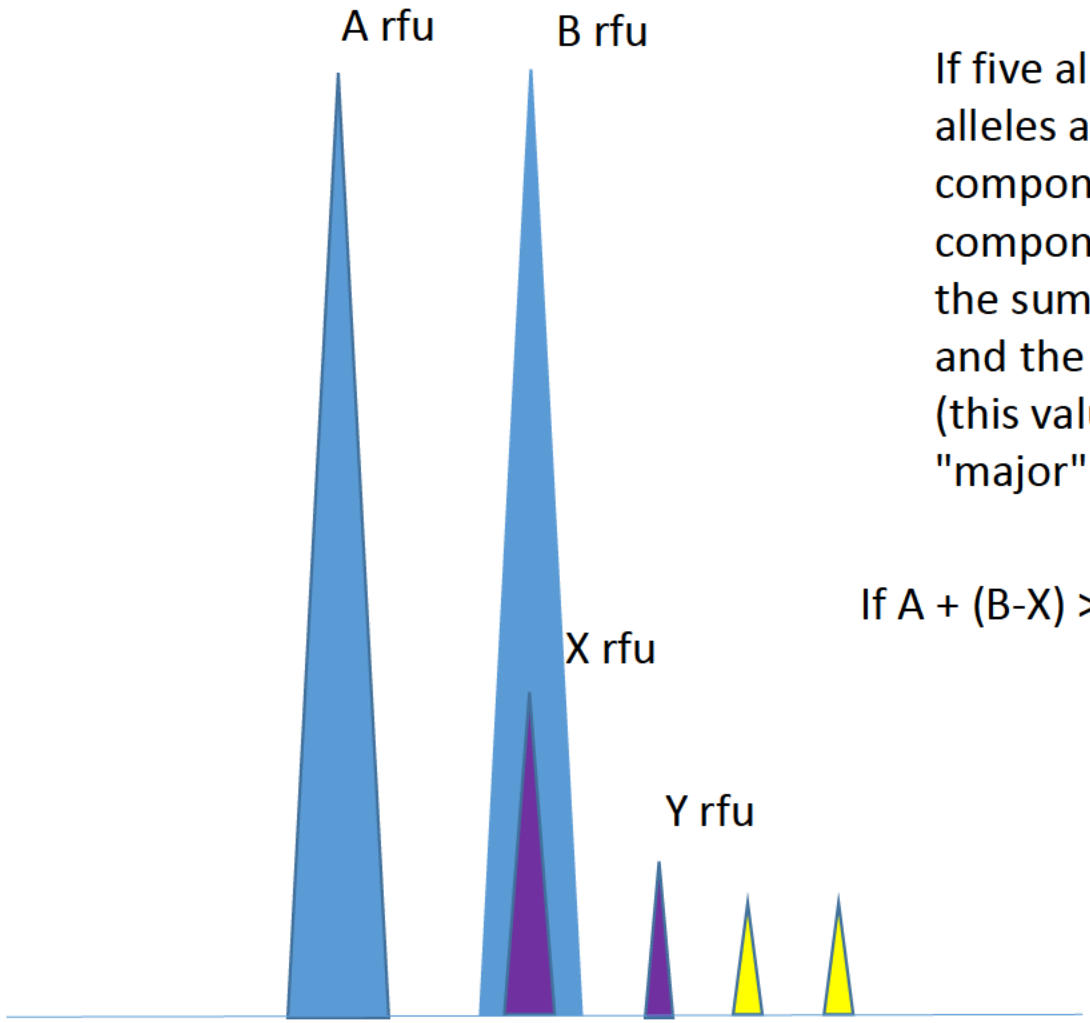
If 6 total alleles are observed, two designated as the "major" component and four as the "minor" component, the "major" component can be distinguished if the sum of the rfus from the "major" component peaks are four times greater than the sum of the two highest "minor" component peaks. In this case, stutter peaks in addition to the four designated allelic peaks do not need to be considered as possible alleles.

Six allele loci



If 6 total alleles are observed, one designated as the "major" component and five as the "minor" component and none of the "minor" alleles can pair with the “major" allele, the mixture will be assumed to be the result of at least four contributors and determined not to be suitable for comparison purposes.

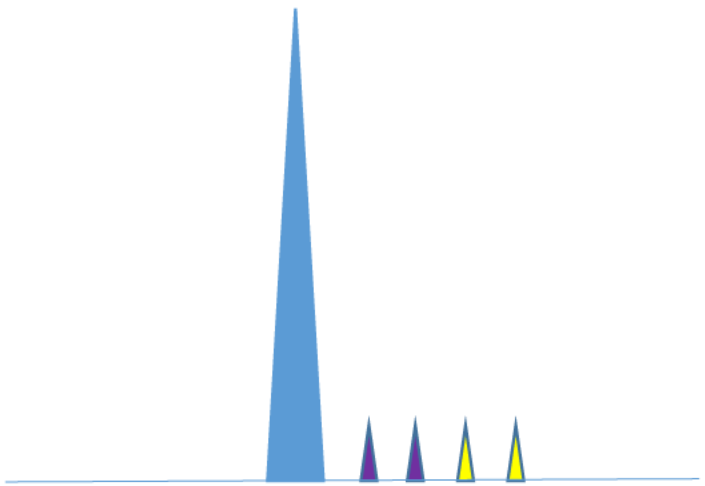
Five allele loci



If five alleles are detected – two "major" component alleles and three "minor" component alleles – the "major" component can be distinguished if the sum of the "major" component allele peak heights is four times greater than the sum of the highest "minor" component peak height and the greatest peak height that can pair with that allele (this value should be subtracted from the sum of the "major" peaks)

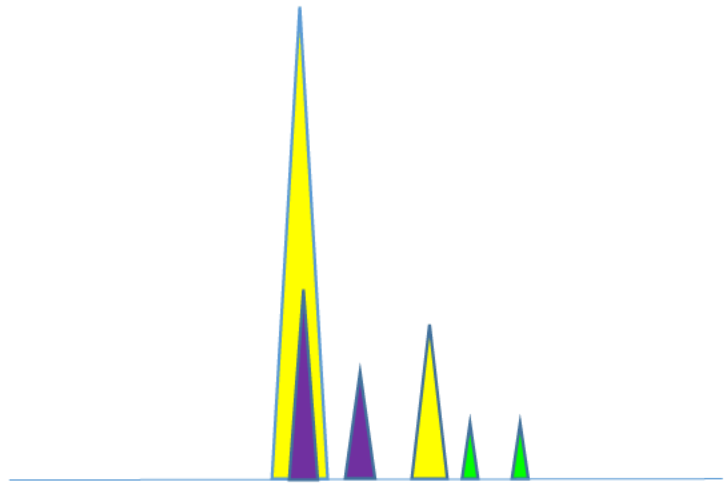
If A + (B-X) > or = 4(X+Y), then distinguishable "major" component

Five allele loci



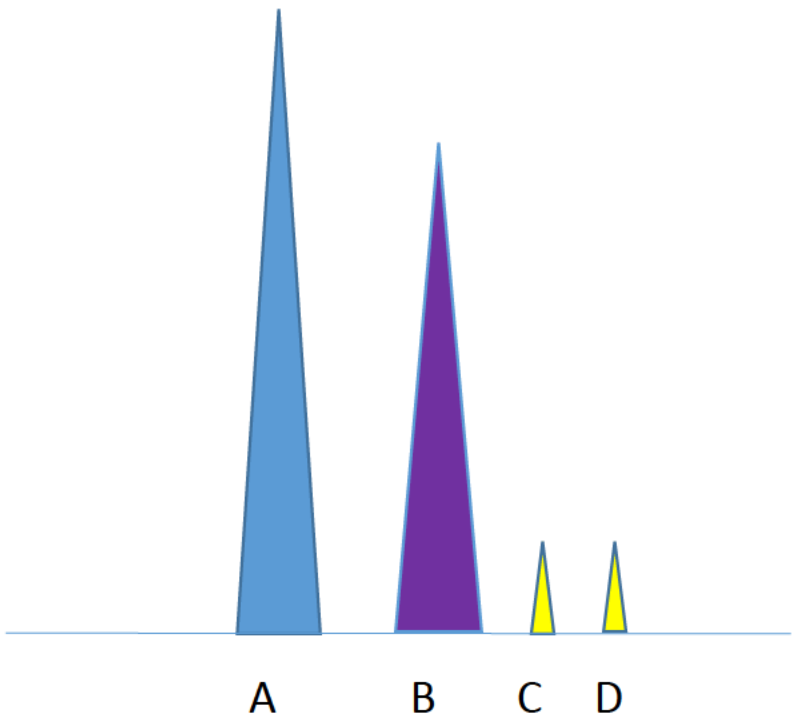
AA + BC + DE

A "major" component can be distinguished if the peak height of the single “major" peak is greater than the sum of the two highest "minor" component peaks ***and*** the sum of the greatest peak heights for possible sister alleles of the two highest "minor" component alleles do not add up to the "major" allelic peak height.



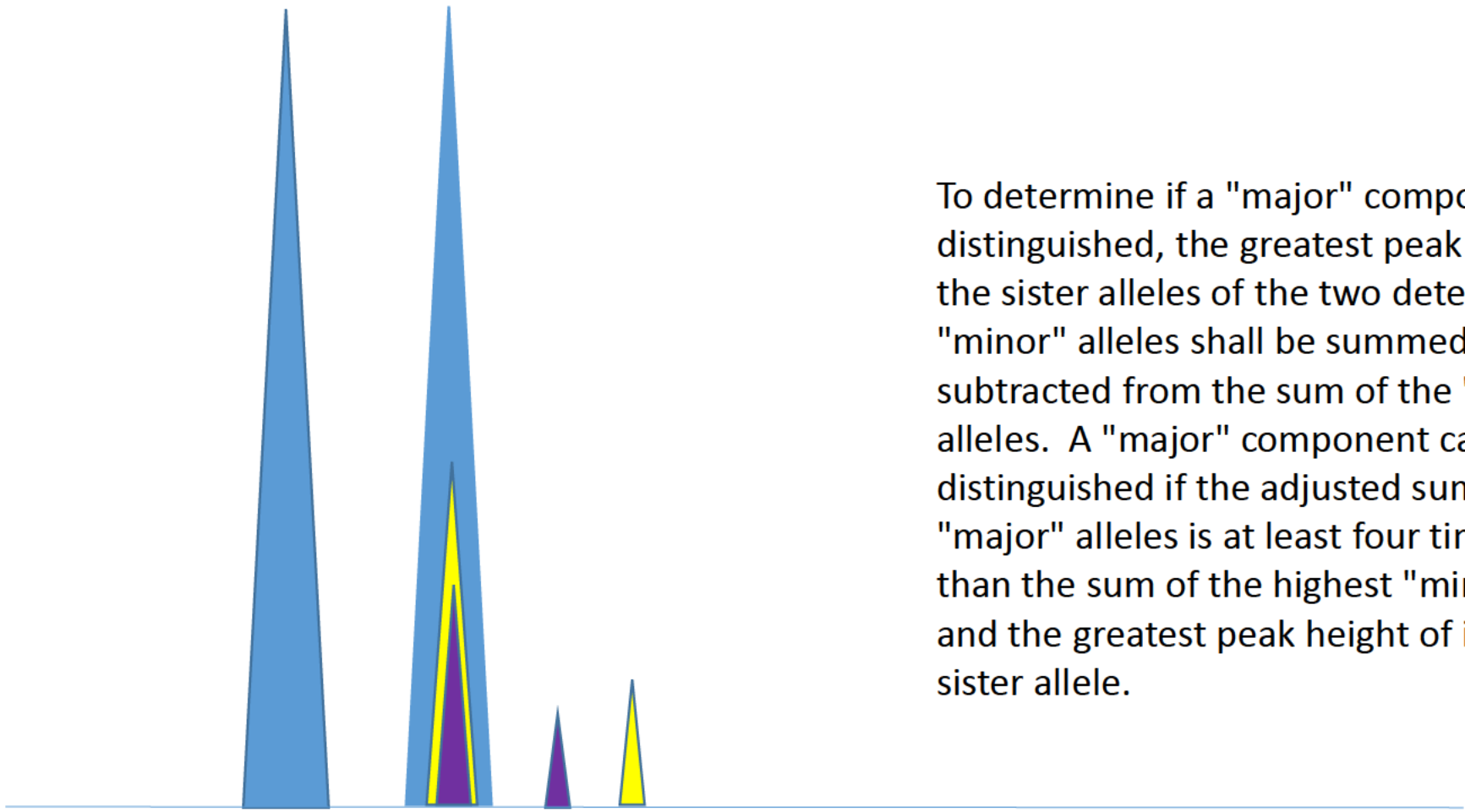
AB + AC + DE

Four allele loci



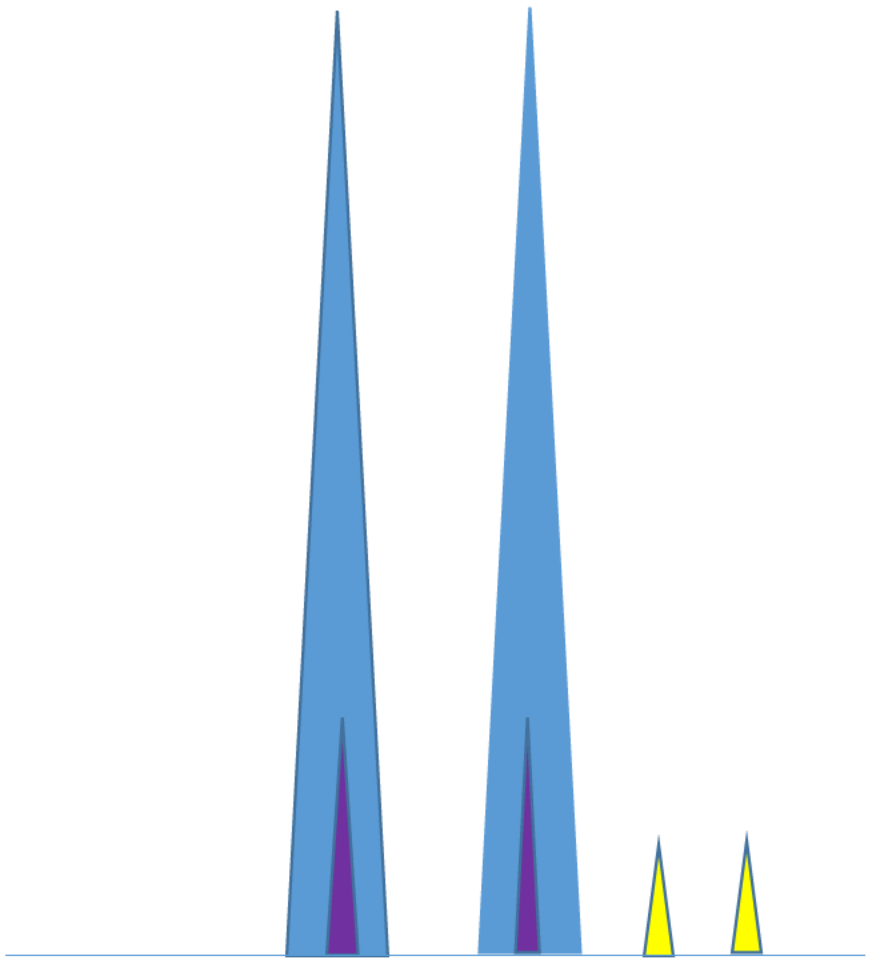
Based on the estimated mixture ratio, the possibility that one of the two "major" sister alleles is a homozygous "minor" component must be reasonably eliminated.

Four allele loci



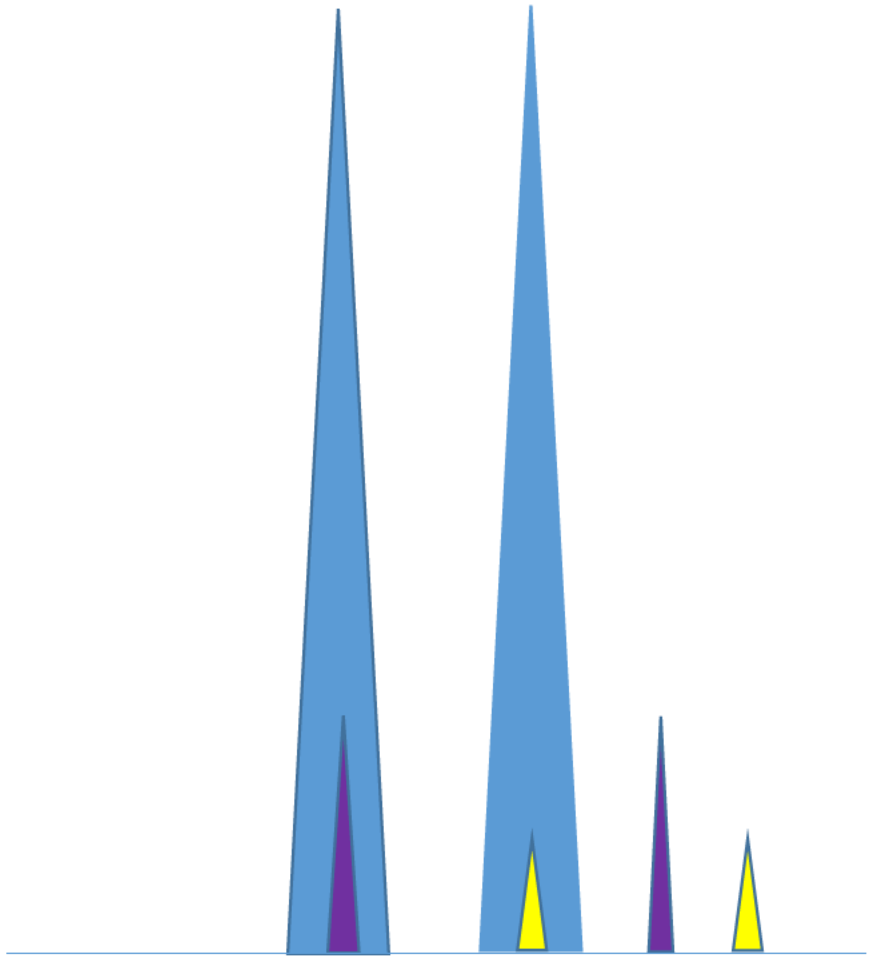
To determine if a "major" component can be distinguished, the greatest peak heights for the sister alleles of the two detected "minor" alleles shall be summed and subtracted from the sum of the "major" alleles. A "major" component can be distinguished if the adjusted sum of the "major" alleles is at least four times greater than the sum of the highest "minor" allele and the greatest peak height of its masked sister allele.

Four allele loci



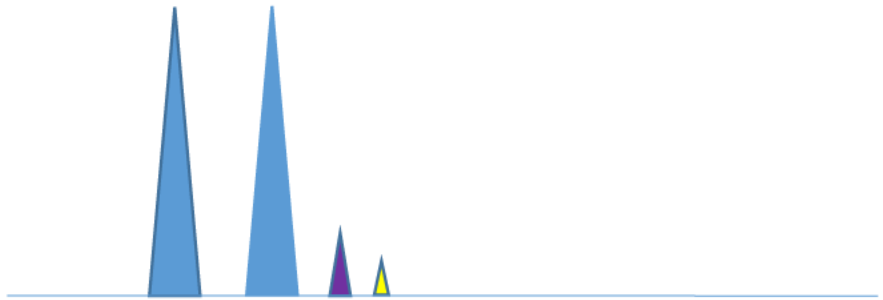
An unknown contribution amount from shared alleles may exist. If a mixture ratio of the third, possibly masked, contributor can be reasonably estimated, then a "major" component can be distinguished if the sum of the "major" alleles are four times greater than the sum of the heights of the two highest "minor" component alleles under the assumption that the alleles are masked by the “major” component alleles. The sum of the "major" alleles shall be adjusted by subtracting the contribution from the masked alleles based on the highest mixture ratio for that component.

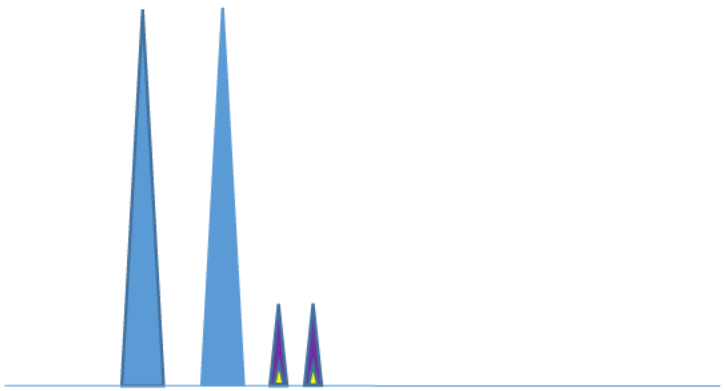
Four allele loci

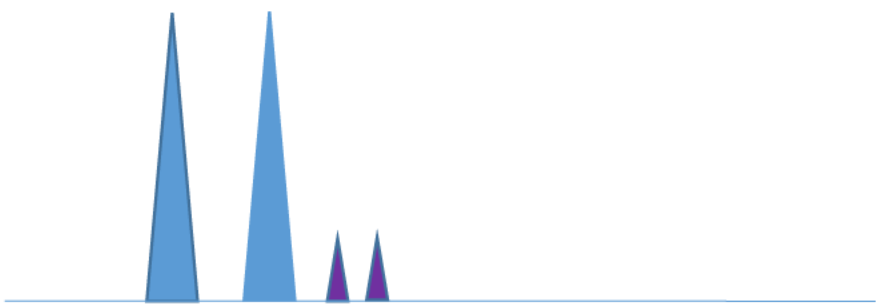


- Determine the greatest possible sister peak heights for the two observed “minor” alleles.
- Adjust the “major” component peak heights by subtracting the sum of the possible sister alleles.
- A “major” component can be distinguished if the adjusted sum of the "major" component is at least four times greater than the sum of the greatest “minor” component pair.

Four allele loci

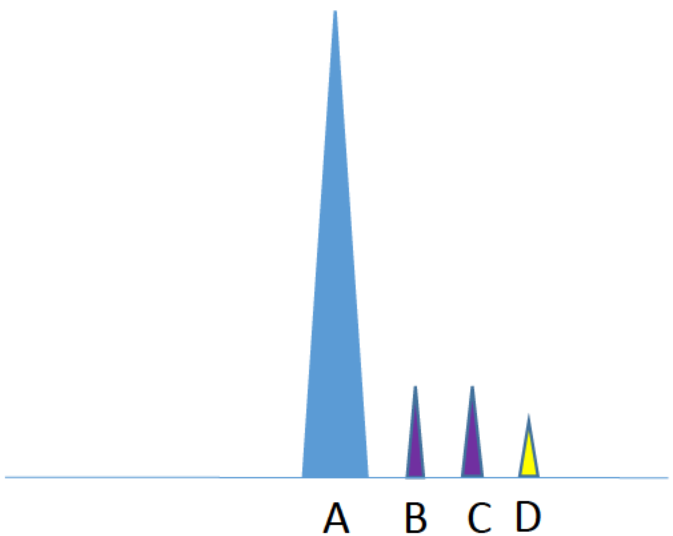






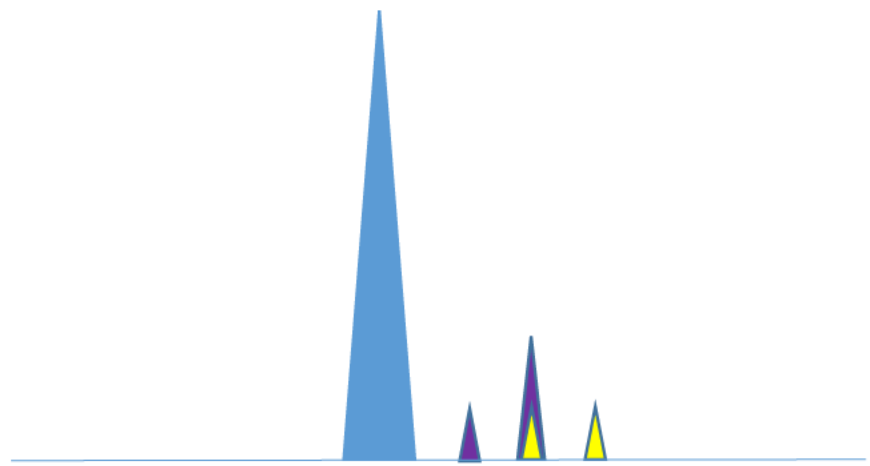
A "major" component can be distinguished if the sum of the "major" alleles is at least four times greater than the sum of the two observed "minor" alleles.

Four allele loci

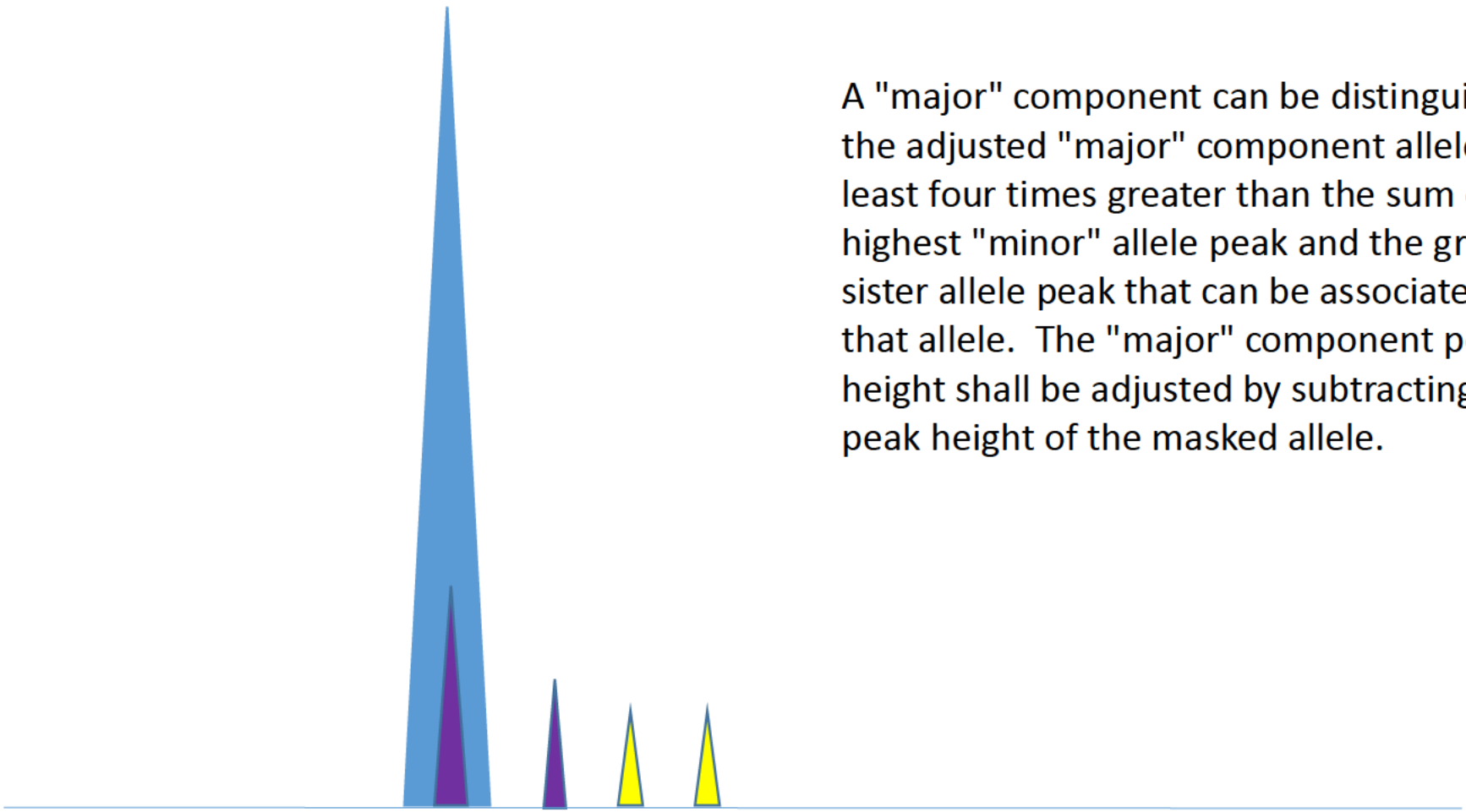


A "major" component can be distinguished if the peak height of the "major" allele is at least four times greater than the sum of the two highest "minor" alleles.

A > or = 4 * (B + C)

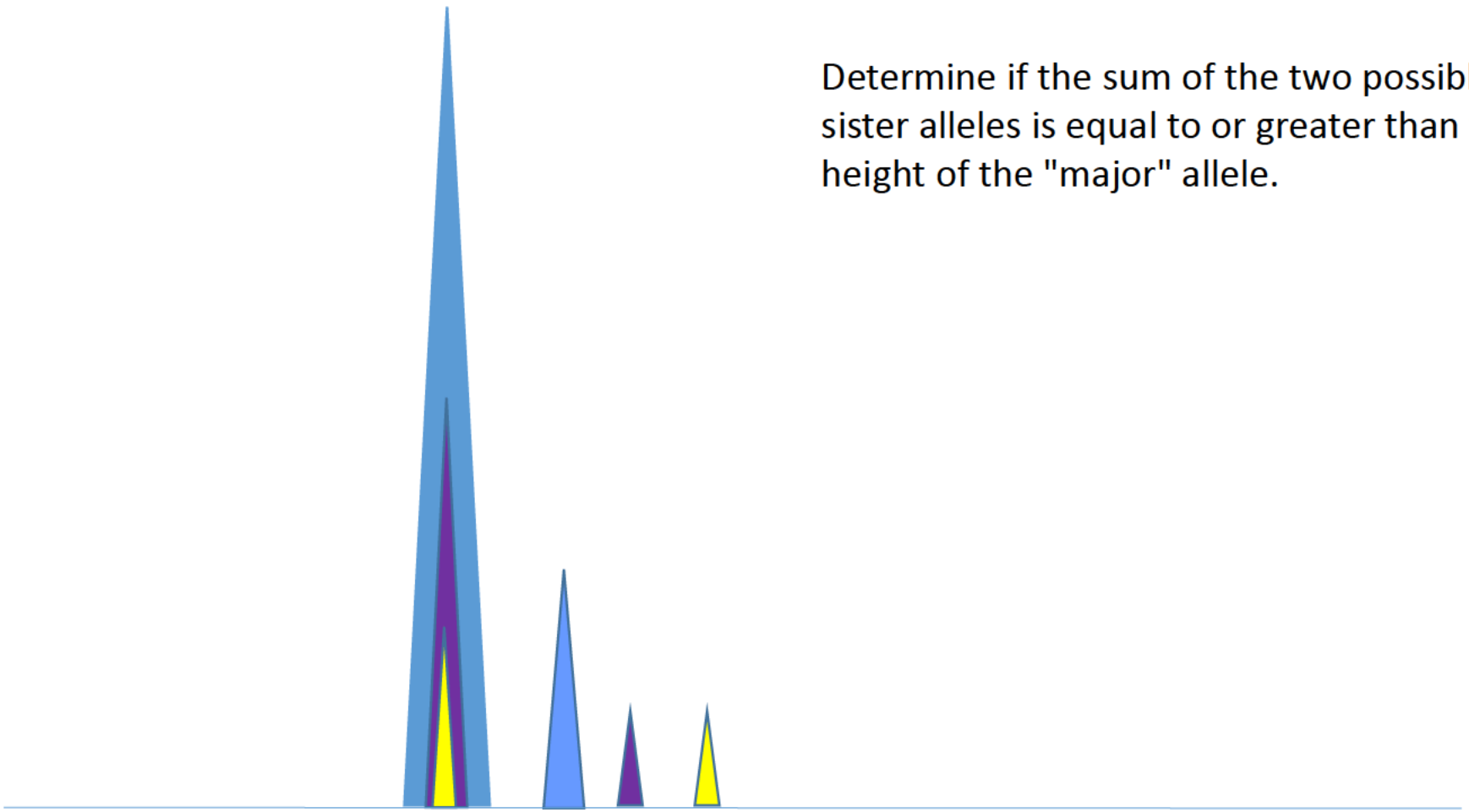


Four allele loci



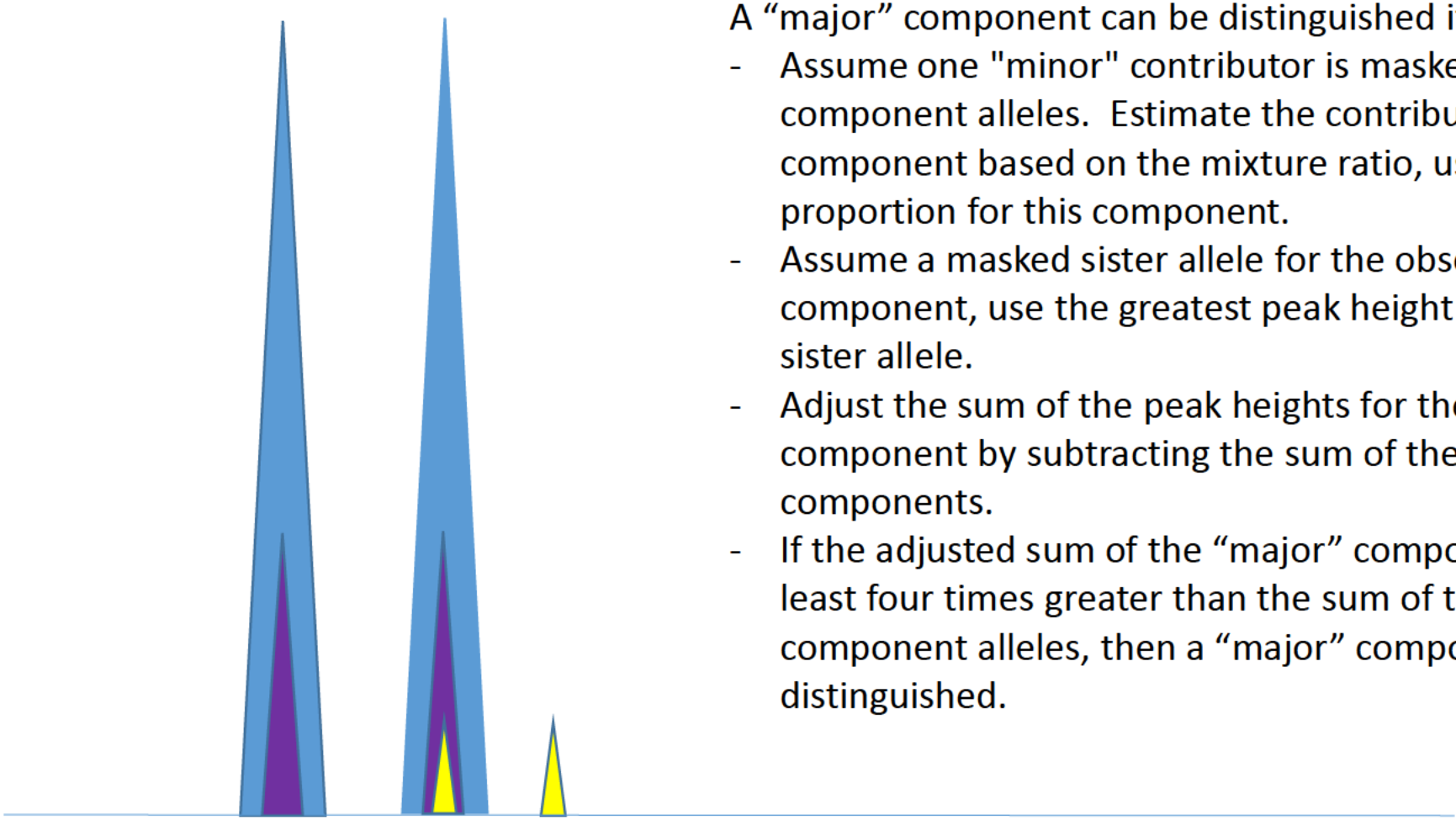
A "major" component can be distinguished if the adjusted "major" component allele is at least four times greater than the sum of the highest "minor" allele peak and the greatest sister allele peak that can be associated with that allele. The "major" component peak height shall be adjusted by subtracting the peak height of the masked allele.

Four allele loci



Determine if the sum of the two possibly masked sister alleles is equal to or greater than the peak height of the "major" allele.

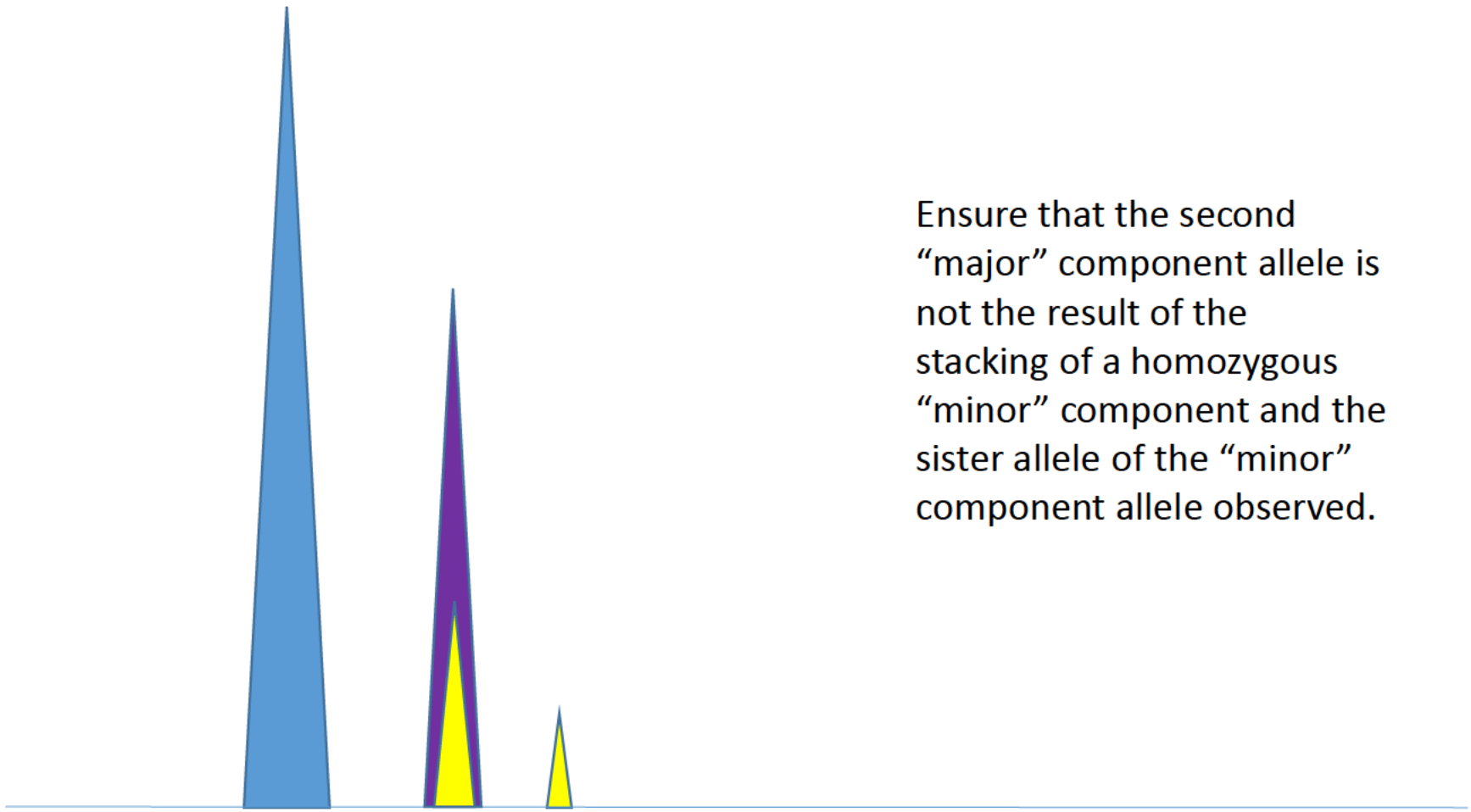
Three allele loci



A “major” component can be distinguished if:

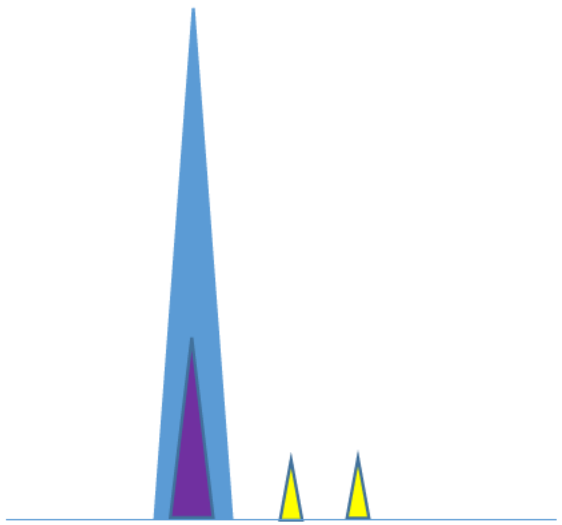
- Assume one "minor" contributor is masked by “major” component alleles. Estimate the contribution of this component based on the mixture ratio, use the highest proportion for this component.
- Assume a masked sister allele for the observed “minor” component, use the greatest peak height for the masked sister allele.
- Adjust the sum of the peak heights for the “major” component by subtracting the sum of the masked “minor” components.
- If the adjusted sum of the “major” component alleles is at least four times greater than the sum of the "minor" component alleles, then a “major” component can be distinguished.

Three allele loci



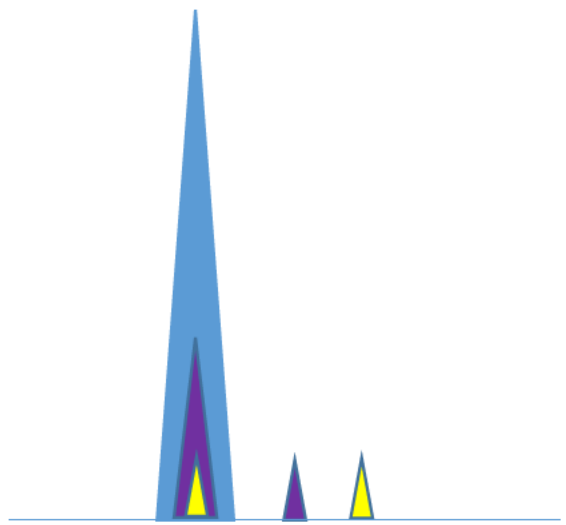
Ensure that the second “major” component allele is not the result of the stacking of a homozygous “minor” component and the sister allele of the “minor” component allele observed.

Three allele loci

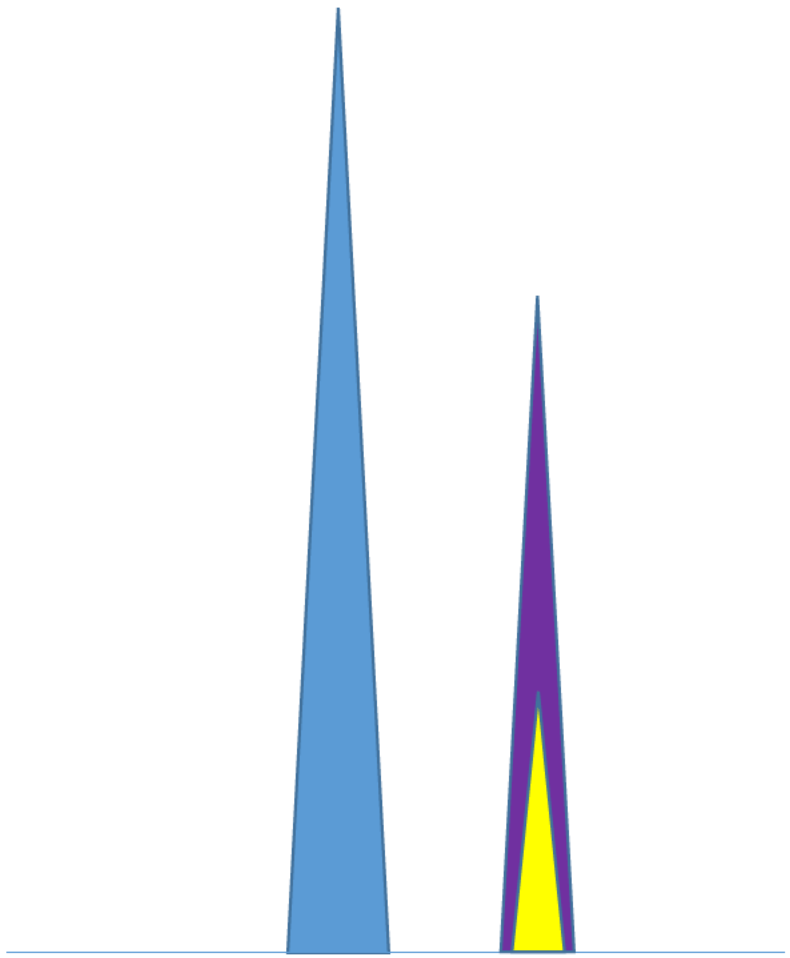


A “major” component can be distinguished if:

- Assuming one of the components is masked by the “major” allele, the greatest proportion for that component is subtracted from the “major” allele peak height and the adjusted “major” component peak is at least four times greater than the greatest “minor” component.
- Assuming the two peaks observed for the “minor” component represent two different contributors, the greatest peak height possible for each of the sister peaks shall be summed and subtracted from the “major” component peak. If adjusted “major” peak is at least four times greater than the greatest "minor" component, the “major” component can be distinguished.

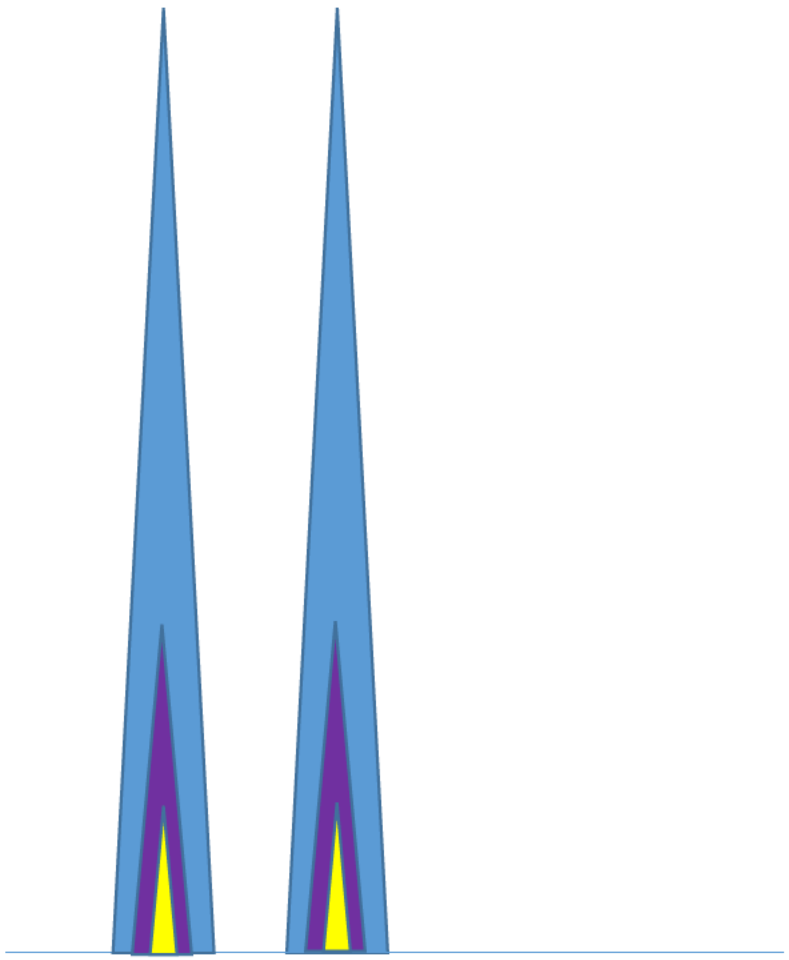


Two allele loci



Ensure that one of the peaks of the “major” component is not the result of stacking of two homozygous "minor" components.

Two allele loci

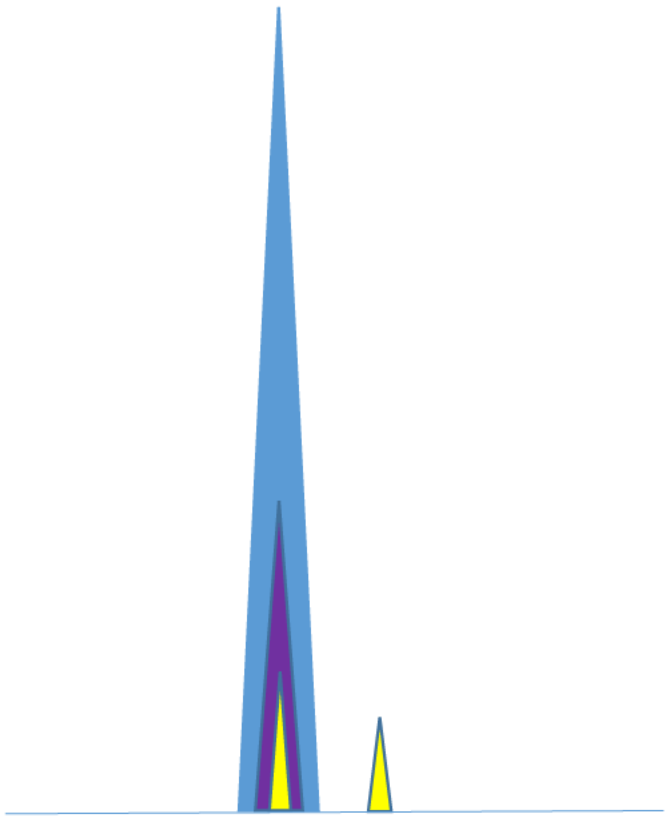


A “major” component can be distinguished if, based on the mixture ratio range calculated from the entire profile, the “major” component is at least four times greater than the highest “minor” component.

Example: Mixture ratio range ***4:2:1*** to 8:2:1, the "major" component ***cannot*** be distinguished.

Example: Mixture ratio range 4:1:1 to 7:1:1, a "major" component ***can*** be distinguished.

Two allele loci



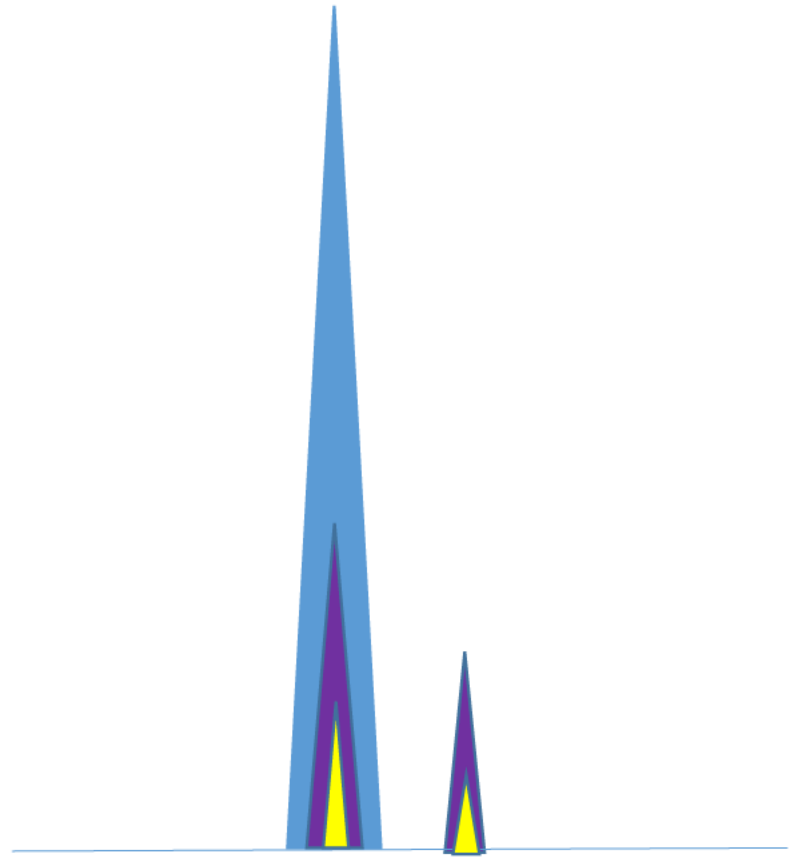
A “major” component can be distinguished if:

- Assume one of the “minor” components is completely masked by the “major” allele and the sister peak of the “minor” allele is masked by the “major” allele. Using the greatest proportion for the completely masked component and the greatest expected peak height for the sister peak, the sum of these contributions to the “major” allele shall be subtracted from the “major” allele peak height. If the adjusted “major” component peak height is at least four times greater than the greater “minor” component, a “major” component can be distinguished.

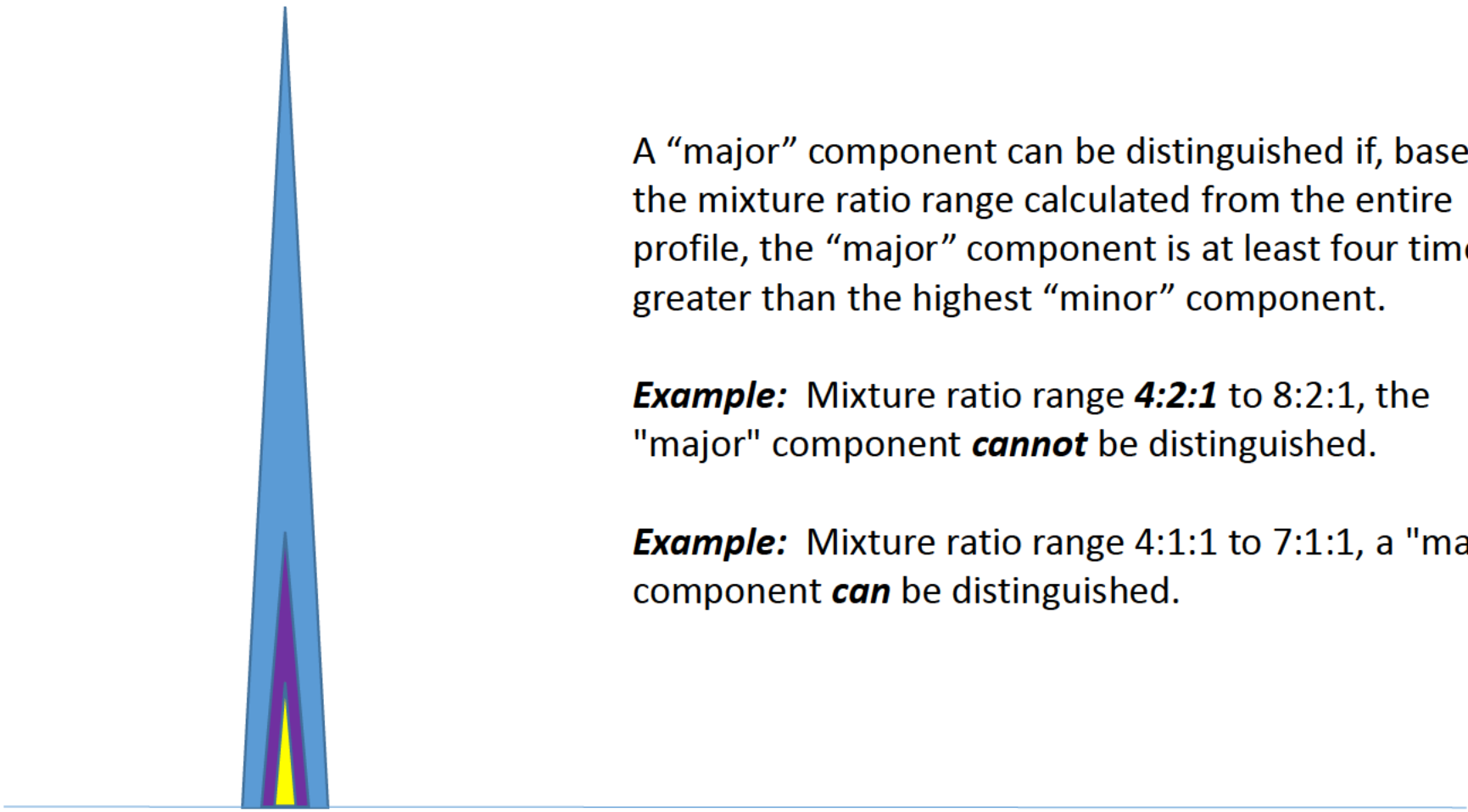
Two allele loci

A “major” component can be distinguished if:

- Assume the “minor” peak detected is a combination of a single peak of a heterozygous pair for each of the two “minor” components. The “minor” peak shall be divided based on the average proportion of the mixture ratio for the “minor” contributors. The greatest expected peak height for each of the sister peaks shall be summed and subtracted from the peak height of the “major” allele. If the adjusted “major” component is at least four times greater than the greatest "minor" component, a “major” contributor can be distinguished.



One allele loci



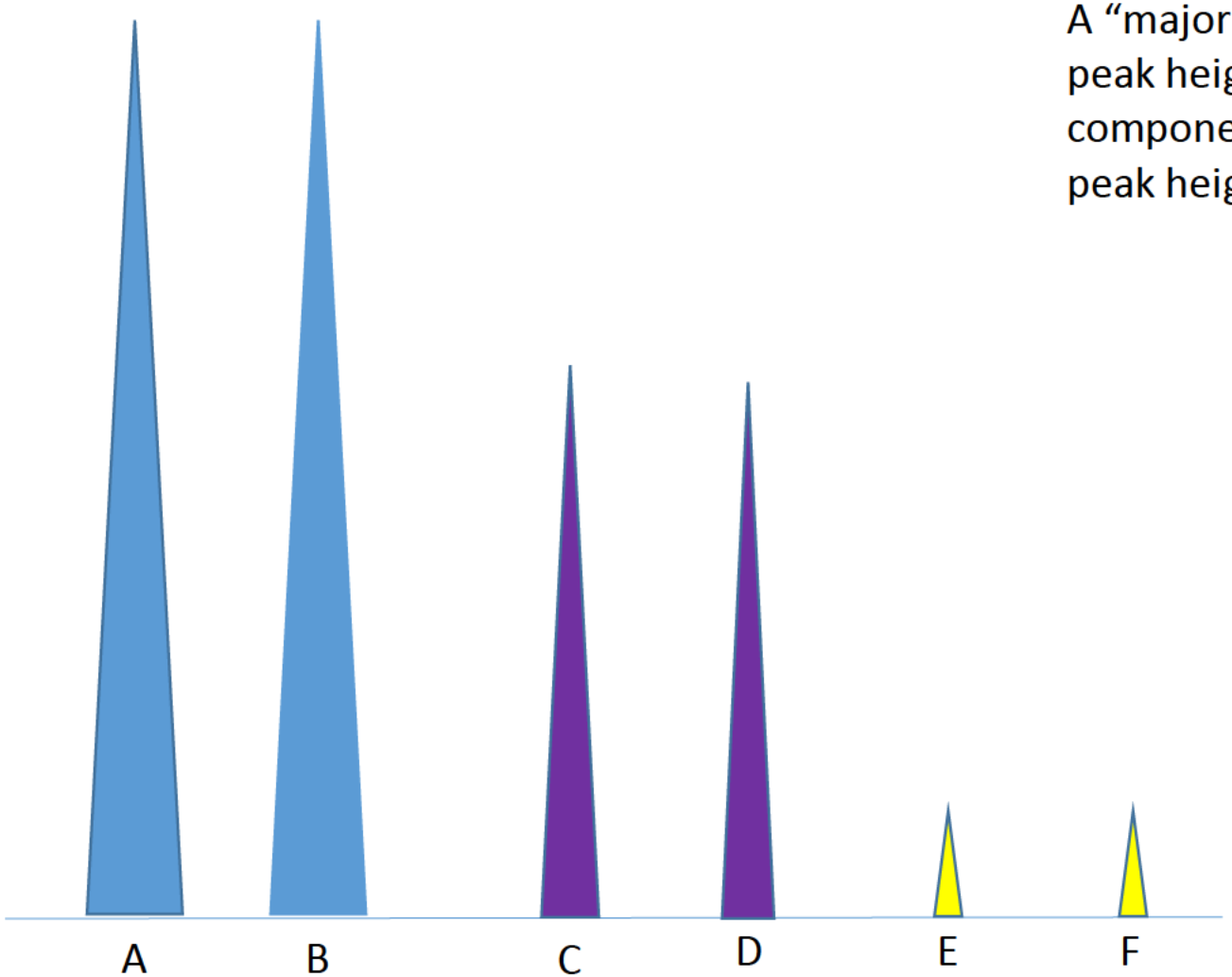
A “major” component can be distinguished if, based on the mixture ratio range calculated from the entire profile, the “major” component is at least four times greater than the highest “minor” component.

Example: Mixture ratio range ***4:2:1*** to 8:2:1, the "major" component ***cannot*** be distinguished.

Example: Mixture ratio range 4:1:1 to 7:1:1, a "major" component ***can*** be distinguished.

Indistinguishable Two-Person "major" in a Three-Person Mixture

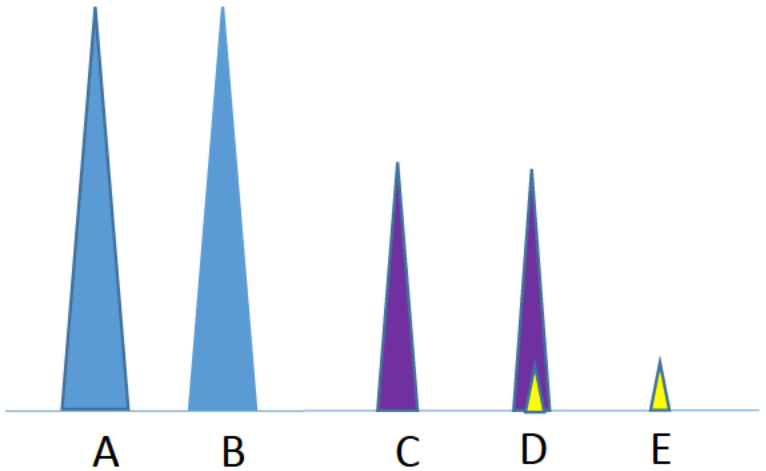
Six allele loci

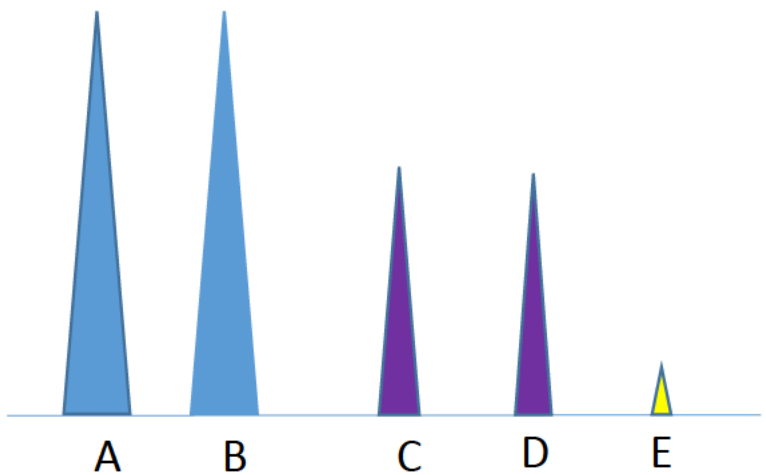


A “major” component can be distinguished if the sum of the peak heights for the lowest pairing peaks of the “major” component is at least four times greater than the sum of the peak heights for the “minor” peaks

$$C + D > \text{or} = 4 * (E + F)$$

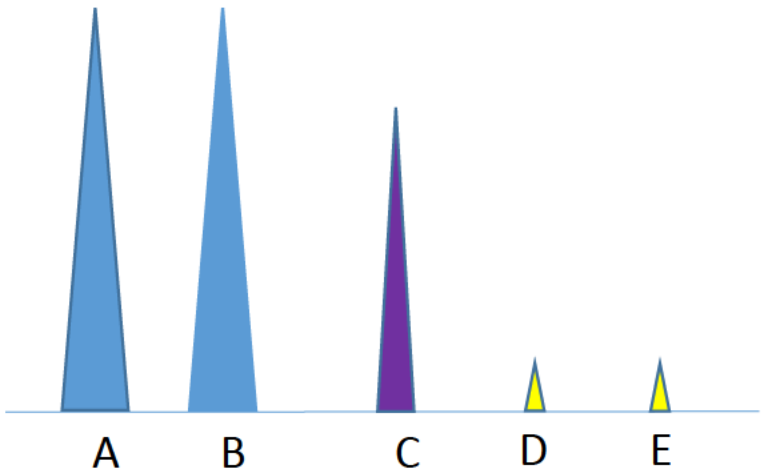
Five allele loci





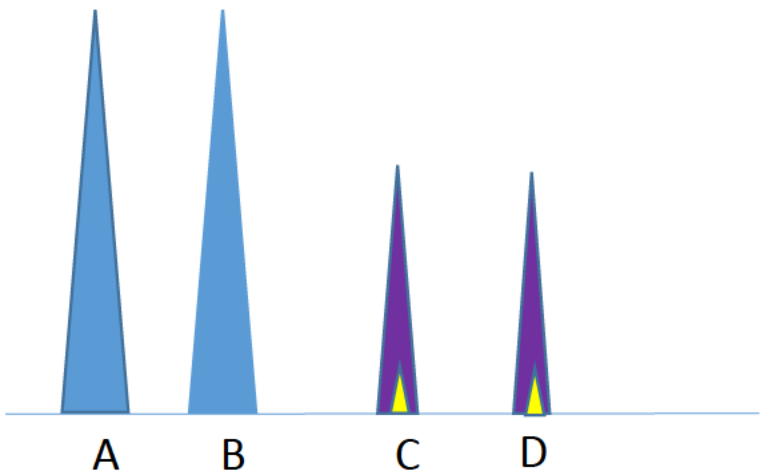
A “major” component can be distinguished if the adjusted sum of the peak heights for the lowest pairing of the “major” peaks is at least four times greater than the sum of the “minor” peaks. The adjusted sum is calculated by subtracting the greatest possible sister peak height for the observed “minor” peak from the sum of the lowest pairing “major” peaks.

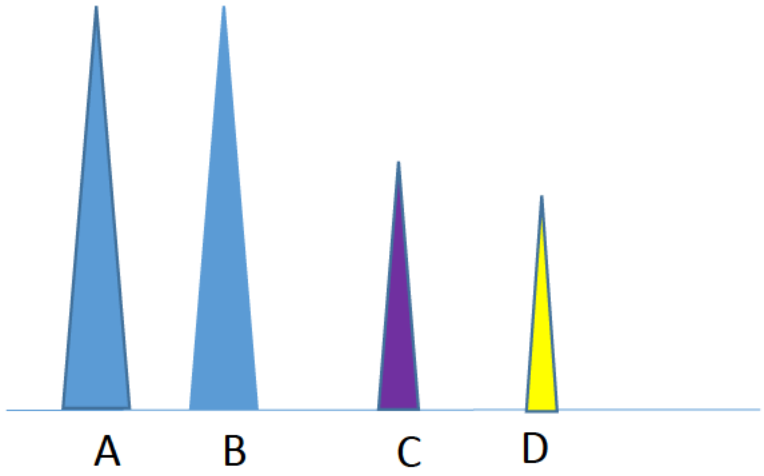
Five allele loci

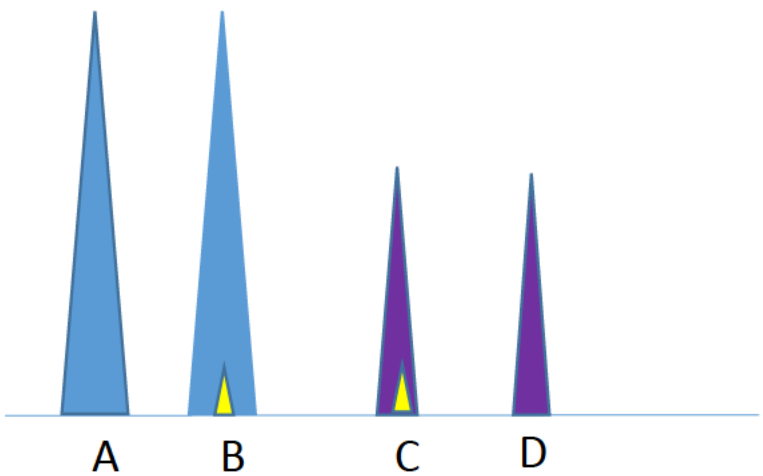


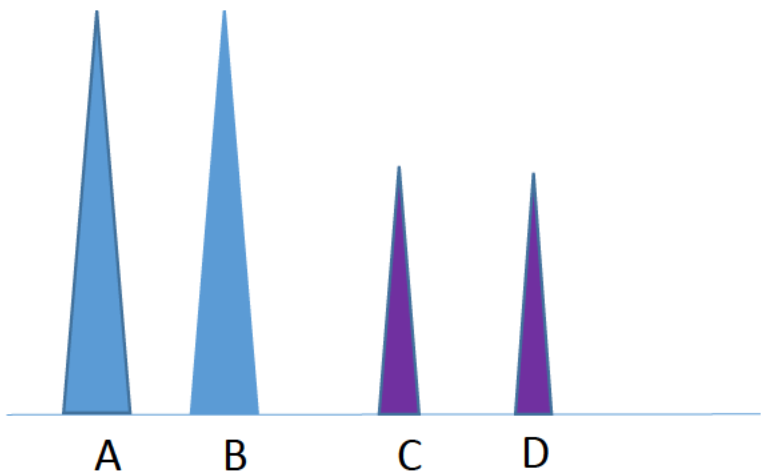
A "major" component can be distinguished if it is assumed that the lowest “major” peak is homozygous and it is at least four times greater than the combined rfu of the “minor” peaks.

Four allele loci





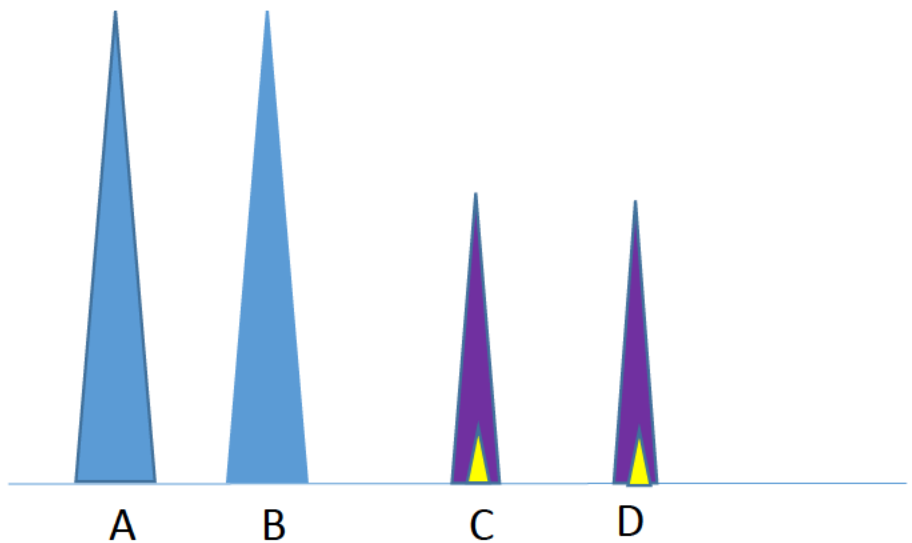




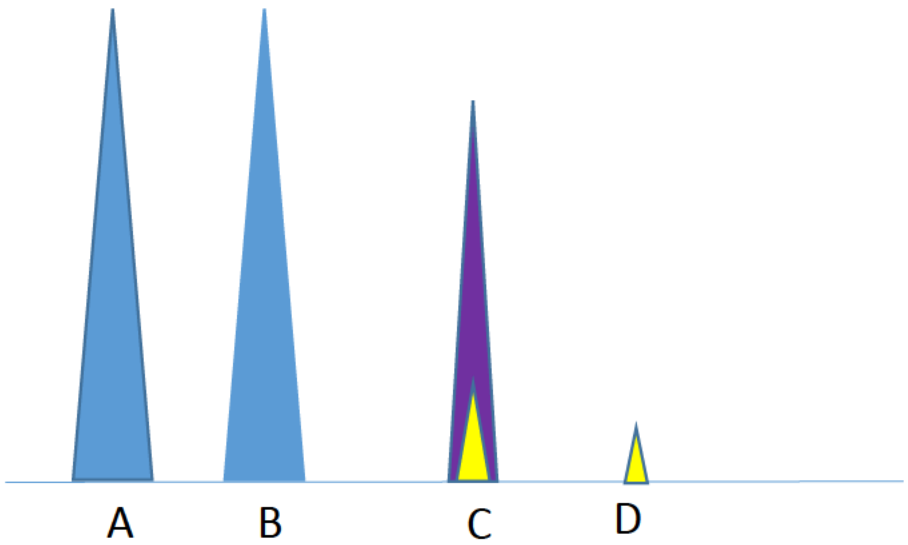
Complete drop-out of third contributor

Four allele loci

- In order to distinguish a "major" component, the adjusted total rfu value for the lower "major" component must be at least four times the maximum expected contribution of the “minor” component. This then would be based on the mixture ratio calculated previously. For example, if the range of the mixture ratio varied from 5:5:1 to 8:7:1, then a two person "major" component can be distinguished. If the mixture ratio ranged from 4:***3:1*** to 8:7:1, then the two person "major" component cannot be distinguished at this locus.

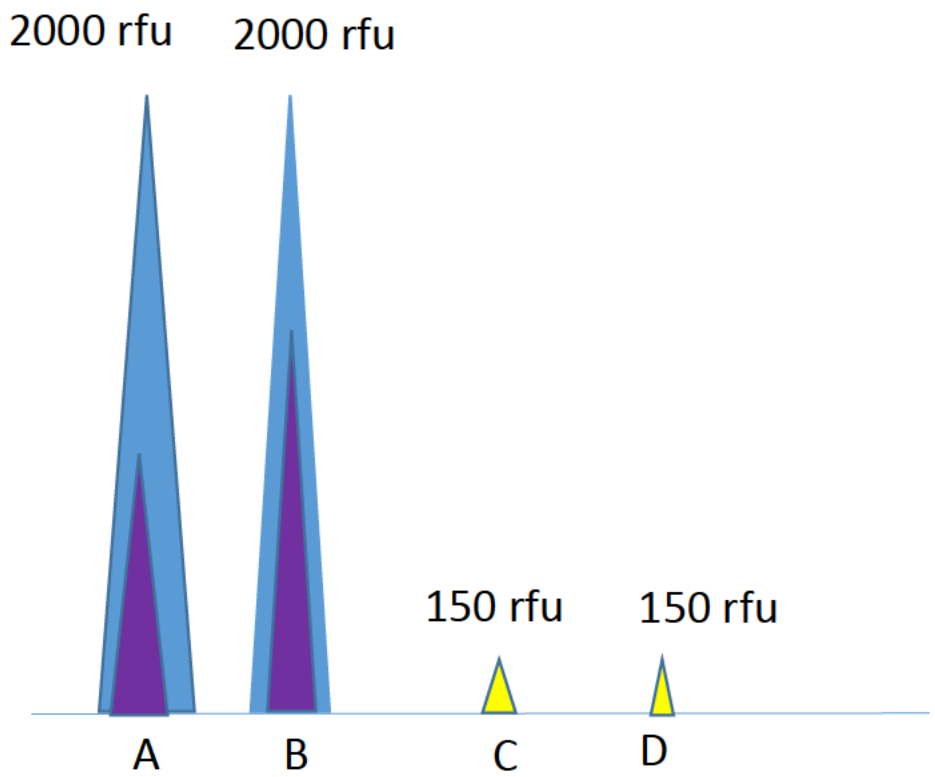


Four allele loci



- If three “major” peaks are observed, then assume the lowest “major” peak is homozygous and adjust the rfu based on the greatest sister peak of the “minor” peak observed. A "major" component can be distinguished if the adjusted peak height is at least four times greater than the combined rfu of the “minor” component.

Four allele loci

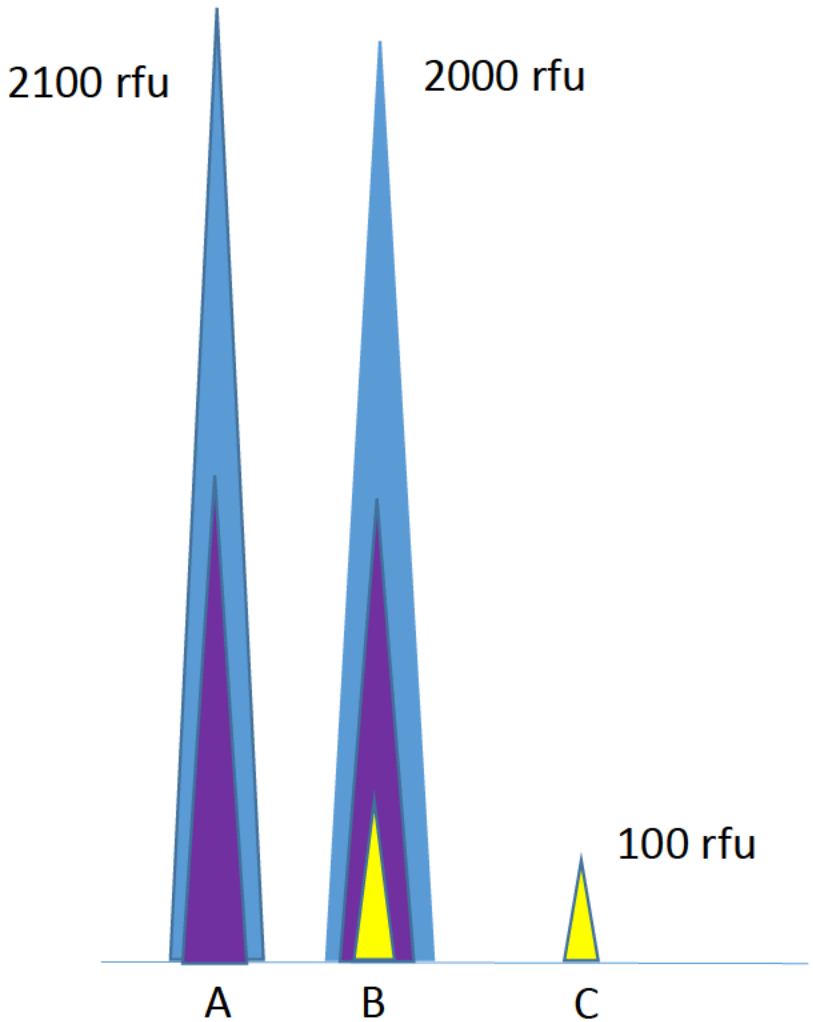


- If two “major” peaks are observed, a "major" component can be distinguished by:
  - Assuming the lowest contribution from one of the components of the “major” contributor
  - If the proportion of the peak heights of the lowest “major” contributor is at least four times greater than the sum of the peak heights of the “minor” contributor, the "major" component can be distinguished.

Example:

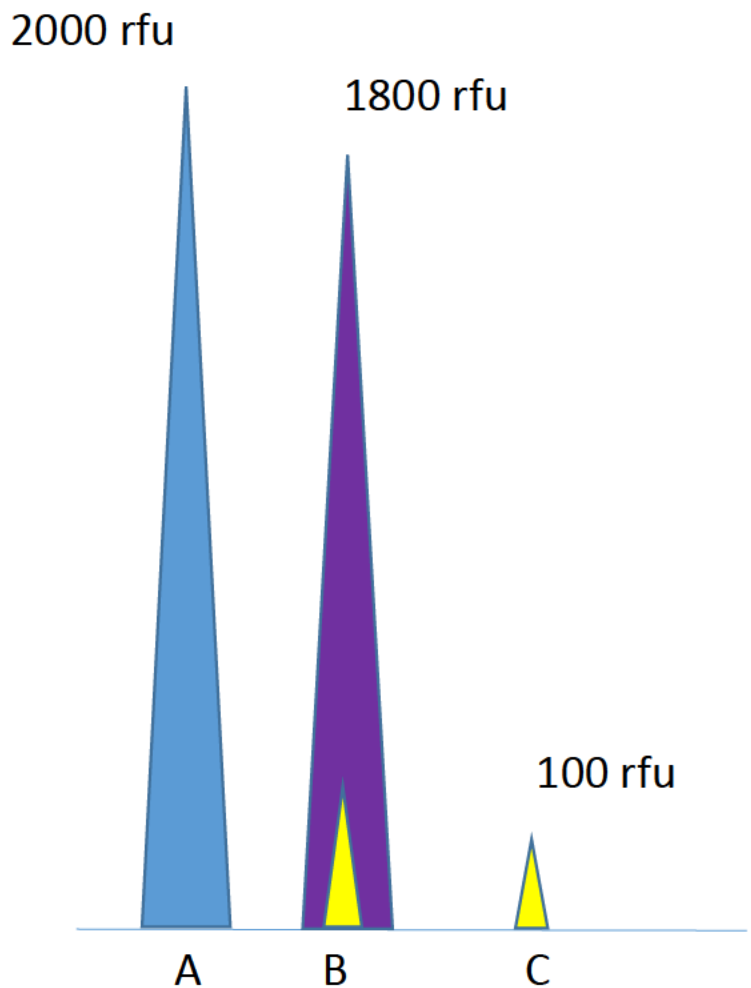
- Range of ratios observed – 6:5:1 to 8:4:1
- Using the 8:4:1 ratio, then 1/3 of the “major” peaks are predicted to be from the middle contributor or approximately 1,333 rfu
- 1,333 is greater than four times (150 + 150)

Three allele loci



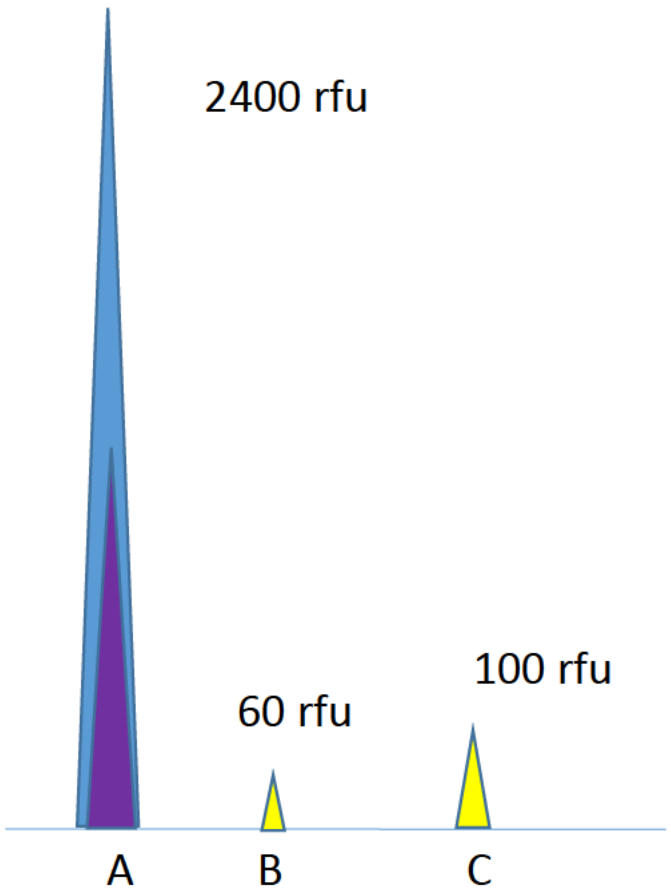
- Based on the lowest contribution of the second “major” contributor from the range of mixture ratios observed, the sum of the “major” component alleles will be adjusted by subtracting the greatest possible sister allele of the observed "minor" allele.
- A "major" component can be distinguished if the adjusted sum of the second “major” component is at least four times greater than the sum of the “minor” component.
- Example: Range of observed mixture ratio – 6:5:1 to 8:6:1.
  - Sum of “major” alleles – greatest possible “minor” sister allele: 4100 – 303 = 3797
  - Proportion of second “major” component based on 6:5 = (3797 / 11) * 5 = 1726 rfu
  - 1726 / (100 + 303) = 4.3
  - Therefore, "major" component ***can*** be distinguished

Three allele loci



- Assume the lowest “major” peak is a homozygous contributor.
- Adjust the peak height by subtracting the greatest possible sister peak of the observed “minor.”
- A "major" component can be distinguished if the adjusted peak height of the “major” allele is at least four times greater than the sum of the “minor” peak heights.
- Example:
  - Lowest “major” – greatest possible sister peak of "minor" = 1800 – 303 = 1497
  - Adjusted peak height / sum of minor peaks = 1497 / 403 = 3.7
  - Therefore, "major" component ***cannot*** be distinguished

Three allele loci

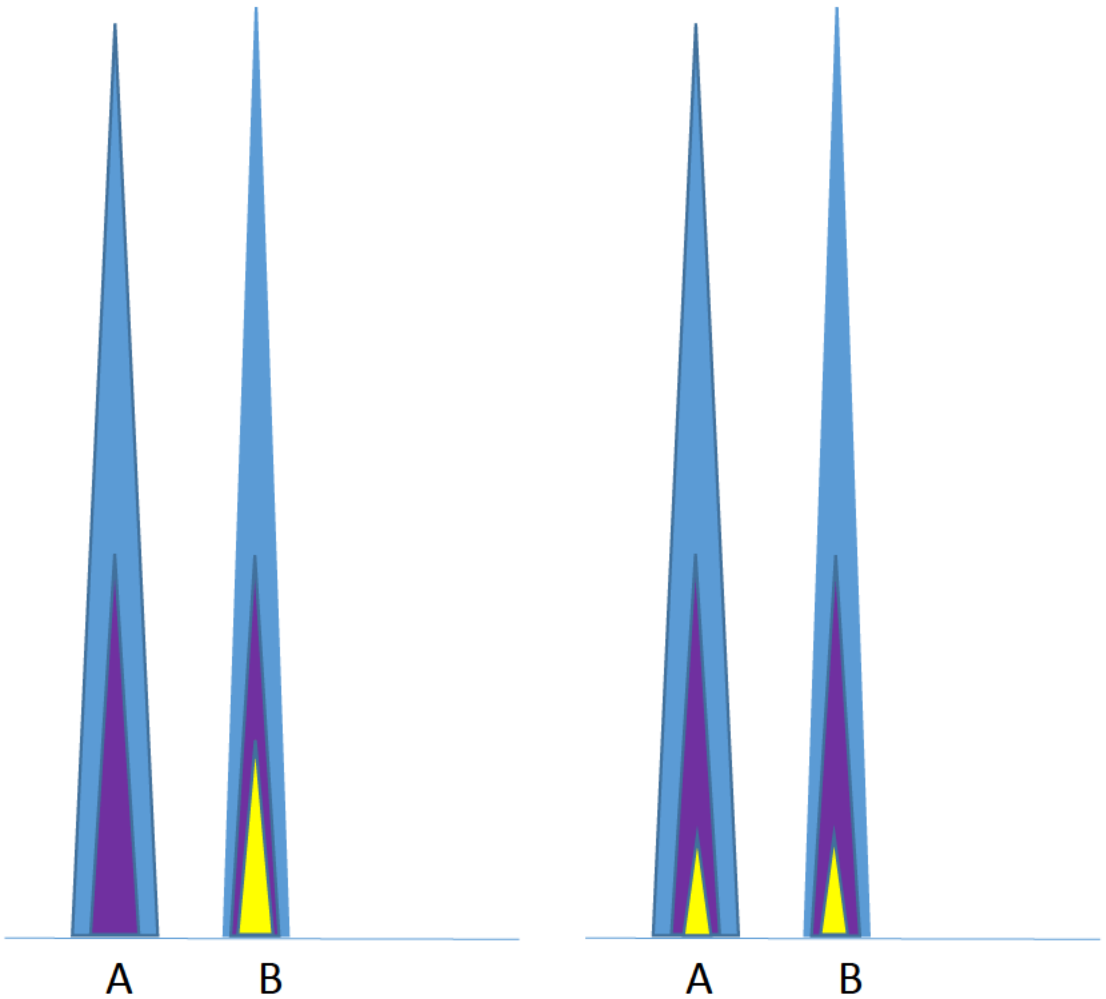


- If one “major” peak is observed, a "major" component can be distinguished by:
  - Assuming the lowest contribution from one of the components of the “major” contributor.
  - If the proportion of the peak height of the lowest “major” contributor is at least four times greater than the sum of the peak heights of the “minor” contributor, the "major" component can be distinguished.

Example:

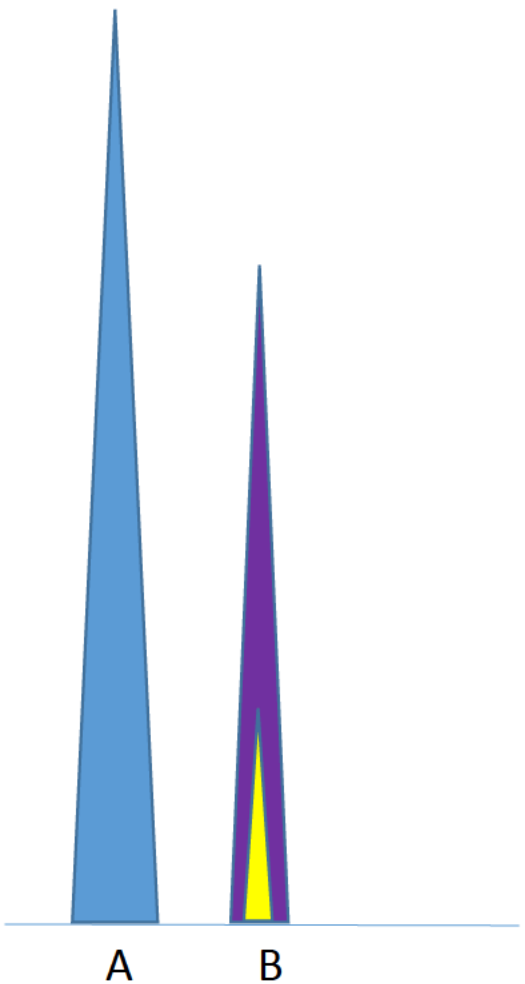
- Range of ratios observed – 6:5:1 to 8:4:1
- Using the 8:4:1 ratio, then 1/3 of the “major” peak is predicted to be from the middle contributor or approximately 800 rfu ((2400 / 12) * 4)
- 800 is greater than four times (60 + 100)

Two allele loci



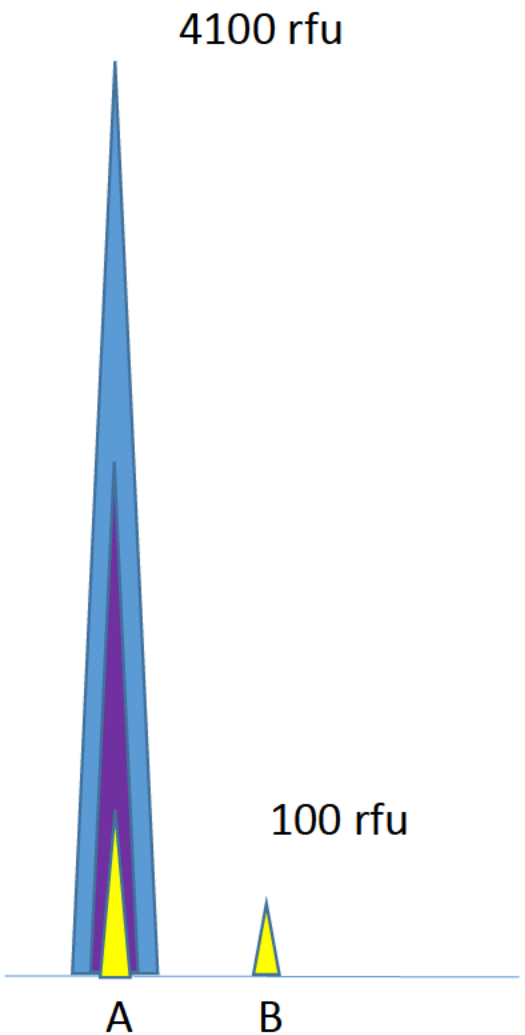
- In order to distinguish a "major" component, the adjusted total rfu value for the lower "major" component must be at least four times the maximum expected contribution of the “minor” component. This then would be based on the mixture ratio calculated previously. For example, if the range of the mixture ratio varied from 5:5:1 to 8:7:1, then a two person "major" component can be distinguished. If the mixture ratio ranged from 4:***3:1*** to 8:7:1, then the two person "major" component cannot be distinguished at this locus.

Two allele loci



- In order to distinguish a "major" component, the adjusted total rfu value for the lower "major" component must be at least four times the maximum expected contribution of the “minor” component. This then would be based on the mixture ratio calculated previously. For example, if the range of the mixture ratio varied from 5:5:1 to 8:7:1, then a two person "major" component can be distinguished. If the mixture ratio ranged from 4:***3:1*** to 8:7:1, then the two person "major" component cannot be distinguished at this locus.

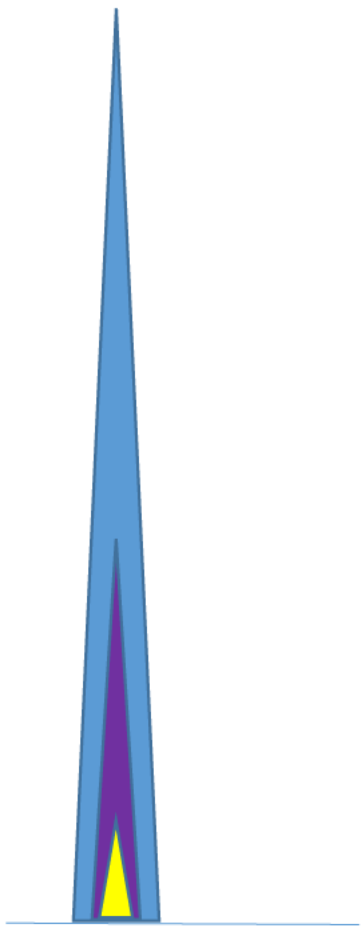
Two allele loci



Based on the lowest contribution of the second “major” contributor from the range of mixture ratios observed, the “major” component allele will be adjusted by subtracting the greatest possible sister allele of the observed "minor" allele.
A "major" component can be distinguished if the adjusted sum of the second “major” component is at least four times greater than the sum of the “minor” component.
Example: Range of observed mixture ratio – 6:5:1 to 8:6:1.

“Major” allele – greatest possible “minor” sister allele:
4100 – 303 = 3797
Proportion of second “major” component based on 6:5 =
(3797 / 11) * 5 = 1726 rfu
1726 / (100 + 303) = 4.3
Therefore, "major" component ***can*** be distinguished

One allele loci



- A “major” component can be distinguished if, based on the mixture ratio range calculated from the entire profile, the lowest “major” component is at least four times greater than the highest “minor” component.

- ***Example:*** Mixture ratio range ***4:3:1*** to 8:5:1, the "major" component ***cannot*** be distinguished.

- ***Example:*** Mixture ratio range 4:4:1 to 7:5:1, a "major" component ***can*** be distinguished.

1. Scope

The following guidelines outline the wording to be used when reporting the results of Forensic Biology analyses. These guidelines cannot encompass all possible circumstances encountered during casework analysis. Therefore, it is assumed that it may be necessary to deviate from the guidelines in certain circumstances.

2. References

2.1 Not applicable

3. Safety / Quality Assurance

3.1 Reports will undergo a technical review in accordance with ATF Laboratory Services ATF-LS-5.9.4A.

3.2 Reports will undergo an administrative review in accordance with ATF Laboratory Services ATF-LS-5.9.4A.

4. Equipment

4.1 Not applicable

5. Procedure

5.1 The general report format will comply with the relevant ATF Policy and Procedures for reporting of results.

5.2 If DNA analysis was performed, the first paragraph in the Results Section shall be stated as follows:

5.2.1 DNA analysis performed at the Bureau of Alcohol, Tobacco, Firearms and Explosives Laboratory utilizes the Polymerase Chain Reaction and the *relevant amplification kit* to examine the following STR loci: *loci.* The methods used during analysis adhere to the Quality Assurance Standards for Forensic DNA Testing Laboratories.

5.3 Results shall be grouped by exhibit.

5.4 Results of serological examinations

5.4.1 If an exhibit was examined for the presence of biological material but no suitable areas were found for testing:

5.4.1.1 No biological stains were observed on the *item* (Exhibit *X*).

5.4.2 If an item is not suitable for forensic biology examination:

5.4.2.1 The *item* (Exhibit *X*) is not suitable for forensic biology examination.

5.4.3 Results of testing for the presence of blood:

5.4.3.1 Blood was indicated on the *item* (Exhibit *X*). No confirmatory testing was performed.

5.4.3.2 No blood was detected on the *item* (Exhibit *X*).

5.4.3.3 Results for the examination of blood were inconclusive on the *item* (Exhibit *X*).

5.5 Evidence sampling

5.5.1 If the evidence is sampled in one or more areas for DNA analysis, the following statement will precede the results statement:

5.5.1.1 The following areas of the *item* were swabbed/sampled for DNA analysis:

5.5.1.2 If all the DNA extracts are combined into a single DNA extract, the following statement shall be made:

5.5.1.2.1 These samples were combined into a single sample during DNA analysis (Exhibit *X.Qcombo*).

5.5.1.3 If a portion of the extracts are combined into a single DNA extract, the following statement shall be made:

5.5.1.3.1 The *swabs/cuttings/etc.* of the *area 1* (Exhibit *X.QX*) and the *swabs/cuttings/etc.* of *area 2* (Exhibit *X.QY*) were combined into a single sample during DNA analysis (Exhibit X.Qcombo).

5.6 If no DNA analysis was performed on the swabs/sampling of the exhibit, the following statement will be used:

5.6.1 No DNA analysis was performed on the swabs/sampling of the *item* at this time.

5.6.2 As per ATF Laboratory policy, the reason for not performing DNA analysis must be stated. For example, the request for examination was withdrawn.

5.7 Results for DNA examination

5.7.1 For known reference samples (blood, buccal swabs, alternate knowns, etc.):

5.7.1.1 A *male/female* DNA profile was obtained from a *cutting/sample/etc.* of the *item* from *John Doe* (Exhibit *X.QX*).

5.7.2 If no DNA profile was obtained for an item:

5.7.2.1 No DNA profile was obtained from *a swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*).

5.7.3 If limited data was obtained for an item:

5.7.3.1 Due to limited results, the DNA profile obtained from *a swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) is not suitable for comparison purposes.

5.7.4 If a partial, **single source** profile was obtained for an item:

5.7.4.1 A partial *male/female* DNA profile was obtained from a *swabbing/cutting/etc.* of the item (Exhibit *X.QX*).

5.7.5 If a complete **single source** profile was obtained for an item:

5.7.5.1 A *male/female* DNA profile was obtained from a *swabbing/cutting/etc.* of the item (Exhibit *X.QX*).

5.7.6 If a partial or full single source profile was obtained from an item, the above statement shall be followed by a conclusion statement.

5.7.6.1 Exclusion

5.7.6.1.1 If the known reference profile(s) is determined to be inconsistent with the unknown DNA profile and therefore the known reference is excluded as a possible contributor of the unknown biological material:

5.7.6.1.1.1 This DNA profile is inconsistent with the DNA profile of *John Doe* (Exhibit *Y.QX*). Therefore, *John Doe* is excluded as the source of the biological material found on the *item*.

5.7.6.2 Inclusion

5.7.6.2.1 If the known reference profile is determined to be consistent with the unknown DNA profile and therefore the known reference is included as a possible contributor of the unknown biological material:

5.7.6.2.1.1 This DNA profile is consistent with the known profile of *John Doe* (Exhibit *Y.QX*). Therefore, *John Doe* cannot be excluded as the source of the biological material found on the *item*.

5.7.6.2.1.2 If a second reference profile is determined to be excluded, the following statement shall be added: *Bob Doe* (Exhibit *Y.QX*) is excluded as the source of the biological material found on the *item.*

5.7.7 If a partial mixed profile was obtained for an item:

5.7.7.1 A partial DNA profile consisting of a mixture of at least *(minimum # of contributors)* individuals was obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*). **NOTE: If an amelogenin Y peak is detected, then add the following: At least one of the contributors is a male.**

5.7.8 If a complete mixed profile was obtained for an item:

5.7.8.1 A DNA profile consisting of a mixture of at least *(minimum # of contributors)* individuals was obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*). **NOTE: If an amelogenin Y peak is detected, then add the following: At least one of the contributors is a male.**

5.7.9 If no conclusions regarding inclusion or exclusion can be drawn due to the complexity or low level nature of the mixture, the following statement will be used:

5.7.9.1 Due to the complex nature of this mixed DNA profile, this DNA profile is not suitable for comparison purposes.

5.7.10 If a partial or complete mixed profile that is suitable for comparison for which a major and minor component cannot be determined was obtained from an item, the initial results statement shall be followed by a conclusion statement:

5.7.10.1 Exclusion

5.7.10.1.1 If it is determined that the known reference profile(s) is not a possible contributor to the unknown DNA mixed profile:

5.7.10.1.1.1 The DNA profile of *John Doe* (Exhibit *X.QX*) is not consistent with being a contributor to this mixture. Therefore, *John Doe* is excluded as a possible contributor to the biological material found on the *item*.

5.7.10.2 Inclusion

5.7.10.2.1 If it is determined that the known reference profile(s) is a possible

contributor to the unknown DNA mixed profile:

5.7.10.2.1.1 The DNA profile of *John Doe* (Exhibit *X.QX*) is consistent with being a contributor to this mixture. Therefore, *John Doe* cannot be excluded as a possible contributor to the biological material found on the *item*.

5.7.11 If a partial or complete mixed profile that is suitable for comparison for which a major and minor component can be determined was obtained from an item, the initial statement above shall be followed by a conclusion statement.

5.7.11.1 Exclusion

5.7.11.1.1 If it is determined that the known reference profile(s) is not a possible contributor to the unknown DNA mixed profile (both the major and minor components):

5.7.11.1.1.1 The DNA profile of *John Doe* (Exhibit *X.QX*) is not consistent with being a contributor to this mixture. Therefore, *John Doe* is excluded as a possible contributor to the biological material found on the *item*.

5.7.11.2 Inclusion

5.7.11.2.1 If it is determined that the known reference profile(s) is a possible contributor to the major component of the unknown DNA mixed profile(s):

5.7.11.2.1.1 The major component of this mixture is consistent with the DNA profile of *John Doe* (Exhibit *X.QX*). Therefore, *John Doe* cannot be excluded as the source of the major component of the biological material found on the *item*.

OR

5.7.11.2.1.2 The DNA profile of *John Doe* (Exhibit *X.QX*) is consistent with being a contributor to the minor component of this mixture. Therefore, *John Doe* cannot be excluded as a possible contributor to the minor component of the biological material found on the *item*.

5.8 Alternatively, the results and conclusions can be reported in a table format.

5.9 If a known reference is included as a possible contributor to a single source profile or mixed profile and the result is probative, a statistical result shall be reported.

5.9.1 A Random Match Probability calculation shall be reported if the unknown profile is a single source or is a major component of a distinguishable mixture:

5.9.1.1 Assuming a single contributor, the probability of randomly selecting an unrelated individual with a DNA profile consistent with the DNA profile obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) is listed below for the following populations:

1 in *X (billion, trillion, etc.)* US Caucasians
1 in *X (billion, trillion, etc.)* US African Americans

1 in *X (billion, trillion, etc.)* US Southwest Hispanics

OR

5.9.1.2 Assuming the major component is a single contributor, the probability of randomly selecting an unrelated individual with a DNA profile consistent with the major component of the DNA profile obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) is listed below for the following populations:

1 in *X (billion, trillion, etc.)* US Caucasians
1 in *X (billion, trillion, etc.)* US African Americans
1 in *X (billion, trillion, etc.)* US Southwest Hispanics

5.9.2 A Random Match Probability calculation shall be reported if the unknown profile is a mixture with indistinguishable major and minor components or a mixture with a distinguishable major and minor component for which the minor component is probative:

5.9.2.1 Assuming *N* number of contributors, the probability of randomly selecting an unrelated individual who could be included as a contributor to the mixture obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) is listed below for the following populations:

1 in *X (billion, trillion, etc.)* US Caucasians
1 in *X (billion, trillion, etc.)* US African Americans
1 in *X (billion, trillion, etc.)* US Southwest Hispanics

OR

5.9.2.2 Assuming *N* number of contributors, the probability of randomly selecting an unrelated individual who could be included as a contributor to the minor component of the mixture obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) is listed below for the following populations:

1 in *X (billion, trillion, etc.)* US Caucasians
1 in *X (billion, trillion, etc.)* US African Americans
1 in *X (billion, trillion, etc.)* US Southwest Hispanics

5.9.3 When stating the statistical results for a partial profile (single source or a mixture), any loci not used for the calculation due to inconclusive results shall be listed. Alternatively, the loci used for the calculation may be listed.

5.9.3.1 The following loci were not used in the statistical calculation because the loci did not meet the required interpretation criteria: *Locus A, Locus B, Locus C, etc.*

OR

5.9.3.2 The following loci were used in the statistical calculation: *Locus A, Locus B, Locus C, etc*. The remaining loci did not meet the required interpretation criteria.

5.9.4 The disposition of all DNA extracts shall be noted in the Disposition of Evidence section of the report.

5.9.4.1 DNA Extracts Retained: The following DNA extracts will be retained by ATF Laboratory: Exhibits *1.Q1, 2.Q1, etc.*

5.9.4.2 DNA Extracts Consumed in Analysis: The following DNA extracts were consumed in analysis: Exhibits *1.Q1, 2.Q1, etc.*

5.9.5 If any potential biological evidence is remaining on an exhibit, the following statement shall be made at the end of the report

5.9.5.1 **Note: Exhibit *X* may potentially contain biological evidence subject to specific storage and preservation requirements. Please reference ATF O 3400.1D and the *ATF Property Taken into Bureau Custody Manual* to review the storage and preservation requirements of this evidence for the purposes of possible future DNA analysis.**

5.10 CODIS Eligibility

5.10.1 NDIS Eligible

5.10.1.1 At least 10 of core 13 loci (at least one locus meeting the requirements for statistical analysis) and meets other eligibility criteria (i.e. directly associated with crime).

5.10.1.1.1 The DNA profile obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) will be entered into the Combined DNA Index System (CODIS) and searched at the national level.

5.10.2 SDIS Eligible

5.10.2.1 At least 8 of core 13 loci (at least one locus meeting the requirements for statistical analysis) and meets other eligibility criteria (i.e. directly associated with crime).

5.10.2.1.1 The DNA profile obtained from a *swabbing/cutting/etc.* of the item (Exhibit *X.QX*) will be entered into the Combined DNA Index System (CODIS). Due to the profile's limited nature, it can only be searched against DNA profiles generated by the ATF and other federal law enforcement agencies.

5.10.3 LDIS Eligible

5.10.3.1 At least 6 of the 15 loci, at least one locus meeting the requirements for statistical analysis, meets other eligibility criteria (i.e. directly associated with crime)

5.10.3.1.1 The DNA profile obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) will be entered into the Combined DNA Index System (CODIS). Due to the profile's limited nature, it can only be searched against other ATF-generated DNA profiles.

5.10.4 For PGI only, non-PGI, etc.

5.10.4.1 Low-level samples going into the PGI

5.10.4.1.1 Due to limited results, the DNA profile obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) cannot be entered into

the Combined DNA Index System (CODIS).

5.10.5 Mixture violating 4x4 rule

5.10.5.1 Due to the nature of this mixture, the mixture DNA profile obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) cannot be entered into the Combined DNA Index System (CODIS).

5.10.6 If waiting for a reference before making determination regarding uploading / association with crime / entry into LDIS / etc.

5.10.6.1 At this time, the DNA profile obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) cannot be entered into the Combined DNA Index System (CODIS).

5.10.7 Unable to determine if "directly associated with a crime".

5.10.7.1 Due to the nature of the evidence, the DNA profile obtained from a *swabbing/cutting/etc.* of the *item* (Exhibit *X.QX*) cannot be entered into the Combined DNA Index System (CODIS) at this time.

5.10.8 Solved forensic profile (matches submitted suspect, for example).

5.10.8.1 Use appropriate CODIS eligibility verbiage from above.

5.11 Evidentiary Profiles Associated with Contamination or Failed Controls

5.11.1 For evidentiary samples associated with contamination or failed controls where it is reasonable to assume that the integrity of the DNA analysis was not affected, the results shall be reported as described above.

5.11.2 For evidentiary samples associated with contamination or failed controls where it is reasonable to assume that the integrity of the DNA analysis may have been affected, the results shall be reported as described above. In addition, a note shall be made in the report briefly describing the issue and stating the results should be interpreted with caution.

5.11.3 For single source evidentiary samples demonstrating the presence of a staff member or a known contaminant, a note shall be made in the report describing the issue.

5.11.4 For mixed DNA evidentiary profiles demonstrating the presence of a staff member or known contaminant, a note shall be made in the report describing the issue. The LR may be calculated conditioning on the presence of the staff member or known contaminant based on the analyst's discretion and with DNA Technical Leader Approval.

5.12 Comparisons to reference DNA profiles from another Laboratory

5.12.1 If a reference DNA profile generated by another laboratory was used for comparison to an ATF-generated evidentiary profile, a note shall be made in the report describing the source of the reference DNA profile.

5.12.1.1 Example: "The DNA profiles obtained from the evidence in this case were compared to DNA profiles identified as coming from Jonathan Doe (SERI Exhibit 2A) and Christopher Doe (SERI Exhibit 4) analyzed and reported by the Serological Research Institute, SERI (see SERI report dated May 5, 2014,

signed by Thomas Brown). SERI was accredited by the American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD/LAB) at the time the analysis was conducted."

5.13 Reporting multiple consistent profiles from the same exhibit

5.13.1 If multiple DNA profiles are obtained from the same exhibit, but each has a different statistical weight, the group of profiles may be reported together using the lowest statistical weight to describe the entire group.

5.13.1.1 Example: "Assuming a single contributor, the probability of randomly selecting an unrelated individual with a DNA profile consistent with the DNA profiles obtained from the *swabbings/cuttings/etc.* of the *item* (Exhibit *X.QX*) is listed below for the following populations:

At least 1 in *X (billion, trillion, etc.)* US Caucasians
At least 1 in *X (billion, trillion, etc.)* US African Americans
At least 1 in *X (billion, trillion, etc.)* US Southwest Hispanics

CONTENTS

1. Scope 2
2. References 2
3. Equipment 3
4. User Roles and Responsibilities 3
5. Specimen Categories 5
DNA Index of Special Concern (DISC) 6
Figure 1: NDIS Categories 8
Figure 2: SDIS Categories 9
Figure 3: LDIS Categories 10
6. Determining CODIS Eligibility 10
Figure 4: ATF DNA Profile Calculator ≥95% Logic Tree 12
7. Entering Specimens in CODIS 12
8. CODIS Entry Documentation for Case Files and Uploading Profiles 14
Technical Review of CODIS Data 14
Deleting Specimens 15
Uploading Profiles 16
9. Searching The CODIS Indexes 16
10. Evaluating Matches 18
11. Match Confirmation Process 21
Offender and External Forensic Hits 21
CODIS Hit Documentation and Review 22
Conviction Matches 23
Internal Forensic Hits 24
Staff and Contamination Hits 25
Internal Forensic Hits Involving the pgi (PGI Matches) 25
12. CODIS Access and Data Security 26
Backups 27
13. Known Sample Inquiry 27
Appendix 1 – Offender / Arrestee Match Data Request Form 28
Appendix 2 – Casework Match Data Response Form 29

Appendix 3 – Offender and Arrestee Hit Memorandum 30
Appendix 4 – Forensic Hit Memorandum 31
Appendix 5 – NDIS Keyboard Search Request Form 32
Appendix 6 – SDIS Keyboard Search Request Form 34
Appendix 7 – Federal Convicted Offender / Arrestee Sample Possession Inquiry 36
Appendix 8 – PGI Match Notification 37

1. SCOPE

The Combined DNA Index System (CODIS) is a collection of DNA databases from forensic DNA casework and databasing laboratories throughout the United States that enables federal, state, and local DNA laboratories to compare DNA profiles and exchange information in order to link crimes to one another and to offenders.

There are three different levels of CODIS:

1. Local DNA Index System (LDIS). All eligible forensic casework DNA profiles generated by the ATF Laboratory are entered and searched in LDIS.
2. State DNA Index System (SDIS). The Federal Bureau of Investigation (FBI) serves as the ATF's SDIS Laboratory. Eligible profiles at SDIS are searched against profiles from the FBI's Federal Forensic DNA Database Unit (FDDU) and Forensic DNA Casework Unit (DCU).
3. National DNA Index System (NDIS). NDIS is the highest level of CODIS, is maintained and operated by the FBI, and consists of applicable DNA profiles contributed by all SDIS laboratories.

The use of CODIS by the ATF will be in accordance with current versions of the ATF Forensic Biology Training Manual and protocols, the NDIS Operational Procedures Manual, the CODIS Training Manual, and CODIS technical notes provided by the FBI and its contractor.

The 13 Original Core CODIS Loci are: CSF1PO, FGA, THO1, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11. The expanded Core CODIS Loci are: D1S1656, D2S441, D2S1338, D10S1248, D12S391, D19S433, and D22S1045.

2. REFERENCES

i. NDIS Operational Procedures Manual, current version
ii. CODIS Administrator's Handbook, current version
iii. The FBI Quality Assurance Standards for Forensic DNA Testing Laboratories, effective July 1, 2020

iv. ATF Forensic Biology Methods of Analyses

3. EQUIPMENT

i. CODIS software, most recent version
ii. CODIS server
iii. CODIS workstation(s)

4. USER ROLES AND RESPONSIBILITIES

i. *ATF CODIS Administrator*: Manages the day-to-day operations of the ATF CODIS system, serves as the central point of contact for CODIS, and is the gatekeeper for DNA records entered into CODIS by the ATF Laboratory
    a. Responsibilities:
        i. Administer the ATF's local CODIS network
        ii. Schedule and document the CODIS computer training of analysts
        iii. Ensure the security and quality of data stored in CODIS is in accordance with federal law and NDIS operational procedures
        iv. Ensure the matches are dispositioned in accordance with NDIS operational procedures
        v. Authorized to terminate an analyst's or laboratory's participation in CODIS until the reliability and security of computer data can be assured, in the event an issue with the data is identified
        vi. Notify State CODIS Administrator of Add/Remove/Update CODIS User information
        vii. Ensure that all CODIS users have successfully completed the required annual training
        viii. Notify the NDIS Custodian, within five business days, if any of the following occur:
            1. If a CODIS User has been arrested for, or convicted of, a criminal offense;
            2. If the laboratory loses its criminal justice agency status;
            3. If the laboratory loses its accreditation, has its accreditation suspended, or has its accreditation revoked;
            4. If the laboratory loses its capability to perform DNA analysis at its facility;
            5. If the laboratory has fewer than two full-time qualified DNA analysts;
            6. If the laboratory has a vacancy in the Technical Leader position when there is no one qualified (per QAS standards) to serve in the position; or,

7. If the laboratory is not in compliance with the external QAS audit requirements.

ix. Review and/or approve, as appropriate, protocols for the entry, searching, and match resolution of DNA records in the local DNA database
x. Ensure compliance with CODIS security requirements
xi. Ensure compliance with QAS Standards, including Standard 17
xii. Ensure NDIS specimen eligibility is determined by CODIS Users
xiii. Upload profiles to SDIS/NDIS
xiv. Review CODIS generated reports
xv. Backup CODIS data, including periodic restores (to be conducted at least once annually) to ensure backups are working properly
xvi. Review all CODIS materials and changes to NDIS Operational Procedures Manual
xvii. Compile and report Investigations Aided and Hit Statistics to SDIS on a monthly basis
xviii. Complete Annual Audit Certificate

b. Qualifications:
   i. Current or previously qualified ATF Forensic Biologist with mixture interpretation training
   ii. Meets educational and experience qualifications specified in the QAS
   iii. Complete the FBI-sponsored CODIS software training within six months of assuming role
   iv. Complete the FBI's DNA auditor training course within one year of assuming role
   v. Shall attend the regularly scheduled annual CODIS conference, unless unavailable, in which case the ATF Alternate CODIS Administrator shall attend
      1. If neither the ATF CODIS Administrator nor the ATF Alternate CODIS Administrator are available to attend the annual CODIS conference, a request for an excused absence shall be made in writing, signed by the ATF Laboratory Chief, and approved by the NDIS Custodian

ii. *ATF Alternate CODIS Administrator*: Serves as back-up to CODIS Administrator
   a. Responsibilities:
      i. Fulfills the CODIS Administrator role during the absence or unavailability of the CODIS Administrator
      ii. May assist with the maintenance and troubleshooting of hardware and software for CODIS

1. An Alternate CODIS Administrator whose primary responsibility is maintenance of hardware and software for the CODIS system may be referred to as *Alternate CODIS Administrator – IT*

iii. Shall be designated within 90 days of a vacancy in the role

b. Qualifications:

i. Current or previously qualified ATF Forensic Biologist with mixture interpretation training
ii. Meets educational and experience qualifications specified in the QAS
iii. Complete the FBI-sponsored CODIS software training within six months of assuming role
iv. Complete the FBI's DNA auditor training course within one year of assuming role
v. May attend the regularly scheduled annual CODIS conference, either in conjunction with, or in place of, the ATF CODIS Administrator

Note: From herein, the term CODIS Administrator(s) *will be used to describe any function or responsibility that either the ATF CODIS Administrator or ATF Alternate CODIS Administrator(s) may perform. The term* ATF CODIS Administrator *shall be used to define any function or responsibility that* only *the ATF CODIS Administrator may perform.*

iii. *ATF CODIS User:* ATF Forensic Biologist who has successfully completed CODIS training, has log-in access to CODIS, and is authorized to add, modify, delete, and read DNA records

a. Responsibilities:

i. Enter and search specimen data
ii. Mark specimens for upload
iii. Complete CODIS paperwork
iv. Complete Annual Review of DNA Data Accepted at NDIS training

b. Qualifications:

i. Must be a qualified ATF Forensic Biologist
ii. Must have successfully completed CODIS section of ATF Forensic Biology Training Manual

5. SPECIMEN CATEGORIES

i. NDIS Categories: See Figure 1 for specific category criteria and eligibility

a. Forensic, Unknown
b. Forensic Partial
c. Forensic Mixture
d. Forensic Targeted

ii. SDIS Categories: See Figure 2 for specific category criteria and eligibility

a. SDIS Only
b. SDIS Only FT

iii. Forensic LDIS Only: See Figure 3 for specific category criteria and eligibility
   a. Possession crimes (i.e. Felon-in-Possession) may be eligible for entry at the LDIS level only
      i. For non-possession crimes, suspect DNA recovered on evidence collected from the suspect's person or personal property is considered a deduced suspect profile and is not eligible for entry at LDIS

iv. Profiles Generated Index (PGI): Database of ATF laboratory-generated DNA profiles that are not eligible for CODIS entry, and is used to aid in the detection of contamination; See Figure 3 for additional information
   a. Technically an index within LDIS, but treated as a separate entity
   b. See *Contamination Event Work Instructions* for more information on how to handle contamination events
   c. The PGI is purged on an annual basis of any specimens that are more than one year older than the date that the purge is performed
      i. The delete report for each purge will be maintained

v. Staff: The Staff Index contains DNA profiles from ATF Laboratory personnel, ATF and other law enforcement personnel, and visitors requiring entry into laboratory workspace

<u>DNA INDEX OF SPECIAL CONCERN (DISC)</u>

The DISC is an index that allows specific forensic profiles to be searched in real-time against arrestee profiles that are entered at an approved Rapid Booking Station. Profiles in the DISC are also referred to as "Rapid-Enabled."

i. To be DISC-eligible, a profile must:
   a. Be a Forensic, Unknown profile
   b. Have a Source ID of **No**
   c. Be from an unsolved case of significant public safety concern, which are classified as four distinct categories:
      i. Rape/sexual assaults
      ii. Homicides
      iii. Kidnappings
      iv. Terrorism, including domestic terrorism

ii. If the Source ID is ever changed to **Yes**, the specimen will automatically be removed from the DISC

iii. The Statue of Limitations must be obtained from the investigating agent/prosecutor by the Examiner and documented in the case Communication Log
   a. Once the Statute of Limitation expires, the specimen will automatically be removed from DISC

iv. The examiner must complete the *DISC Enrollment Form*, which will be maintained by the CODIS Administrators. The *DISC Enrollment Form* contains the following casework metadata fields, and must be technically reviewed prior to DISC-enabling a profile:

a. Investigating Agency ID: DCATF0000, the ORI for the ATF JSOC desk, will be used for all specimens
    i. The Unsolicited DNA Notification (UDN) messages containing hit information will be routed through this ORI
b. Investigative Case Tracking ID: The Investigation Number (IN)
c. Investigative Case Alias: Case Title
d. Investigator Email Address: The investigating agent's email address
e. Investigator Phone #: The cell phone number of the investigating agent
f. Statute of Limitation: Entered in MM-DD-YYYY format, this is the charge filing deadline for the crime
g. Offense Description: Brief (<64 characters) description of type of crime that the evidence is associated with
    i. Generally, we will use one of the following descriptions, but may choose to expand depending on case scenario:
        1. Homicide
        2. Terrorism
        3. Kidnapping
        4. Rape/sexual assault (rare)
    ii. May wish to enter additional information if high priority case (i.e. Austin Bombings, Florida serial shooter, etc.)
h. Extradition Information: The ATF must be willing to extradite in order to allow for DISC-enabling
    i. The minimum accepted input is "Yes"
    ii. This must be confirmed with the investigating agent and documented in the case Communication Log
    iii. Also possible to enter PENDING EXTRADITION DETERMINATION if the ATF is unable to commit to extradition because it is not ultimately their decision
i. Investigative Agency Contact Information: Any additional contact information or comments specific to the case
    i. Always enter: "JSOC 24/7 phone number: 202-648-7200"

FIGURE 1: NDIS CATEGORIES

Level	*Specimen Category*	*MME*	*MRE*	*Minimum # of Loci*	*Eligibility Requirements*
NDIS	Forensic, Unknown	≥ 1.0 x10^7	---	13 Original Core	Single source (SS) DNA profile, or fully-deduced SS component in a mixture, from a putative perpetrator
					One tri-allele core locus permissible
					Acceptable to enter ≥95% profile based on STRmix™ genotype weightings
					No locus or allelic drop-out allowed at the 13 Core loci
	Forensic Partial	≥ 1.0 x10^7	---	≥ 8 of the 13 Original Core	SS DNA profile, or fully-deduced SS component in a mixture, from a putative perpetrator
					Locus **OR** allelic drop-out at one or more of the 13 Original Core loci
					Acceptable to enter ≥95% profile based on STRmix™ genotype weightings
					One tri-allele core locus permissible
	Forensic Mixture	≥ 1.0 x10^7	---	≥ 8 of the 13 Original Core	Profile containing DNA from more than one source, or a component with a ≥95% profile composed of several genotype combinations, from a putative perpetrator
					Acceptable to enter ≥95% profile based on STRmix™ genotype weightings
					No more than 4 alleles per locus
	Forensic Targeted	---	≥ 1.0 x10^7	≥ 8 of the 13 Original Core	DNA profile from a putative perpetrator that does not meet the MME threshold for entry into NDIS
					Partial loci and loci with more than 2 alleles are searched at *Moderate* stringency; all other loci are searched at *High* stringency
					Only searched against full, SS profiles (Forensic, Unknown; Convicted Offender; Arrestee; Detainee; Juvenile, and; Legal Indexes)
					Acceptable to enter ≥95% profile based on STRmix™ genotype weightings
					No more than 4 alleles per locus
					Specimen category of last resort

FIGURE 2: SDIS CATEGORIES

Level	*Specimen Category*	*MME*	*MRE*	*Minimum # of Loci*	*Eligibility Requirements*
S D I S	SDIS Only	≥ 1.2 x10^6	---	≥ 7 Core loci (not necessarily Original Core), **excluding** SE33 and D22S1045	DNA profile from a putative perpetrator that does not meet the eligibility requirements for entry into NDIS
					Acceptable to enter ≥95% profile based on STRmix™ genotype weightings
					No more than 4 alleles per locus
	SDIS Only FT	---	≥ 1.2 x10^6	≥ 7 Core loci (not necessarily Original Core), **excluding** SE33 and D22S1045	DNA profile from a putative perpetrator that does not meet the MME threshold for entry into NDIS or SDIS
					Acceptable to enter ≥95% profile based on STRmix™ genotype weightings
					No more than 4 alleles per locus

FIGURE 3: LDIS CATEGORIES

Level	*Specimen Category*	*MME*	*MRE*	*Minimum # of Loci*	*Eligibility Requirements*
LDIS	Forensic LDIS Only	≥ 1.0 x10^4	---	≥ 6 loci, **excluding** sex-determining loci	DNA profile from a putative perpetrator that does not meet the eligibility requirements for entry into NDIS or SDIS
					Acceptable to enter ≥95% profile based on STRmix™ genotype weightings
					No more than 4 alleles per locus
					Certain possession cases are permissible in LDIS, although not eligible in NDIS/SDIS:
					- If the item is recovered from a suspect or their personal property, but is linked to a possession crime, the resulting profile can be entered into LDIS only
OTHER	PGI	≥ 1.0 x10^4	---	≥ 6 loci, **excluding** sex-determining loci	Profile does not meet eligibility requirements for entry into LDIS, SDIS, or NDIS for one or more of the following reasons:
					- Evidence cannot be linked to a crime and/or putative perpetrator
					- Profile is of undetermined source (i.e. contaminant in a control sample)
	Staff	---	---	Full profile should be attempted*	DNA profiles from the ATF Laboratory staff, voluntary samples from ATF and law enforcement personnel, and visitors requiring entry into laboratory workspace
					Profiles maintained indefinitely

**Some staff samples are older and/or low-level, and therefore may exhibit partial or full drop-out at one or more loci. In these instances, if no sample remains for re-testing, a note should be entered in CODIS indicating why a full profile was not entered.*

6. DETERMINING CODIS ELIGIBILITY

i. DNA profiles entered into CODIS must be:
   a. Suitable for comparison and statistical analysis
   b. From crime scene evidence
   c. Only contain alleles that are attributable to the putative perpetrator(s)
      i. Alleles derived from a forensic sample that are unambiguously attributed to the victim or individuals other than the putative perpetrator(s) will *not* be entered into CODIS

d. Derived from analysis of NDIS-accepted PCR loci/systems

ii. The ATF CODIS Administrator has the final say on determining the eligibility of a DNA profile for CODIS entry

iii. Generally, the >=99% Contributor Summary should be the starting-off point for all entries

a. If the >99% profile is not suitable for NDIS, then a ≥95% Profile should be evaluated for CODIS entry. The ≥95% profile will be calculated from the STRmix™ deconvolution output by adding the genotype weights (percentages) in the Component Interpretation section of the STRmix™ output. The DNA Profile Calculator may be used to aid in the calculation of the ≥95% profile for CODIS entry. Alternatively, the calculation can be performed manually using the logic tree below (Figure 4).

i. CODIS entries that do not meet the ≥95% threshold, or ≥95% profiles that deviate from the logic tree, must be approved by a CODIS Administrator.

b. CODIS entries should contain enough information to be uploaded to Forensic, Unknown, Forensic Partial, or Forensic Mixture Categories if possible. If the >99% profile is only eligible for Forensic Targeted or SDIS categories, a ≥95% profile should be calculated to evaluate if additional data can be added to the CODIS entry.

iv. When multiple samples yield consistent >99/95% profiles eligible for CODIS entry, only the most complete DNA profile will be entered into CODIS

v. Obligate alleles should be designated in CODIS (the plus "+" sign) for alleles that are definitively attributed to a putative perpetrator

a. Only one obligate allele can be selected per locus

b. Using obligate alleles can improve MME and Match Rarity Estimate (MRE) calculations

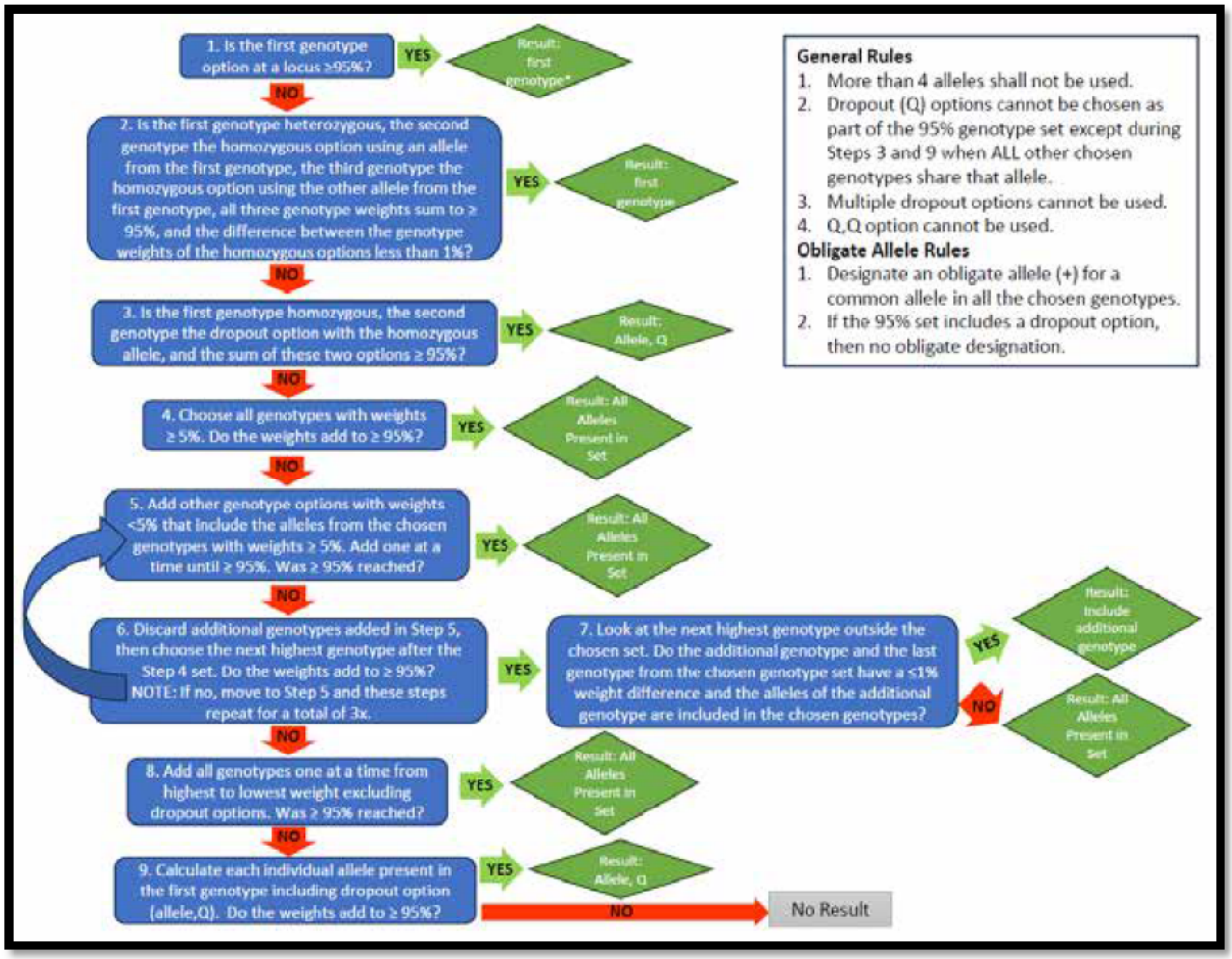
vi. Conditioning mixtures that are not well resolved may assist in calculating a ≥95% profile eligible for CODIS entry

a. See *How to Condition a ≥95% Contributor Profile from a Mixture Work Instructions* for guidance on conditioning

b. A profile may be conditioned on a known reference profile or an unknown >99% component profile from another evidence sample if there is support for inclusion

c. If the unconditioned data produces a CODIS entry eligible for Forensic, Unknown, Forensic Partial, or Forensic Mixture categories, then it is not necessary to condition the profile and the unconditioned deconvolution should be used for CODIS entry.

FIGURE 4: ATF DNA PROFILE CALCULATOR ≥95% LOGIC TREE



7. ENTERING SPECIMENS IN CODIS

i. *Specimen ID*: Unique identifier for the DNA profile

a. Will be in the format of "Case Number_Sub-Exhibit Number_Brief Description_Contributor Designator"

i. Example: 21W0123_2.1_GRIP_C1

ii. If the DNA profile is an indistinguishable two-person mixture, the designator of "MIX," or "C1C2" shall be used

1. Example: 21W0123_3.1_AMMOMAG_MIX

iii. If the DNA profile is from a single source sample, no designator needs to be used

iv. In instances where conditioning was performed, the contributor designator shall align with the contributor position in the *original* deconvolution

b. For elimination samples submitted as evidence, the designator of "CSI" or "LEO" shall be used
    i. Example: 23W0123_3.1_CSI
    ii. Only elimination samples submitted from individuals involved in the collection and/or packaging of evidence will be entered into PGI
c. For staff elimination samples that are entered into the Staff Index, the staff sample will be assigned the next available anonymous number aligning with the appropriate category (i.e. FSLW-XXX, SA-XXX)

ii. *Specimen Category*: The category where the profile will reside
    a. See Section 5 Specimen Categories
    b. If the Specimen Category is changed after a final report is issued, the customer will be notified why the change was made and what the new category is
    c. Additional categories are available and may be used on rare occasions

iii. *Source ID*: Conveys whether a known individual is included as a contributor to the profile
    a. Select **Yes** if an individual was included as a possible contributor to the profile
    b. Select **Yes** if the profile is from an elimination or staff reference sample and being entered into PGI
    c. Select **No** if the source is unknown
    d. Note: if a CODIS hit to an offender sample occurs, the Source ID will automatically change from No to Yes once the match disposition has been updated to reflect the hit confirmation

iv. *Partial Profile*: Designates if <u>allelic</u> drop-out may have occurred in a specimen
    a. Based on the completeness of allelic information at each locus, *not* the completeness of the DNA profile as a whole
        i. Full locus dropout is accounted for by the Specimen Category of Forensic Partial
    b. When Partial Profile is **Yes**, the locus/loci demonstrating possible allelic dropout shall be designated as Partial – **Yes**
        i. Changing the Partial indicator at the locus level to Yes will automatically change the Partial Profile designator to Yes

v. *Case ID*: Optional field to provide additional information about DNA profiles in PGI/LDIS
    a. May be one of the following designations:
        i. **REF**: Used for elimination reference samples
        ii. **NATURE**: Used for PGI casework samples not eligible for LDIS due to case circumstances (i.e. cannot be linked to a crime and/or a putative perpetrator)
        iii. **POSSESSION**: Used for Forensic LDIS Only profiles obtained from evidence recovered directly from the putative perpetrator or from the

personal property of the putative perpetrator and therefore may only be entered in LDIS

vi. *STR/Y-STR Comments*: Optional field to provide additional information for the CODIS Administrators to be aware of
    a. This field does not get uploaded with the specimen, and is only viewable by the ATF Laboratory

8. CODIS ENTRY DOCUMENTATION FOR CASE FILES AND UPLOADING PROFILES

i. The *CODIS/PGI Specimen Entry Log* shall be used to document all profiles entered in CODIS
    a. The Justification section shall clearly state how each profile qualifies for the category in which it is being entered
        i. Supporting documentation must exist to support justification, and may include:
            1. Reports of Investigation (ROI)
            2. E-mail or phone communication with investigators
            3. Supporting documentation provided on or with the Laboratory Exam Request Form

ii. The ATF DNA Profile Calculator Profile Comparisons Table shall be included in the case file just before the CODIS section. The Profile Comparisons Table shows which profiles will be used for CODIS entry.

iii. The *CODIS/PGI Specimen Entry Log* will be the first page of the CODIS section, followed by the following documentation (per profile):
    a. Specimen Details Report
    b. Match Details Report(s) (Short Report)
        i. If the specimen hits to another sample in PGI during the initial search (see Section 9 Searching CODIS), the disposition of the match must be documented in writing by the analyst on the Match Details Report
    c. Additionally, at the analyst's discretion, documentation supporting eligibility may be included with the CODIS paperwork in the case file
        i. Examples:
            1. Relevant pages of the ROI associating the evidence with a crime/putative perpetrator
            2. E-mails between the analyst and the investigator discussing eligibility

TECHNICAL REVIEW OF CODIS DATA

i. The technical reviewer shall document their CODIS review by initialing all documentation discussed in Section 8.ii.

ii. If an error is discovered on the Specimen Details Report during technical review, the original profile will be corrected and re-searched
    a. The corrected Specimen Details Report and Match Details Report will then be reviewed by the Technical Reviewer
    b. The original versions shall remain in the case file and appropriately marked as "Not Used"
iii. In rare instances, a one-time Local Keyboard Search may be performed
    a. In these instances, the Local Match Details Report will be maintained in the case file and subjected to technical review

DELETING SPECIMENS

i. A specimen may be deleted at two different points in the lifespan of a case:
    a. Before report is issued
        i. A specimen may be deleted after initial entry due to too many adventitious matches (discuss with CODIS Administrators), because a more complete and consistent profile was chosen for entry, or due to a staff hit
        ii. The original Specimen Details Report (and Match Details Reports, if applicable), will be maintained and clearly marked as "Deleted" along with the date of deletion
        iii. It is not necessary to maintain the Specimen Delete Report in the case file, as it is stored within the CODIS software
        iv. The reason the specimen was deleted from CODIS should be documented on the *CODIS/PGI Specimen Entry Log*
    b. After report has been issued
        i. Although rare, specimens may be deleted during or after a report has been issued
            1. The most common reasons for deleting a specimen after report issuance are a change in the eligibility criteria for the category in which the specimen exists, or that the specimen was later linked to an elimination sample (such as CSI or victim samples submitted at a later date)
        ii. The Specimen Delete Summary Report will be maintained in the case file, along with the reason for the deletion
            1. For profiles existing in LDIS, SDIS, or NDIS: If the specimen is deleted after the issuance of the final report, communication with the investigating agent as to why the specimen was deleted must also be documented in the case file
        iii. Deletion of forensic data in CODIS after a laboratory report has been issued will only be performed by a CODIS Administrator

ii. When the annual PGI purge is performed by the ATF CODIS Administrator, the Specimen Delete Summary Report will be maintained; no record of the deletion will be placed in the relevant case files

UPLOADING PROFILES

Following a completed technical review, eligible samples will be marked by the analyst for upload to SDIS. The date and signature of the analyst marking the profile for upload will be documented on the *DNA Case Record Review Form*.

i. Forensic LDIS Only, PGI, and Staff samples will not be marked for upload
ii. Once marked, eligible DNA profiles will be automatically uploaded to SDIS and potentially NDIS, depending on the category
iii. Laboratory reports must indicate which level of CODIS each eligible sample is being searched in, if applicable
    a. See *ATF-LS-FB34 Reporting the Results of Forensic Biology Analysis using STRmix*™ for additional CODIS reporting guidance
iv. Uploads to SDIS are automatically scheduled to occur daily, generally Monday through Friday
    a. SDIS sets the schedule for automatic uploads to NDIS, which generally also occur daily Monday through Friday
v. Early Uploads
    a. In some instances, profiles may be marked for upload prior to the completion of a case
    b. Approval for early uploads must be obtained from a DNA Section Chief
    c. The CODIS Specimen Entry Log for Priority Upload and Search shall be used to document the technical review of the relevant paperwork and data associated with the profile(s) prior to upload

9. SEARCHING THE CODIS INDEXES

i. *Searcher*: This module is utilized to perform the initial search of a profile against all other profiles in LDIS and PGI
    a. All samples entered into PGI/LDIS must undergo a Local Search prior to technical review
    b. The following CODIS Indexes will be searched as part of the Local Search: Forensic, Forensic Partial, Forensic Mixture (to include SDIS Only categories), Forensic Targeted, Forensic LDIS Only, Missing Persons, Staff, and PGI
    c. The default Searcher parameters at ATF are as follows:
        i. Minimum Number of Loci to Report a Match: **6**
        ii. Include Candidate Specimens that Match at all but **1** locus

iii. Search all 21 autosomal Globalfiler STR loci at **Moderate** stringency, if possible, and Amelogenin

d. On a quarterly basis, a CODIS Administrator will perform the following search to potentially benefit profiles with fewer loci
   i. Minimum Number of Loci to Report a Match: **5**
   ii. Include Candidate Specimens that Match at all but **0** locus
   iii. Search all 21 autosomal Globalfiler STR loci at **Moderate** stringency, if possible, and Amelogenin

ii. *Autosearcher*: Allows for a scheduled search of the specified categories
   a. Autosearches of LDIS/PGI is performed weekly by the ATF CODIS Administrator
      i. During the weekly autosearch, the following CODIS Indexes will be searched: Forensic, Forensic Partial, Forensic Mixture (to include SDIS Only categories), Forensic Targeted, Forensic LDIS Only, Missing Persons, Staff, and PGI
   b. Autosearches of SDIS are typically conducted daily, Monday through Friday
      i. The SDIS Only FT search is performed once per month
   c. Autosearches of NDIS are typically conducted daily, Sunday through Thursday
   d. All matches resulting from autosearches are automatically sent to Match Manager

iii. *Keyboard Searches:* One-time, immediate search of LDIS, SDIS, and/or NDIS; should only be used when an EUSR is not possible due to CODIS being unavailable (such as being offline or during maintenance)
   a. NDIS and FBI SDIS Keyboard Search Requests
      i. Can be made for urgent, high-priority cases
      ii. Approval must be obtained from a DNA Section Chief
      iii. The request will be made to, and initiated by, a CODIS Administrator and routed through the FBI SDIS Administrator
      iv. See Appendix for Keyboard Search Request form
   b. SDIS Keyboard Search Requests
      i. There may be instances where it is appropriate to request the search of a specific state's SDIS
      ii. These search requests will be initiated by the ATF CODIS Administrator to the individual SDIS Administrator with prior approval from the FBI SDIS Administrator
      iii. See Appendix for Keyboard Search Request form
   c. Keyboard Search Requests of the ATF's LDIS
      i. One-time search requests of the ATF's LDIS made by other law enforcement agencies will be evaluated by the ATF CODIS Administrator, DNA Technical Leader, and/or DNA Section Chief(s)
         1. The PGI and Staff Index will not be searched

iv. *Emergency Upload and Search Requests (EUSR)*: One-time, instant search of NDIS
   a. Can be made for urgent, high-priority cases where there is an imminent threat to public safety
   b. Approval must be obtained from a DNA Section Chief
   c. SDIS will be searched automatically prior to the search at NDIS; however, profiles eligible for SDIS Only and SDIS Only FT will have to undergo a keyboard search request (see prior section)
   d. The *CODIS Specimen Entry Log for Priority Upload and Search* shall be used to document the technical review of the relevant paperwork and data associated with the profile(s) prior to upload
   e. The profile must be marked for uploaded before performing an EUSR
   f. Only the CODIS Administrators have the ability to perform an EUSR

10. EVALUATING MATCHES

Matches are typically evaluated and pursued by the CODIS Administrators

i. *Match Stringency*: Term used to describe the strength of the match
   a. There are three possible match stringencies:
      i. High Stringency: When the allele calls in each profile are the same
      ii. Moderate Stringency: When two DNA profiles have a different number of alleles, but all of the allele calls for the profile with the fewest number of alleles are found in the profile with the greatest number of alleles
      iii. Low stringency: At least one of the alleles at a locus in one profile must match at least one of the alleles at that locus in the other profile
   b. Match stringency at the locus level refers to the way in which alleles for a given locus are compared between two profiles
   c. Match stringency at the specimen level refers to the lowest allowable locus-level match stringency for all loci involved in the match
      i. At NDIS:
         1. Forensic, Unknown profiles are searched at High Stringency
         2. Forensic Mixture and Forensic Partial profiles are searched at Moderate Stringency (specimen level)
         3. Forensic Targeted profiles search stringency is dependent on the information present at each locus
      ii. At SDIS:
         1. SDIS Only and SDIS Only FT profiles are searched at Moderate Stringency
      iii. At LDIS:
         1. Forensic LDIS Only profiles are searched at Moderate Stringency
ii. *Match Manager*: Module in CODIS where matches are sent for evaluation

a. Any matches resulting from the initial search of a specimen during entry into LDIS will *not* be sent to Match Manager until the weekly autosearch of LDIS is performed
b. All matches are automatically labeled as Candidate Match until the match has been evaluated and the disposition has been changed (see *Match Disposition* section below)
c. For the purpose of this protocol, the ATF Laboratory will be referred to as the **Target Laboratory**, and the database lab (for offender and arrestee hits) or casework lab (for forensic hits) will be referred to as the **Candidate Laboratory**
d. When assessing a match, the following should be evaluated:
    i. Locus level stringencies
    ii. The specimen category for each profile involved in the match, including that the ATF specimen(s) is/are in the appropriate category and that eligibility is satisfied
        1. LDIS hits will be officially dispositioned during the weekly autosearch that occurs after initial entry
    iii. The Source ID for each specimen involved in the match
    iv. Whether or not a STRmix™ LR from Previous calculation needs to be performed to the Candidate specimen
        1. An LR from Previous calculation may need to be performed for any matches where the ATF profile is an ambiguous mixture
        2. When the lowest 99.0% 1-sided Lower HPD value is greater than or equal to the size of the database that the hit occurred in, the match will be verified, and the hit confirmation can be pursued
        3. LRs that support inclusion will be stored in the CODIS section of FireTOSS for the relevant case
        4. LRs that result in the exclusion of the Candidate specimen will be reviewed by the CODIS Administrator who did not perform the calculation
            a. The Input file must be carefully checked to ensure appropriate alleles were entered when creating the digital reference profile
            b. Once the exclusion has been verified, the CODIS Administrator performing the review will update the disposition to No Match with a note in the Comments field of “No Match via STRmix reviewed by (initials)” to notify the Candidate lab of why this disposition was selected
            c. The Match Details Report and LR from Previous do <u>not</u> have to be maintained in the case file for NDIS and SDIS No Match dispositions

e. If the match is evaluated and determined to be a hit, the CODIS Administrator will document the verification of the hit on the Match Details Report, along with her/his initials and date (when applicable, add a note that the match was verified via STRmix™ on the Match Details Report)
    i. The Match Disposition should then be changed to Pending
    ii. See Section 11 Match Confirmation Process for additional steps
    iii. **Do not notify investigators about potential CODIS hits until both laboratories involved have confirmed the hit and exchanged relevant information**

iii. *Match Dispositions*: The official type of match/hit between two samples; will be changed after the match confirmation process has been completed
    a. **Candidate Match**: Default disposition
    b. **No Match**: Samples do not match
    c. **Pending**: Match confirmation or evaluation is in process
    d. **Offender Hit**: Confirmed match between an unsolved forensic sample and a convicted offender
        i. Unsolved: Source ID is **No**
    e. **Arrestee Hit**: Confirmed match between an unsolved forensic sample and an arrestee
    f. **Forensic Hit**: Confirmed match between two unsolved forensic samples, or one solved to one unsolved forensic sample
        i. Solved: Source ID is **Yes**
    g. **Investigative Information**: Confirmed match between two solved forensic samples
    h. **Conviction Match**: Confirmed match between a solved forensic sample and a convicted offender or arrestee specimen
    i. **Benchwork Match**: Confirmed match between samples from cases where there is previous knowledge of a potential connection
    j. **Staff Hit**: Confirmed match between staff and casework samples
    k. **PGI Match**: Confirmed match between an LDIS sample and a PGI sample or two PGI samples where contamination is determined to be unlikely
    l. **Contamination:** Confirmed match between an LDIS sample and a PGI sample, two PGI samples, or two LDIS samples that is determined to be contamination
    m. **Same Case**: Confirmed match between two samples originating from the same case, or entries targeting different contributors from the same case that match due to an overlap in genotype possibilities

11. MATCH CONFIRMATION PROCESS

The match confirmation process is initiated and followed through by a CODIS Administrator. The term Offender from herein refers to arrestees, convicted offenders, detainees, multi-allelic offenders, juvenile, and legal index samples.

OFFENDER AND EXTERNAL FORENSIC HITS

i. Once a match has been determined to be a true match, a CODIS Administrator will initiate the match confirmation process with the Candidate Laboratory
    a. For Forensic Hits, including Investigative Information hits (i.e. solved-to-solved), the CODIS Administrator may utilize the *Match Data Response Form*

ii. The match confirmation request will formally be made via e-mail to the Candidate Laboratory's LDIS or SDIS Administrator
    a. Suggested e-mail wording for Offender Hits: "Our *Specimen Category* sample **21W0123_1.1_GRIP-C1** has hit to your Candidate Lab's *Specimen Category* specimen **DB2101234**; please begin the match confirmation process."
    b. Suggested e-mail wording for Forensic Hits: "Our *Specimen Category* sample **21W0123_1.1_GRIP-C1** has hit to your Candidate Lab's *Specimen Category* specimen **CW012345**; our case is *solved/unsolved*. Attached is our Match Data Response form. Please send your case information when you have a moment."
    c. The casework analyst should be copied on this initial e-mail as notification that a hit occurred in her/his case
    d. The Candidate Laboratory may request a Match Data Request form (See Appendix 1)

iii. The CODIS Administrator will update the CODIS Hit Counting Spreadsheet with the basic hit information for tracking purposes

iv. The Candidate Laboratory, which may be the ATF Laboratory in the case of a Forensic Hit, is responsible for making a good faith effort to confirm a candidate match within 30 business days
    a. If it has been over 30 business days, the CODIS Administrator will contact the Candidate Laboratory's CODIS Administrator(s) to check the status of the confirmation

v. Once the personally identifiable information (PII) is provided by the Candidate Laboratory to the ATF, the PII must be released to the investigating agent within 14 days of receipt
    a. Typically, the information is initially relayed via phone call to the investigating agent
        i. This communication must be documented
        ii. A CODIS Hit Memo will be sent via mail

1. If requested by the investigating agent, an email containing a copy of the signed CODIS Hit Memo may be sent internally to the ATF agent

vi. The CODIS Administrator will update the disposition in Match Manager, and may adjust the Investigations Aided field to "1," if appropriate
   a. An Investigation can only be "aided" once
   b. Only investigations of unsolved cases may be aided
   c. If multiple investigations are aided by one hit, then the Investigations Aided field may be "2" or more
   d. In the instance of an unsolved ATF specimen hitting to both an Offender sample *and* a Forensic sample, an Offender Hit disposition takes precedence over a Forensic Hit disposition when the hits occur during the same search
      i. The Investigation Aided count will be assigned to the Offender Hit
      ii. The Forensic Hit will be dispositioned as Investigative Information

vii. The CODIS administrator will ensure that the Source ID for the ATF specimen has been changed to **Yes** for Offender Hits, and will print the new Specimen Details Report reflecting the change

CODIS HIT DOCUMENTATION AND REVIEW

A CODIS Hit Memo will be created and shall include all of the PII and any other relevant information provided by the Candidate Laboratory

i. For Offender and Arrestee Hits, the *Offender and Arrestee Hit Memo Template* may be used
ii. For Forensic Hits, the *Forensic Hit Memo Template* may be used
   a. Note: For Investigative Information hits, this information can be sent in the body of an e-mail to the investigating agent
      i. Only a self-review by the author of the e-mail notification of Investigative Information needs to be performed
      ii. Note: Investigative Information hits between two solved cases are not typically tracked in the CODIS Hit Counting Spreadsheet
      iii. When and Offender Hit and a Forensic Hit both occur upon the initial search of an unsolved profile, both the Offender Hit and Forensic (Investigative Information) Hit will be tracked in the CODIS Hit Counting Spreadsheet and the Forensic Hit (Investigative Information) will be sent to the agent as a hit memo.
iii. The CODIS hit packet shall contain:
   a. Copy of reviewed CODIS Hit Memo
   b. The original Match Details Report indicating the hit with initials and date of the verifier

c. The e-mail communication from the ATF Laboratory to the Candidate Laboratory, including any attachments
d. The response from the Candidate Laboratory, including any attachments
e. The updated Specimen Details Report with Source ID of **Yes**, if applicable

iv. The administrative review of the CODIS hit packet shall be performed by a CODIS Administrator, DNA Section Chief, or a designee. The administrative review shall consist of:
   a. Verifying the information has been accurately transcribed from the Candidate Laboratory's response to the ATF CODIS Hit Memo;
   b. Confirming that the mailing address and the ATF's investigation information are correct;
   c. Ensuring that all items listed in section iii above are present;
   d. Reviewing any STRmix™ LR from Previous Calculations, if applicable; and,
   e. Reviewing the correspondence with the investigator, including documentation that investigative information was provided and a known sample was requested, if applicable

v. Once the review has been completed, the CODIS Hit Memo shall be initialed and dated by the administrative reviewer of the packet as an indication that the review has been completed and the information is correct

vi. The CODIS Hit Memo will be signed by the author, and a copy will be stapled to the front of the CODIS hit packet

vii. The CODIS hit packet will be page-numbered and given to the assigned Forensic Biologist to be placed in the corresponding case file

viii. The CODIS Hit Memo will be sent to the investigating agent via USPS

CONVICTION MATCHES

When a solved ATF sample hits to an Offender sample, an exchange of PII is performed by the CODIS Administrators from each lab

i. The ATF Laboratory will contact the Offender Laboratory and provide the name associated with the ATF's forensic sample to determine if there are any discrepancies
   a. Suggested e-mail wording for Conviction Matches: "Our *Specimen Category* sample **21W0123_1.1_GRIP-C1** has hit to your Candidate Lab's *Specimen Category* specimen **DB2101234**; I would like to verify this as a conviction match. Our putative perpetrator's name is John Doe."
   b. If the name of the putative perpetrator was provided to the ATF Laboratory via an Offender Hit confirmation (i.e. the ATF has not performed a direct comparison from a known sample to the forensic sample), the following suggested wording may be used:
      i. "Our *Specimen Category* sample **21W0123_1.1_GRIP-C1** has hit to your Candidate Lab's *Specimen Category* specimen **DB2101234**; I would like

to verify this as a conviction match. Our putative perpetrator's name, which we received through a hit confirmation with another laboratory, is John Doe."

ii. If the Offender Laboratory verifies the name, then the match shall be dispositioned as a Conviction Match in Match Manager
    a. If there are discrepancies, the CODIS Administrators from each lab will investigate
iii. Conviction Match packets shall include:
    a. The e-mail correspondence with the Offender Laboratory, verifying the hit as a Conviction Match
    b. The original Match Details Report indicating the hit with initials and date of the verifier
iv. The Conviction Match packet shall be stapled, page-numbered, and placed in the case file
    a. Paperwork associated with a Conviction Match is not required to undergo a review
v. Note: Conviction Matches are not tracked in the CODIS Hit Counting Spreadsheet

<u>INTERNAL FORENSIC HITS</u>

A confirmed hit between two forensic profiles in the ATF's LDIS

i. With LDIS hits between two different cases, make sure that all staff involved in the examination of the evidence have been entered in the Staff Index
    a. Also, evaluate whether the same investigating agent or Field Office is involved in each case, and whether the hit could be potential contamination
ii. Case information will be exchanged between examiners (if applicable), and may utilize the *Match Data Response Form*
    a. If one of the cases is solved, the name of the putative perpetrator may be exchanged
iii. Once Technical Review for **<u>both</u>** cases involved in the match has been completed, the primary investigator(s) will be contacted about the hit, and all relevant case information will be provided both verbally and in an official CODIS Hit memo
    a. However, if both cases are solved, this information will be considered Investigative Information and can be sent in the body of an e-mail to the investigating agent, so long as it does not contain PII
        i. Only a self-review by the author of the e-mail notification of Investigative Information needs to be performed
iv. The match disposition will be changed to Forensic Hit in Match Manager, and the Investigation Aided and Source ID fields will be updated, if applicable
v. The investigator(s) shall be informed that a CODIS hit is an investigative lead, and should contact each other to exchange relevant information, if applicable

vi. The CODIS Hit packet(s) shall contain the same documentation outlined in the Offender and External Forensic Hits section above, and subjected to the same review process

STAFF AND CONTAMINATION HITS

Confirmed matches between an ATF casework sample and a staff sample, or between two ATF casework samples that are determined to be contamination

i. Candidate matches between casework and PGI samples should be carefully evaluated to determine if they may be due to contamination
ii. Confirmed internal contamination will be documented in the *Contamination Event Log*
iii. Confirmed external contamination (i.e. prior to the evidence being received by the ATF Laboratory, such as originating from an investigator) will be documented in the *External Contamination Event Log*
iv. See *Contamination Event Work Instructions* on how to handle contamination events, as well as how to fill out the *ATF Internal Contamination Event Form*, if applicable
v. If a match between two PGI and/or LDIS specimens cannot be verified as contamination, see the CODIS Administrator(s) and DNA Technical Leader or DNA Section Chief and the section below

INTERNAL FORENSIC HITS INVOLVING THE PGI (PGI MATCHES)

A confirmed match between two PGI specimens or one PGI specimen and one LDIS specimen where contamination has been determined to be unlikely

i. Candidate matches between casework and PGI samples should be carefully evaluated to determine if they may be due to contamination
ii. The CODIS Administrators will evaluate eligibility for both profiles involved in the match
iii. Once the case files for both specimens involved in the match have completed Technical Review, the CODIS Administrator will create a PGI Match Notification containing the case information associated with each profile involved in the match (See Appendix 8)
iv. The PGI Match packet shall contain (each case will have its own PGI Match Packet):
    a. Copy of reviewed PGI Match Notification
    b. The original Match Details Report indicating the hit with initials and date of the verifier and note of "laboratory contamination unlikely due to_____ (time/space separation, etc.)"
    c. If one of the profiles involved in the match has a Source ID of Yes, the Source ID will not be changed for the other profile until a direct comparison is made to a reference sample
        i. PII will <u>not</u> be released for PGI Matches
v. The administrative review of the PGI Match Packet shall be performed by a CODIS Administrator, DNA Section Chief, or a designee. The administrative review shall consist of:

a. Confirming that the mailing address and the ATF's investigation information are correct;
b. Ensuring that all items listed in section iv above are present; and,
c. Reviewing any STRmix™ LR from Previous Calculations located in FireTOSS, if applicable

vi. Once the review of both PGI Match Packets has been completed, the following items will be sent through Qualtrax for Legal review/approval prior to dissemination to the involved investigating agents (each case will have its own Qualtrax workflow):
a. Reviewed and signed PGI Match Notification
b. Lab Exam Request Form and ROI
c. Match Details Report, including note of "laboratory contamination unlikely"

vii. After approval for dissemination of PGI Match Notification has been obtained from Legal:
a. Each investigating agent will be called by a CODIS Administrator, who will explain what a PGI Match is and how it differs from CODIS hits
b. The following information will be documented in each case's communication log:
i. The Disclosure Authorization from Qualtrax
ii. A summary of the conversation with the investigating agent surrounding the PGI Match, including that the information should be handled with caution
c. The PGI Match Notifications will be mailed to the investigating agents
i. Note: If the investigating agent is the same for each case, only one PGI Match Notification needs to be written and reviewed
1. Each case will still have its own PGI Match Packet
d. The Disclosure Workflow in Qualtrax shall be closed

viii. The PGI Match Packet for each case will be page numbered and placed in the case file

12. CODIS ACCESS AND DATA SECURITY

i. The ATF CODIS server and workstations are located in a controlled-access room
a. Only authorized personnel will have access to the CODIS server and workstations

ii. CODIS users are responsible for the security of the software, and must log out of the system when finished

iii. Granting of access rights and the level of access will be determined by the ATF CODIS Administrator

iv. The ATF CODIS Administrator will periodically verify the status of unmarked specimens

v. Antivirus software is installed on the CODIS server and workstations
a. The antivirus software is maintained by the FBI

vi. Hit counting statistics, including Investigations Aided, are reported to the SDIS Administrator on a monthly basis

BACKUPS

i. Data within the ATF CODIS system is automatically backed-up onto at least two separate storage media (external hard drives or data tape cartridges), at least once weekly
    a. One set of backup data for the ATF Laboratory will be maintained in the ATF Fire Research Laboratory, which is separated from the ATF Forensic Science Laboratory building by fire doors and walls
        i. Backup data media will be rotated on a monthly basis
ii. A CODIS Administrator will perform a periodic restore to ensure that the backups are working properly
iii. One full backup of the entire CODIS folder and the SQL database shall be performed each calendar month
iv. CODIS backups and the entire CODIS folder on the data drive shall be encrypted
    a. Additionally, CODIS backup data (CODIS SQL server database and the entire CODIS folder) at rest on an external drive shall be encrypted
        i. Data is at "rest" when it is stored on a hard drive, removeable media, DVD, or backup tapes
v. The ATF Laboratory shall retain the monthly CODIS backup data for a minimum of six months
vi. The ATF Laboratory shall maintain the following log files for a minimum period of six months:
    a. Windows Event Viewer Logs
        i. Application
        ii. Security
        iii. System
        iv. CODIS
        v. CODIS Messaging
        vi. NGISS
        vii. RAPID
    b. SQL Logs
        i. CODIS_log.ldf

13. KNOWN SAMPLE INQUIRY

i. If an investigator inquires about an individual's status in CODIS, instruct them to contact the appropriate state or federal CODIS administrator
    a. For state inquiries, the investigator should contact the State CODIS administrator directly
    b. For federal inquiries, the *Federal Convicted Offender/Arrestee Sample Possession Inquiry Form* should be used (see Appendix 7)

APPENDIX 1 – OFFENDER / ARRESTEE MATCH DATA REQUEST FORM

CODIS DNA MATCH DATA REQUEST
OFFENDER / ARRESTEE MATCH

To: _____ Date: _____

From:
Agency: ATF
ORI: [redacted]
Telephone:
Fax: 202-648-6199
Email:

ATF specimen ID # _____

has matched to your specimen ID # _____

Please begin the match confirmation process. Once the process is completed, respond with the match disposition and the putative perpetrator's identifying information.

Feel free to contact me with any questions.

Sincerely,

NAME
(Alternate) CODIS Administrator

APPENDIX 2 – CASEWORK MATCH DATA RESPONSE FORM

CODIS DNA MATCH DATA RESPONSE
CASEWORK MATCH

To: _____ Date: _____

From: _____
Agency: ATF
ORI: [redacted]
Telephone: _____
Fax: 202-648-6199
Email: _____

ATF specimen ID # _____
has matched to your specimen ID # _____

Our case is ☐ SOLVED ☐ UNSOLVED.

Investigator's contact information:

Agency: ATF
Name:
Address: Bureau of Alcohol, Tobacco, Firearms and Explosives
Telephone:
Email:
Title of Investigation:
Investigation #:
ATF Lab #:

Comments:

Feel free to contact me with any questions.

Sincerely,

NAME
(Alternate) CODIS Administrator

APPENDIX 3 – OFFENDER AND ARRESTEE HIT MEMORANDUM

(On agency letterhead)

Choose an item. Click or tap here to enter text.
Bureau of Alcohol, Tobacco, Firearms and Explosives
Click or tap here to enter text.
Click or tap here to enter text.
Click or tap here to enter text.

Click or tap to enter a date.

Choose an item. Click or tap here to enter text.,

A search of the Choose an item. resulted in a match stringency match between an unsolved ATF case and the Choose an item. listed below. The hit was the result of a search of the national database based on biological evidence recovered from crime scenes.

ATF Forensic Specimen
Investigation number (title): Investigation Number (Title of Investigation)
ATF Laboratory identifier: Lab Case Number/ Lab Ex. Parent Ex. #(ATF Ex. Agent's Ex. #) / Brief description of evidence

Name of Offender Lab Type of Specimen ***Specimen***

Name:	DOB:
FBI #:	Sex:
SID #:	Race:
Last Known Whereabouts:	Qualifying Offense:

This information is provided only as an investigative lead, and any possible connection or involvement of the individual to the case must be determined through further investigation.

Please submit two buccal (cheek) swabs from the above-named individual to the ATF Laboratory for comparison.

If you have any questions, please contact me at Enter your phone number here.

Name.
Choose an item. Admin Review:

APPENDIX 4 – FORENSIC HIT MEMORANDUM

(On agency letterhead)

Choose an item. Click or tap here to enter text.
Bureau of Alcohol, Tobacco, Firearms and Explosives
Click or tap here to enter text.
Click or tap here to enter text.
Click or tap here to enter text.

Click or tap to enter a date.

Choose an item. Click or tap here to enter text.,

A search of the Choose an item. resulted in a Match Stringency match between an unsolved ATF case and solved/unsolved Name of Candidate Forensic Lab case listed below. The hit was the result of a search of the national database based on biological evidence recovered from crime scenes.

The following information will also be provided to the Name of Investigating Agency investigator:

ATF Forensic Specimen
Investigation number (title): Investigation Number (Title of Investigation)
ATF Laboratory identifier: Lab Case Number/ Lab Ex. Parent Ex. #(ATF Ex. Agent's Ex. #) / Brief description of evidence

Name of Investigating Agency ***Investigator's Information***
{ENTER ALL INFO RECEIVED FROM CANDIDATE LAB HERE}

This information is provided only as an investigative lead, and any possible connection between these cases must be determined through further investigation.

OPTIONAL: Choose this option if a name is provided by the candidate lab, otherwise, delete

If you have any questions, please contact me at Enter your phone number here.

Name.
Choose an item. Admin Review:

APPENDIX 5 – NDIS KEYBOARD SEARCH REQUEST FORM

(On agency letterhead)

Dear NDIS Custodian:

I, INSERT NAME, CODIS Administrator, request a manual keyboard search of NDIS for the DNA record set forth below.

Point of Contact:

Name: _____
Agency: ATF **ORI:** [redacted]
Address: 6000 Ammendale Road
Beltsville, MD 20705
Telephone: _____
E-mail: _____

DNA Record **Specimen ID #:** _____

☐ Forensic, Unknown ☐ Forensic Mixture ☐ Forensic Partial

☐ Forensic Targeted ☐ SDIS Only ☐ LDIS Only

☐ I confirm that this record was searched at SDIS and resulted in no matches.

Reason for Search: *Brief case scenario.*

Origin of DNA Record: *Description of evidence item the profile originated from.*

STR Data

Locus	Alleles	Stringency	Locus	Alleles	Stringency
D3S1358			**D13S317**		
VWA			**D7S820**		
FGA			**D16S539**		
D8S1179			**TH01**		
D21S11			**TPOX**		
D18S51			**CSF1PO**		
D5S818			Amelogenin		
D1S1656			D10S1248		
D2S441			D12S391		
D22S1045			**Match Estimate(13 Core):**	**1 in**	**Million**

Minimum of 7 original CODIS core loci are required for a search with a match estimation greater than 1 in 10 million

☐ I certify that the identity of the contributor of the DNA profile is an unknown putative perpetrator.

☐ If this is a forensic mixture or forensic partial DNA profile, I certify that it satisfies a statistical threshold for match rarity of approximately one in 10 million.

I further certify that the DNA record was developed in accordance with the provisions of the Federal DNA Identification Act (as amended) as indicated below (a checkmark in the box indicates compliance).

☐ 1. DNA record(s) generated by or on behalf of a criminal justice agency.
A "criminal justice agency" is an agency or institution of a federal, state or local government, other than the office of the public defender, which performs as part of its principal function, activities relating to the apprehension, investigation, prosecution, adjudication, incarceration, supervision or rehabilitation of criminal offenders.

☐ 2. DNA record(s) generated in compliance with the FBI Director's "*Quality Assurance Standards for Forensic DNA Testing Laboratories"*

☐ 3. DNA record(s) prepared by a laboratory that is accredited by a nonprofit professional association of persons actively involved in forensic science that is nationally recognized within the forensic science community.
Name of accrediting agency: ANAB

☐ 4. DNA record(s) maintained by Federal, state and local criminal justice agencies (or the Secretary of Defense in accordance with 10 U.S.C. § 1565) pursuant to rules that allow disclosure of stored DNA samples and DNA analyses only-

(A) to criminal justice agencies for law enforcement identification purposes;
(B) in judicial proceedings, if otherwise admissible pursuant to applicable statutes or rules;
(C) for criminal defense purposes, to a defendant, who shall have access to samples and analyses performed in connection with the case in which such defendant is charged; or
(D) if personally identifiable information is removed, for a population statistics database, for identification research and protocol development purposes, or for quality control purposes.

☐ 5. If the DNA record(s) is submitted by a state or local criminal justice agency, the applicable state provides for the expungement of a DNA record of a person whose qualifying conviction has been overturned or whose qualifying arrest has been dismissed, resulted in acquittal or no charges were filed within the applicable time period.

☐ 6. DNA record(s) generated in accordance with all applicable NDIS Operational Procedures.

☐ 7. The Laboratory participates in annual audits and external audits every two years.

Sincerely,
(Signature)
CODIS Administrator

APPENDIX 6 – SDIS KEYBOARD SEARCH REQUEST FORM

(On agency letterhead)

DATE

Dear SDIS ADMINISTRATOR,

I, ADMIN NAME, CODIS Administrator, request a manual keyboard search of STATE NAME's database for the DNA record set forth below.

Point of Contact:

Name: _____

Agency: ATF **ORI:** [redacted]

Address: 6000 Ammendale Road

Beltsville, MD 20705

Telephone: _____

E-mail: _____

DNA Record **Specimen ID #:** _____

☐ Forensic, Unknown ☐ Forensic Mixture ☐ Forensic Partial

☐ Forensic Targeted ☐ SDIS Only ☐ LDIS Only

☐ Add to Batch Target File

Reason for Search: *Brief case scenario.*

Origin of DNA Record: *Description of evidence item the profile originated from.*

STR Data

Locus	Alleles	Stringency	Locus	Alleles	Stringency
D3S1358			D19S433		
vWA			**TH01**		
D16S539			**FGA**		
CSF1PO			D22S1045		
TPOX			**D5S818**		
Y-Indel			**D13S317**		
Amelogenin			**D7S820**		
D8S1179			SE33		
D21S11			D10S1248		
D18S51			D1S1656		
DYS391			D12S391		
D2S441			D2S1338		
MME:			**MRE:**		

☐ I certify that the identity of the contributor of the DNA profile is an unknown putative perpetrator.

I further certify that the DNA record was developed in accordance with the provisions of the Federal DNA Identification Act (as amended) as indicated below (a checkmark in the box indicates compliance).

☐ 1. DNA record(s) generated by or on behalf of a criminal justice agency.
A "criminal justice agency" is an agency or institution of a federal, state or local government, other than the office of the public defender, which performs as part of its principal function, activities relating to the apprehension, investigation, prosecution, adjudication, incarceration, supervision or rehabilitation of criminal offenders.

☐ 2. DNA record(s) generated in compliance with the FBI Director's "*Quality Assurance Standards for Forensic DNA Testing Laboratories"*

☐ 3. DNA record(s) generated in accordance with all applicable NDIS Operational Procedures.

Sincerely,
(Signature)
CODIS Administrator

APPENDIX 7 – FEDERAL CONVICTED OFFENDER / ARRESTEE SAMPLE POSSESSION INQUIRY

(On agency letterhead)

(Date)

Federal DNA Database
FBI Laboratory
2501 Investigation Parkway
Quantico, VA 22135
[redacted]

Dear _____:
We are inquiring if your state has a known DNA profile for a particular convicted offender / arrestee in your state DNA database.

Full Name:_____

AKA:_____

Social Security #:_____

Date of Birth:_____

Race:_____

Sex:_____

FBI#: _____

Please advise if you are in possession of the sample specified and if it has been analyzed. This information is requested in furtherance of an official criminal investigation.

Sincerely,

(Name)
(Title)

Phone number
Fax number
Email address

APPENDIX 8 – PGI MATCH NOTIFICATION

(On agency letterhead)

Choose an item. Click or tap here to enter text.
Bureau of Alcohol, Tobacco, Firearms and Explosives
Click or tap here to enter text.
Click or tap here to enter text.
Click or tap here to enter text.

Click or tap to enter a date.

Choose an item. Click or tap here to enter text.,

A routine search for contamination between the ATF's local DNA database and the Profiles Generated Index (PGI) resulted in a possible link between the two ATF cases listed below. The PGI is comprised of DNA profiles from known individuals (i.e. elimination samples), as well as DNA profiles from evidence which are not eligible for entering into CODIS because they do not meet policy requirements, quality criteria, or are not directly related to a crime.

Due to separation of the time and space in the DNA Analysis of the two samples involved, laboratory contamination is unlikely to be the cause of this match.

This is not a CODIS hit. Any possible connection between these cases must be determined through further investigation.

ATF Local DNA Database Specimen

Investigation number (title):	Investigation Number (Title of Investigation)
ATF Laboratory identifier:	Lab Case Number/ Lab Ex. Parent Ex. #(ATF Ex. Agent's Ex. #)
Case Agent:	Special Agent John Doe
Case Agent Phone:	301-555-1234

ATF PGI Specimen

Investigation number (title):	Investigation Number (Title of Investigation)
ATF Laboratory identifier:	Lab Case Number/ Lab Ex. Parent Ex. #(ATF Ex. Agent's Ex. #)
Case Agent:	Special Agent Jane Smith
Case Agent Phone:	301-867-5309

If you have any questions, please contact me at AAA-BBB-CCCC.

Name
Choose an item.

cc: Special Agent Jane Smith
Bureau of Alcohol, Tobacco, Firearms and Explosives
123 Main Street
Suite 1
Anywhere, US 12345

Bureau of Alcohol, Tobacco, Firearms and Explosives
Laboratory Services
Forensic Biology
FB20 – Forensic Biology Case Note Glossary

This glossary is intended for use as a guide for ATF Forensic Science Laboratory Forensic Biologists. It is not intended to be all inclusive. Those abbreviations that are widely recognized and/or generally accepted in forensic laboratories may not be included in this document.

Addit'l or Add'l	Additional
AL or A/L	At least
ALS	Alternate light source
Approx.	Approximately
Art	Artifact
b/c	Because
b/w	Between
BPB	Brown paper bag
c:	Containing
CB	Cardboard
CBB	Cardboard box
Com or Cpx	Complexity of the mixture
Comm	Communications
CR	Central receiving
c/w or C/W	Consistent with
C[#]	STRmix™-generated contributor order number, e.g. C1, C2, etc.
D	Date/Dated
DBLR	Component not suitable due to excessive H1 true and H2 true overlap
DNU	Did not use/analyze, data not used
e.	Evidence
EPG	Electropherogram
ET	Evidence tape, can be used with R(red), G(green), B(blue), Y(yellow), P(purple), e.g. RET
ETS	Evidence tape sealed
Ex.	Exhibit
Exp.	Expiration
FS	Forward stutter
FTC	Found to contain
GM or GMIDX	GeneMapper® or GeneMapper® ID-X
H/F	Hairs/fibers
Het	Heterozygote
Homo	Homozygote
I	Initials/Initialed
K	Known
KM	Kastle-Meyer
LIP	Labeled in part

Bureau of Alcohol, Tobacco, Firearms and Explosives
Laboratory Services
Forensic Biology
FB20 – Forensic Biology Case Note Glossary

L.P.P. or LPP	Latent Print Processing
LRes or Lim Res	Limited results
M2M	Mix to Mix
ME	Manila envelope
MLG	Minimum Locus Guidelines
NCN	No calculation necessary
NEG	Negative control, negative amplification control
NOC	Number of contributors
NR	No results
NSFC	Not suitable for comparison
OFTC	Open, found to contain
OL	Off ladder
OLA	Off-ladder allele
PCR	Polymerase Chain Reaction
Ph or Phth or PHE	Phenolphthalein
Pop^{n}	Population
POS or PC	Positive control, positive amplification control
PR/Prtl/PL	Partial
PRD/S or PRDS	Possible ridge detail/smudging
PU	Pull-up
Q	Questioned
RA	Re-amp
RBS	Reddish-brown stain(s)
RI	Re-injection
RP	Re-plate
S	Suspect
S/	Sealed with
SBPB	Sealed brown paper bag
S/F	Swabs from
SFC	Suitable for comparison
SL	StarLims
SLS	Secure lab storage
St	Stutter
SS	Single source
STC	Said to contain
STR	Short tandem repeats
SWE	Sealed white envelope
SME	Sealed manila envelope
Tem	Template (referring to theSTRmix™-generated value)
US	Unsealed
V	Victim
w/	With
w/d	Wet/dry

Bureau of Alcohol, Tobacco, Firearms and Explosives
Laboratory Services
Forensic Biology
FB20 – Forensic Biology Case Note Glossary

w/o	Without
w/w	Wet/wet
WE	White envelope
ZL	Ziplock
[ ]	Concentration

1. Scope

This method describes how to sample evidence items for potential biological material suitable for DNA analysis. Evidence sampling should be performed by qualified personnel who have been trained and tested to competency in the DNA collection process.

Laboratory Services Forensic Biology functions are housed at the Forensic Science Laboratory – Washington (FSL-W); however, each of the ATF forensic science laboratories is capable of collecting and preserving potential DNA evidence. The DNA samples, or on occasion the actual items of evidence, are forwarded to the FSL-W for DNA analysis.

2. References

2.1. D. Sweet, M. Lorente, J.A. Lorente, A. Valenzuela, E. Villanueva, An improved method to recover saliva from human skin: the double swab method, J. Forensic Sci. 42 (1997) 320-322.
2.2. ATF-LS-7.1 Review of requests, tenders, and contracts
2.3. ATF Forensic Science Laboratory – Atlanta (FSL-A) Safety Manual.
2.4. ATF Forensic Science Laboratory – Washington (FSL-W) Safety Program and Chemical Hygiene Plan.
2.5. DNA Swabbing Guidelines and Examples, ATF training PowerPoint, current version.

3. Equipment

3.1. Disposable gloves
3.2. Lab coat
3.3. 10% bleach solution
3.4. 70% EtOH
3.5. Bench paper
3.6. Sterile water or other approved wetting solution
3.7. Sterile cotton-tipped applicators or other approved collection device
3.8. Appropriate packaging material (swab box, paper envelope, plastic tube)
3.9. Evidence tape
3.10. Scissors/tweezers
3.11. Sterile scalpels or scalpel blades

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling swabs and evidence.
4.2. Lab coat should be worn at all times while performing this procedure.
4.3. When practical, only one item of evidence shall be open at a time.
4.4. Reference samples shall be examined in a dedicated workspace.
4.5. Any utensils used to cut or manipulate the evidence must be cleaned between uses with 10% bleach solution and followed by 70% ethanol or alcohol wipe.
4.6. The laboratory bench surface shall be cleaned before and after each use with 10% bleach solution or other sanitizing agent and may be followed by 70% ethanol. Fresh bench paper shall then be placed on the surface prior to evidence examination.

5. Procedure

5.1. DNA collection methods such as swabbing, cutting, or scraping may be used depending on the type of evidence.

5.2. Examiners/Technicians should refer to the most recent copy of the *DNA Swabbing Guidelines and Examples* presentation for specific examples of how to swab evidence, where to swab evidence, and the approximate number of swabs to take.

5.2.1. As no set of examples or instruction can cover all possible scenarios, the *DNA Swabbing Guidelines and Examples* presentation is intended to be used as a reference guide. Examiners and Technicians are encouraged to use their best judgement when sampling evidence for DNA in order to maximize the quality of the DNA sample, while preserving the evidence for further analyses.

5.3. At the Examiner's/Technician's discretion, in certain circumstances one set of swabs may be used to swab areas from two or more exhibits of evidence if it can be reasonably assumed (based on information from the investigating agent, Forensic Chemist, etc.) that the exhibits originated from the same source. For example, multiple fragments of an end cap from a pipe bomb that were submitted as separate exhibits may be swabbed with a single set of swabs.

5.4. In general, a new sterile scalpel or scalpel blade shall be used to cut DNA samples.

5.5. Due to the properties of copper, in general, DNA will not be collected from brass-colored cartridges and cartridge cases or from copper evidence unless select criteria have been met. If the cartridges or cartridge cases are to be sampled, then ATF-LS-FB37 will be used to collect the DNA.

5.6. **Touch Evidence**

5.6.1. The Double Swab Technique

5.6.1.1. Disposable gloves will be worn throughout the swabbing process. The Examiner/Technician must take care not to touch the swab tips and/or come into direct contact with the evidence. The Examiner/Technician must change gloves when moving from exhibit to exhibit or more often as needed.

5.6.1.2. A sterile cotton-tipped swab is moistened with sterile water (or other approved wetting solution). The area with potential DNA evidence is then swabbed to remove biological material from the questioned area. Analysts should attempt to concentrate the collected material on the swab tip. Avoid contact of the sterile swab tip with surfaces other than the areas of interest.

5.6.1.3. A dry swab is then used to swab over the same area of interest covered using the initial wet swab, still attempting to concentrate the collected material on the swab tip.

5.6.1.4. The swabs should be placed in an envelope, swab box, or other breathable container.

5.6.1.4.1. For swabs used to collect potentially low quantities of biological material, the swabs should be placed in a swab box that minimizes contact between the swab tips and the sides of the container.

5.6.2. Other methods, approved by the DNA Technical Leader, may be used for the collection of touch evidence (e.g. tape-lifting, scraping, etc.).

5.7. **Hair Roots**

5.7.1. Prior to processing any hairs for DNA, consult with a trace evidence examiner.

5.7.2. When appropriate, gently rinse hair with 70% ethanol followed by sterile water prior to taking a cutting.

5.7.3. Cut a 0.5-1 cm piece of the root end of the hair and put it in a microcentrifuge tube.

5.7.4. If hair is not cleaned with ethanol and water, generate a control portion of hair by cutting another 0.5-1 cm piece of hair (non-root) and process it alongside the sample.

5.8. **General Biological Stains (e.g. blood, saliva, etc.)**

5.8.1. Cuttings may be taken from biological stains.

5.8.1.1. Cut an approx. 5 mm x 5 mm portion of the stain/substrate (clothing, carpet, etc.) and place in a clean microcentrifuge tube (or in a clean NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket with tube if proceeding directly to extraction).

5.8.1.2. Some specimens may require cuttings of differing sizes depending on the type, condition and concentration of the biological material. Larger sized cuttings may be cut into smaller pieces before placing them into the tube.

5.8.2. Biological stains may also be swabbed.

5.8.2.1. Wet a sterile swab with sterile water (or other approved wetting solution) and swab the stain, attempting to concentrate the collected material onto the swab tip.

5.8.2.1.1. Following the wet swab with a dry swab is not usually necessary when a dried bloodstain is swabbed.

5.8.2.2. The swab(s) should be placed in an envelope, swab box, or other breathable container.

5.8.2.2.1. For swabs used to collect potentially low quantities of biological material, the swabs should be placed in a swab box that minimizes contact between the swab tips and the sides of the container.

5.8.3. For liquid samples, place a portion of the liquid (1-100 µL) directly into a clean microcentrifuge tube.

5.8.4. Other collection methods approved by the DNA Technical Leader may be used.

5.9. **Reference Samples**

5.9.1. Cut a small portion of a blood card (up to 5 mm^2), swab tip, or other reference material and place in a clean microcentrifuge tube (or in a clean NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket with tube if proceeding directly to extraction).

5.10. In order to maximize the recovery of information from each item of evidence, sampling for DNA will be done in conjunction with Latent Print, Explosive, Fire Debris, or Firearms/Toolmark examinations as applicable.

5.10.1. When processing an item in conjunction with a **Latent Print** examination:

5.10.1.1. **Cyanoacrylate fuming**

5.10.1.1.1. The textured areas of the exhibit will be swabbed prior to any chemical treatment (e.g. cyanoacrylate fuming or dye-staining). If this is done, care must be taken when handling the exhibit to avoid destroying any latent fingerprint evidence.

5.10.1.1.2. **OPTIONAL:** For Firearms evidence only, the Examiner/Technician may swab the exhibit (both textured and smooth areas) after the Latent Print examination has been completed.

5.10.1.1.2.1. It may be beneficial to swab one or more areas of the exhibit prior to the Latent Print examination so there is a "safe" place to handle the exhibit (e.g. grips of a firearm).

5.10.1.1.2.2. If a Latent Print examiner swabs an area prior to the Latent Print examination and a DNA analyst will be performing the remaining swabbing, the Latent Print examiner can place the swabs (in a marked and properly sealed swab box) back in the original container along with the evidence and report the swabbing activity in the Latent Print report.

5.10.1.1.3. After the Latent Print examination has been completed, the Examiner/Technician collecting the DNA samples will evaluate the evidence using an alternate light source, such as the latent print laser, to look for areas of possible ridge detail, smudging, smears, or other areas potentially indicating the deposition of biological material on the smooth surfaces. The alternate light source should use a wavelength appropriate for the dye used during the latent print process. Additional swabs will be taken from these areas, if located.

5.10.1.1.4. The presence or absence of possible ridge detail/smudging is only one factor in deciding what additional areas to swab. The type of evidence and how an item is typically used or handled should also be evaluated. For example, the entire surface of a lighter should be swabbed even if there is no possible ridge detail or smudging observed.

5.10.1.1.5. In some cases, the Examiner/Technician may perform additional swabbing between cyanoacrylate fuming and dye-staining. If this is done, care must be taken when handling the exhibit to avoid destroying any latent fingerprint evidence.

5.10.1.2. **Ninhydrin**

5.10.1.2.1. DNA samples may be taken from evidence that has undergone ninhydrin treatment if the chemical was applied with a spray or squirt bottle. DNA swabs or samples shall not be taken from evidence that has been immersed in ninhydrin solution, or if ninhydrin was applied to the evidence with a previously used brush.

5.10.1.2.2. Cuttings or swabs may be taken depending on the evidence or case scenario. Swabbing paper may cause the paper to tear, therefore care should be taken to minimize the damage to the evidence as much as possible during swabbing.

5.10.1.3. **Fingerprint Powder**

5.10.1.3.1. DNA swabs or samples shall not be taken from evidence that has been dusted with fingerprint powder unless a new brush was used and the powder was not previously used on other evidence.

5.10.1.4. **Identifiable Prints**

5.10.1.4.1. Prints designated "identifiable" by a latent print examiner shall be swabbed for DNA analysis. **Latent print photography must be completed before any identifiable prints are swabbed.**

5.10.1.4.2. When feasible, each identifiable print shall be swabbed separately as its own sample. Under certain circumstances (e.g. large number of identifiable prints), multiple prints may combined and sampled together if it is reasonable to assume they originated from the same source. See the *DNA Swabbing Guidelines and Examples* presentation for examples.

5.10.1.4.3. Prints that have been identified as originating from a specific individual at the time of DNA swabbing do not need to be swabbed unless the case circumstances suggest it may be informative (e.g. a bloody fingerprint that was identified to the suspect, but may contain the victim's DNA in the blood).

5.10.2. When processing an item in conjunction with an **Explosive** examination:

5.10.2.1. DNA samples should be taken at the earliest possible time that will not interfere with further analysis during the Explosives examination.

5.10.2.2. The evidence should be sampled prior to subjecting the exhibit to UV radiation.

5.10.3. When processing an item in conjunction with a **Fire Debris** examination:

5.10.3.1. The DNA swabs may be taken before or after the Fire Debris examination unless a solvent wash will be used as part of the Fire Debris examination. DNA swabs should be taken prior to a solvent wash.

5.10.4. When processing an item in conjunction with a **Firearms/Toolmark** examination:

5.10.4.1. In general, evidence will not be sampled for DNA analysis after it has been subjected to a Firearms/Toolmark examination.

5.10.4.2. Exceptions may be made based on the specific circumstances of the case/evidence. Firearms/Toolmark examiners will contact a Forensic Biologist if questions regarding potential DNA evidence arise.

6. Writing DNA Collection Notifications

6.1. Once DNA collection activities have been completed for a case or submission, a *DNA Collection Notification* will be generated.

6.1.1. Examiners from other disciplines authorized to perform DNA collection can include the *DNA Collection Notification* within laboratory reports of examinations.

6.1.2. If DNA analysis will proceed shortly after DNA collection, then a DNA Collection Notification is not required.

6.2. **DNA Collection Notifications are not reports of scientific findings; rather they are a means of communicating to the submitter that the request for DNA collection has been fulfilled.**

7. Independent DNA Collection Notifications

7.1. Independent *DNA Collection Notifications* will be issued to the submitter via email (see Appendix 1 for an example). This email will include the following sections:

7.1.1. **Header** – This section contains the ATF Laboratory Number, the ATF Investigation Number, and the Title of Investigation.

7.1.2. **Introduction Statement** – This section contains the date on which the evidence was delivered to the laboratory.

7.1.3. **Exhibits section** – This section contains the exhibits evaluated for DNA Collection.

7.1.4. **DNA Sample Collection section** – This section contains the exhibit numbers that were sampled for DNA, along with sub-exhibits generated.

7.1.4.1. If an exhibit does not meet the criteria for DNA collection, it will be noted in this section along with the reason. The following reasons will be used:

7.1.4.1.1. "**due to the condition of the evidence**"

This statement is used when the evidence is badly damaged, rusted, burnt, or consists of numerous, very small pieces. If it were in better/different condition, this type of evidence would normally be sampled for DNA. A description of the condition will be inserted so the investigating agent knows why the exhibit could not be sampled for DNA.

7.1.4.1.2. "**"Brass/copper evidence must meet select criteria to be sampled for DNA analysis at the ATF Laboratory. Exhibits __ appear to be constructed of brass/copper, but do not meet the required criteria ; therefore, these exhibits were not sampled for DNA analysis."**"

This statement is used when the evidence consists of brass-colored cartridges or other evidence that appears to be made of brass or copper.

7.1.4.1.3. "**no areas suitable for DNA collection were observed**"

This statement is used when an exhibit has been evaluated and no areas of possible ridge detail, smears or smudging are observed. In addition, there are no areas where someone would normally handle the exhibit. This reason should be used rarely.

7.1.5. **Disposition section** – This section contains a description of what was done with the collected DNA samples and the evidence that was sampled.

7.1.5.1. DNA samples or evidence will typically be "forwarded to the Forensic Science Laboratory – Washington for DNA analysis" or "returned to the investigating agent." Other wording may be used as appropriate.

7.1.5.2. If a DNA sample or evidence that could reasonably be expected to still have DNA present (i.e. a shirt) is returned to the investigating agent, a statement explaining retention of biological evidence will be included.

7.1.5.2.1. NOTE: Exhibit *X* may potentially contain biological evidence

subject to specific storage and preservation requirements. Please reference the current version of ATF O 3400.1 to review the storage and preservation requirements of this evidence for the purposes of future DNA analysis.

8. DNA Collection Notifications within a Laboratory Report

8.1. When *DNA Collection Notification* is included as part of a laboratory examination report, the DNA collection activities shall be listed in the Examination/Analysis and Interpretation of Results Section (see Appendix 2 for an example).

8.1.1. If an exhibit does not meet the criteria for DNA collection, it will be noted in this section along with the reason. See 7.1.4.1 for approved reasons.

8.2. The Disposition Section of the Laboratory Report will contain the disposition of the evidence and the DNA samples taken during DNA collection.

8.2.1. DNA samples or evidence will typically be "forwarded to the Forensic Science Laboratory – Washington for DNA analysis" or "returned to the investigating agent." Other wording may be used as appropriate.

8.2.2. If a DNA sample or evidence that could reasonably be expected to still have DNA present (i.e. a shirt) is returned to the investigating agent, a statement explaining retention of biological evidence will be included.

8.2.2.1. NOTE: Exhibit *X* may potentially contain biological evidence subject to specific storage and preservation requirements. Please reference the current version of ATF O 3400.1 to review the storage and preservation requirements of this evidence for the purposes of future DNA analysis.

9. Technical/Administrative Review of Notifications

9.1. Prior to sending the notification email or the laboratory report containing a *DNA Collection Notification* to the investigating agent, the printed draft notification email or the draft laboratory report and all associated DNA collection case records shall undergo a technical review and an administrative review using the *DNA Collection Review Form* (ATF-LS-F-5.9.4 C).

9.2. The technical review shall be conducted by an individual qualified to perform DNA Collection activities. The administrative reviewer shall be a Section Chief or an employee authorized by laboratory management. The technical and administrative reviews for DNA collection can be conducted by the same individual.

9.3. The completion of the technical and administrative review is documented by the initials of the reviewer(s) on the completed *DNA Collection Review Form*.

9.3.1. If the notification is through email, the initials of the technical and administrative reviewer(s) shall also be included on the printed draft notification email. After completion of the review, the email will be sent. A copy of the draft and sent emails will be maintained with the case record.

9.3.2. If the notification is included as part of a laboratory examination report, the *DNA Collection Review Form* will be used in addition to the appropriate review form for the laboratory examination.

Appendix 1: Sample DNA Collection Notification

Email Subject: DNA Collection Notification for ATF Lab# (ATF IN)

ATF Laboratory Number: xxxxx
ATF IN: xxxxx
Title of Investigation: Attempted Murder

Special Agent / Task Force Officer xxxxx,

The evidence described below was received on August 2, 2016:

EXHIBITS

1 One 9mm Glock pistol, Serial number 12343
2 One gas can
3 One cloth

DNA SAMPLE COLLECTION

Exhibits 1 and 3 were sampled for DNA analysis. The DNA samples were designated 1.1 through 1.4, 3.1 and 3.2.

The gas can (Exhibit 2) is not suitable for DNA Collection due to the melted/burned condition of the evidence.

DISPOSITION OF EVIDENCE

The DNA samples listed above will be forwarded to the ATF Forensic Science Laboratory – Washington for DNA analysis. The remaining evidence will be returned to the investigating agent.

NOTE: Exhibit 3 may potentially contain biological evidence subject to specific storage and preservation requirements. Please reference the current version of ATF O 3400.1 to review the storage and preservation requirements of this evidence for the purposes of future DNA analysis.

Joe Swabber
Job Title
Bureau of Alcohol, Tobacco, Firearms and Explosives
Forensic Science Laboratory
Office: (xxx) xxx-xxxx

Appendix 2: Sample Laboratory Examination Report including DNA collection

Special Agent xxxxx
1000 1st Avenue, NE
Suite 101
Beltsville, MD 20705

Date of Report: xxxxxx
Lab Number: xxxxxxx
Reference: xxxxxx-xx-xxxx
Title: Attempted Murder
Type of Exam: Fingerprint

The evidence described below was received on xx/xx/xx:

EXHIBITS

1 One 9mm Glock pistol, Serial number 12343
2 One gas can
3 One cloth

EXAMINATION/ANALYSIS AND INTERPRETATION OF RESULTS

Exhibits 1 and 2 were processed and examined for the presence of identifiable latent prints with negative results.

DNA SAMPLE COLLECTION

Exhibits 1 and 3 were sampled for DNA analysis. The DNA samples were designated 1.1 through 1.4, 3.1 and 3.2.

The gas can (Exhibit 2) is not suitable for DNA Collection due to the melted/burned condition of the evidence.

DISPOSITION OF EVIDENCE

The DNA samples listed above will be forwarded to the ATF Forensic Science Laboratory – Washington for DNA analysis. The submitted exhibits are being returned to the contributor.

Joe Analyst
Fingerprint Specialist

REVIEWED BY:

Bob Smith
Chief, Identification Section

1. **Scope**

The Staff Index is a database of DNA profiles from the Bureau of Alcohol, Tobacco, Firearms and Explosives (ATF) staff members who may come in contact with evidence in the course of their routine duties. ATF staff members may include: laboratory staff, special agents, explosive enforcement officers, task force officers, contractors, and interns involved in processing scenes and handling evidence, as well as building maintenance personnel and visitors (e.g. maintenance contractors or instrument repair personnel, attorneys and local officers) who may enter a room where evidence is processed. The purpose of the DNA Staff Index is to detect contamination of DNA profiles resulting from individuals who handled the evidence during, or after, the investigation or those who may enter evidence processing laboratories during the course of routine duties. Comparing and eliminating the DNA profiles of ATF staff members augments the value and integrity of the potential leads that may be derived from the DNA collected at crime scenes.

The DNA Staff Index is maintained electronically on the Laboratory's Combined DNA Index System (CODIS) server. There are three levels of CODIS: the National DNA Index System (NDIS), the State DNA Index System (SDIS), and the Local DNA Index System (LDIS). The ATF DNA Staff Index is stored at the LDIS level and can only be accessed by the members of the ATF Laboratory's DNA Unit. Each ATF Staff DNA profile is assigned an anonymous identifier. There are no names maintained on the CODIS server, only the anonymous identifiers. A separate computer contains the secure file that coordinates the anonymous identifiers with the names of the ATF staff members.

2. **References**

2.1. The genetic information nondiscrimination act (2008), 42 U.S.C. § 2000FF-1 (b)(6).
2.2. Federal Bureau of Investigation, Quality Assurance Standards for Forensic DNA Testing Laboratories, (current version).
2.3. Federal Bureau of Investigation, Quality Assurance Standards for Databasing Laboratories, (current version).
2.4. ATF-LS-FB19 CODIS Manual.

3. **Equipment**

3.1. Disposable gloves
3.2. Eye protection
3.3. Lab coat

4. **Safety/Quality Assurance**

4.1. All samples must be handled, prepared and processed in the dedicated reference areas of the laboratory.
4.2. Disposable gloves shall be worn when handling swabs.

4.3. Lab coat and eye protection must be worn at all times while processing reference samples in the lab.

5. Procedure

5.1. Each sample will be assigned an anonymous ATF Staff Index Specimen ID number for tracking on the CODIS server.

5.2. The list of names and corresponding Staff Index Specimen ID numbers will be maintained in a secure file, separate from the CODIS server or workstation(s).

5.3. **In-house processing of Staff Index samples**

5.3.1. All Staff Index samples will be processed and analyzed according to current ATF protocols.

5.3.2. A data review will be conducted on all of the data from the Staff Index samples and associated control samples.

5.3.2.1. This data review shall be conducted by a qualified examiner other than the one who conducted the analysis.

5.3.2.2. The data review will be documented on the *Staff Index Sample Review Form*.

5.3.2.3. To successfully pass the data review, the following conditions must be met:

All DNA types must be supported by the raw or analyzed data (electropherograms).

Correct types must be obtained for the amplification positive control sample(s).

Possible allelic peaks in the reagent blank or amplification negative control will be evaluated according to current interpretation protocols.

5.3.3. Samples that successfully pass the data review shall be entered into CODIS in the Staff Index. The samples may be exported from the GeneMapper® IDX software and imported into CODIS or entered by hand. The Specimen Category of "Staff" shall be used for the DNA profiles of all ATF Staff members and others included in the Staff Index.

5.3.4. Following entry into CODIS, but prior to the next search conducted, the sample data will be verified by a qualified DNA analyst with access to CODIS, other than the one who conducted the data review. This verification ensures that the DNA types in CODIS match the DNA types from the data for that sample. This verification is documented on the *Staff Index Sample Review Form*.

5.3.5. The *Staff Index Sample Review Form* will be maintained with the sample data.

5.3.6. Staff Index samples shall remain at the LDIS level and cannot be uploaded to the SDIS or NDIS levels.

5.4. **Outsourcing of Staff Index samples**

5.4.1. On occasion, it may be necessary to outsource Staff Index Samples to a qualified vendor. The requirements for qualifying a vendor may be found in ATF-LS-FB26 Outsourced Casework Samples.

5.4.2. Preparing Staff Samples

5.4.2.1. The samples being sent to the vendor laboratory for analysis will be labeled with the ATF Staff Index Specimen ID number only.

5.4.2.2. No identifying markings (name, badge number, post of duty, etc.) will be included on the samples sent to the vendor laboratory. The samples should be placed in new packaging if necessary.

5.4.3. Sending Samples for Analysis

5.4.3.1. Samples sent to the vendor laboratory will be organized into batches. A list of the ATF Staff Index Specimen ID numbers for the samples in each batch will be compiled and provided to the vendor laboratory along with the samples.

5.4.3.2. Each batch of samples sent for analysis shall include at least one ATF Staff sample previously analyzed by ATF. These previously analyzed samples will serve as Quality Assurance (QA) samples.

5.4.3.3. The samples will be delivered to the vendor laboratory by a secure, traceable means (such as FedEx) or hand delivered. If the samples are hand delivered, an acknowledgement of receipt (including date) shall be obtained.

5.4.4. Reviewing Data Received

5.4.4.1. A review will be conducted on all of the data from the Staff Index samples and associated control samples processed by the vendor laboratory. The control samples should include, but are not limited to, the reagent blanks, positive and negative amplification controls, internal sizing standards and allelic ladders.

5.4.4.2. The data review shall be conducted by a DNA analyst, currently or previously qualified in the technology, analysis platform and amplification kit used to generate the DNA data.

5.4.4.3. The data review will be documented on *the Staff Index Sample Review Form*.

5.4.4.4. To successfully pass the data review, the following conditions must be met:

All DNA types must be supported by the raw or analyzed data (electropherograms).

Correct types must be obtained for the amplification positive control sample(s).

Correct types must be obtained for the QA samples.

No true peaks observed in the reagent blank(s) or amplification negative control samples.

5.4.5. After successfully passing the initial data review, the samples will be entered into the Staff Index in CODIS by importing the electronic file supplied by the vendor laboratory.

5.4.6. Following entry into CODIS, but prior to the next search conducted, the sample data will be verified by a qualified DNA analyst with access to CODIS. This verification ensures that the DNA types in CODIS match the DNA types from the data for that sample. This verification is documented on the *Staff Index Sample Review Form*.

5.4.7. The *Staff Index Sample Review Form* will be maintained with the sample data.
5.4.8. Samples or batches that do not pass the technical data review will be evaluated for return to the vendor laboratory for re-analysis.
5.4.9. The remainder of the samples sent to the vendor laboratory will be returned to the ATF Laboratory after analysis.

1. Scope

This protocol is used to extract and purify DNA from low template evidence samples, hair root material, and high template/reference samples. DNA can be extracted from biological material efficiently using the QIAamp® DNA Investigator Extraction Kit on the QIAcube® and QIAcube® Connect Robotic Workstation using the custom ATF DNA purification protocol. First, the biological material is digested and the cells are lysed in a buffer containing a detergent and a protease. Next, the samples are placed on the QIAcube® or QIAcube® Connect and through automation, the lysate is passed through a membrane that binds the DNA. Contaminants are then washed off the membrane through three washing steps. Finally, the purified DNA is eluted off the membrane in a small volume of an appropriate buffer. The DNA extract should be free of contaminants but further purification methods may be used to remove inhibitory substances that remain, if necessary.

2. References

2.1. Qiagen® QIAamp® DNA Investigator Handbook, December 2007.
2.2. Qiagen® QIAcube® User Manual, June 2008.
2.3. Qiagen® QIAcube® Connect User Manual, January 2022
2.4. Qiagen®, Developmental validation of the QIAamp® DNA Investigator Kit, July 2015, available at: https://www.qiagen.com/us/resources/resourcedetail?id=1d9de4e8-cbd4-4be7-aec2-dd9f79f038fb&lang=en&autoSuggest=true.
2.5. I. Van Niekerek, C. Snyman, J. Van Niekerk, A. Greyling, Efficient Purification of Genomic DNA from Forensic Samples using QIAamp® Kits, Qiagen News 1 (2005) 10-12.
2.6. V. Castella, N. Dimo-Simonin, C. Brandt-Casadevall, P. Mangin, Forensic Evaluation of the QIAshredder®/QIAamp® DNA Extraction Procedure, Forensic Sci. Int. Genet. 156(1) (2006) 70-73.
2.7. D.J. Johnson, L. Martin, K.A. Roberts, STR-Typing of Human DNA from Human Fecal Matter using the Qiagen® QIAamp® Stool Mini Kit, J. Forensic Sci. 50(4) (2005) 802-808.
2.8. N. Vandenberg, R.A. Van Oorschot, Extraction of Human Nuclear DNA from Feces Samples using the QIAamp® DNA Stool Mini Kit, J. Forensic Sci. 47(5) (2002) 993-995.
2.9. J.M. Butler, Sample Collection, DNA Extraction, and DNA Quantitation in: Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers, second ed., Elsevier Academic Press, 2005, pp 42-50.
2.10. P. Gill, A.J. Jeffreys, D.J. Werrett, Forensic Application of DNA Fingerprints, Nature 318 (1985) 557-579.
2.11. R. Higuchi, C. H. Von Beroldingen, G. F. Sensabaugh, H. A. Erlich, DNA Typing from Single Hairs, Nature 332 (1988) 543–546.

2.12. C.T. Comey, B.W. Koons, K.W. Presely, J.B. Smerick, C.A. Sobieralski, D.M. Stanley, F.S. Baechtel, DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis, J. Forensic Sci. 39(5) (1994) 1254-1269.
2.13. S.A. Greenspoon, M.A. Scarpetta, M.L Drayton, S.A. Turek, QIAamp® Spin Columns as a Method of DNA Isolation for Forensic Casework, J. Forensic Sci. 43(5) (1998) 1024-1030.
2.14. K. Sinclair, V.M. McKehnie, DNA Extraction from Stamps and Envelope Flaps using QIAamp® and QIAshredder®, J. Forensic Sci. 45(1) (2000) 229-230.
2.15. I. Van Niekerk, C. Snyman, J. , Van Niekerk, A. Greyling, Efficient Purification of Genomic DNA from Forensic Samples using QIAamp® Kits, Qiagen News 1 (2005) 10-12.
2.16. ATF Laboratory Validation of the Custom ATF DNA Extraction Protocol on the QIAcube® Robotic Workstation.
2.17. ATF Laboratory Performance Check of the QIAcube® Connect Robotic Workstation.

3. Equipment

3.1. Disposable gloves
3.2. Eye protection
3.3. Lab coat
3.4. Sterile swabs
3.5. Sterile water
3.6. Disposable scalpels or razor blades
3.7. Scissors
3.8. Forceps
3.9. 70% ethanol or alcohol wipes
3.10. 10% bleach solution
3.11. QIAcube® Robotic and QIAcube® Connect Workstation containing the following:
  3.11.1. QIAcube® Reagent bottles
  3.11.2. QIAcube® Reagent rack with appropriate labeling strips
  3.11.3. QIAcube® Centrifuge rotor adapters
  3.11.4. QIAcube® 1000ul Filter tips
  3.11.5. QIAcube® 1.5 mL Microcentrifuge elution tubes
3.12. QIAamp® DNA Investigator Kit (Qiagen Catalog # 56504) containing the following:
  3.12.1. QIAamp® MinElute™ columns
  3.12.2. Collection Tubes
  3.12.3. Buffer ATL
  3.12.4. Buffer AL
  3.12.5. Buffer AW1
  3.12.6. Buffer AW2
  3.12.7. Buffer AE
  3.12.8. Carrier RNA

3.12.9. Proteinase K
3.13. Invitrogen Proteinase K (optional)
3.14. 96-100% Ethanol (EtOH)
3.15. Dithiothreitol (DTT)
3.16. Pipettes
3.17. Disposable aerosol-resistant pipette tips
3.18. Microcentrifuge tubes
3.19. NAO™ Baskets/QIAGEN® Investigator Lyse and Spin Baskets and Tubes **(OPTIONAL: DNA IQ™ Spin Baskets)**
3.20. Benchtop hood
3.21. TE^{-4} (10mM Tris-HCl , 0.1mM EDTA, pH 8.0)
3.22. Bench paper
3.23. Thermomixer
3.24. Centrifuge
3.25. Vortexer

4. Safety/Quality Assurance

4.1. Any utensils used to cut or manipulate the swabs or other types of evidence must be cleaned between uses with 10% bleach solution, followed by 70% ethanol or alcohol wipe.
4.2. Disposable gloves shall be worn when handling kit reagents and evidence.
4.3. Extraction steps performed outside of the QIAcube® or QIAcube® Connect Robotic Workstation should be performed in a hood to reduce the risk of contamination. Clean surfaces with 10% bleach solution, followed by 70% EtOH, before and after use. After exiting hood, turn on UV light (automatically set for 15 minutes of exposure).
4.4. Use aerosol-resistant pipette tips when transferring liquids containing DNA.
4.5. Change pipette tips after transferring any liquids potentially containing DNA.
4.6. Record the lot number, and expiration date of each reagent used in notes. Do not use the reagents after the expiration date.
4.7. Initiate the appropriate number of reagent blanks as the final samples of the set of extractions.
4.8. Lab coat and eye protection must be worn at all times while performing this procedure.
4.9. When practical, only tubes associated with one DNA extract shall be open at a time.
4.10. Exercise caution when opening tubes.
4.11. The laboratory bench surface shall be cleaned before use with 10% bleach solution or other sanitizing agent and may be followed by 70% ethanol. Fresh bench paper shall then be placed on the surface prior to evidence examination.
4.12. The QIAcube® or QIAcube® Connect worktable shall be cleaned with 70% ethanol solution before and after use.
4.13. Final DNA extract tubes shall be labeled, at a minimum, with case number, item number, and analyst's initials.

4.14. Samples thought to contain lower levels of DNA will be handled before those thought to contain large amounts of DNA.

4.15. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the technical leader.

4.16. Dedicated QIAcube® or QIAcube® Connect Workstations for use with high template or reference samples shall not be used for low template samples. High template samples and reference samples may be processed on the same dedicated QIAcube® or QIAcube® Connect Workstations, though not at the same time.

5. Procedure

5.1. Check the reagent logs or the reagent bottles to ensure that Buffer AW1, Buffer AW2, and carrier RNA have been appropriately prepared. Prior to use, Buffer ATL must contain no precipitates. If precipitates have formed, gently heat the bottle prior to dispensing the reagent.

Cell Lysis (performed manually off instrument)

5.1.1. Lyse samples according to step 5.2 (touch evidence),5.3 (hair roots), or 5.4 (high template/reference samples):

NOTE: A reagent blank shall be initiated as the last sample in the set of samples. If more than one workstation will be used, the samples and reagent blanks will be set up in subsets per workstation, with the reagent blank(s) at the end of each subset. The reagent blank shall contain all the liquid reagents contained in the evidentiary samples except for the biological material. The reagent blank shall be handled in a manner that is identical to the evidentiary sample(s), and is the most sensitive volumes and steps used with the evidentiary sample(s). For example, if carrier RNA is used with only a few of the evidentiary samples being extracted in a set, carrier RNA will be added to the reagent blank, as well. Additionally, if two or more evidence extracts are combined after extraction during the concentration step, the same number of reagent blank extracts will be combined.

NOTE: If batches containing greater than twelve samples are extracted using QIAcube® and/or QIAcube® Connect workstations at least one reagent blank shall be processed on each of the QIAcube® and/or QIAcube® Connect workstations employed. Reagent blanks initiated on the same day, even if a portion of the batch was completed on a workstation on day one and the remaining samples were processed on a workstation on day two after an overnight incubation, are considered part of a single batch of samples. If the analyst may potentially combine two DNA extracts after quantitation, processed on the same workstation, two reagent blanks will be initiated for that workstation. If multiple workstations are going to be used to process a batch

of samples and there is the possibility of combining samples across workstations, then two reagent blanks will be initiated for each of the workstations. For each workstation involved in combined samples, the two reagent blank extracts will be combined. For example, QIAcubes® 1, 2, and 3 are used to process a batch of samples. Due to the possibility of combining DNA extracts after quantitation, two reagent blanks are initiated for each workstation. A DNA extract from QIAcube® 1 and QIAcubes® 2 are to be combined after quantitation. The two reagent blanks from QIAcubes® 1 are combined and the two reagent blanks from QIAcubes® 2 are combined resulting in two reagent blanks representing the samples processed on QIAcube® 1 and QIAcubes® 2. In this scenario, if no DNA extracts are to be combined from QIAcube® 3, then only one of the reagent blanks needs to be processed further. If, after quantitation, the analyst determines that none of the samples will be combined, then at least one reagent blank from each QIAcube® and/or QIAcube® Connect workstation will be processed with the associated samples. Documentation for the reagent blank(s) associated with the workstation(s) used for the specific case will be retained with the case file.

5.2. Touch Evidence (or other samples expected to contain low quantities of DNA)

5.2.1. Sample the area of interest using the appropriate sampling method for the evidence, and place sample in a clean Qiagen® Investigator Lyse and Spin basket.

5.2.2. To initiate reagent blanks, cut the tips or break off the appropriate swabs into a clean Qiagen® Investigator Lyse and Spin basket. Initiate the appropriate number of reagent blanks based on the sample set being processed.

5.2.3. Add 400 µL of Buffer ATL and 20 µL of ProK (20 mg/mL) to each sample and vortex for approximately 10 seconds. For fired cartridge case (FCC) samples, just add 20 µL of ProK (20 mg/mL) to the existing Qiagen® Investigator Lyse and Spin basket containing the lysate and swabs. Continue at step 5.5.

5.3. Hair Roots

NOTE: Prior to processing any hairs for DNA, consult with trace evidence examiners.

5.3.1. Using a clean pair of forceps and/or scissors (or disposable scalpel/razor blade), cut off a 0.5-1 cm piece of the root end of the hair and place it in a clean 2 mL safe-lock microcentrifuge tube or QIAGEN® Investigator Lyse and Spin tube. Gently rinse hair with 70% ethanol followed by sterile water.

5.3.2. Initiate a reagent blank using a clean 2 mL safe-lock microcentrifuge tube or Qiagen® Investigator Lyse and Spin tube.

5.3.3. Add 400 µL of Buffer ATL, 20 µL of ProK (20 mg/ml), and 20 µL of 1M DTT to each sample and vortex the tubes for approximately 10 seconds. Continue at step

5.4. High Template or Reference Samples

5.4.1. For high template or reference samples, cut a small portion of blood card (typically 5 mm^2), swab tip, or other material and place in a clean microcentrifuge

tube (or in a clean NAO™ Basket/QIAGEN® Investigator Lyse and Spin basket with tube if recovery of lysate is a priority).

5.4.2. Add 400 µL of Buffer ATL and 20 µL of ProK (20 mg/mL) to each sample and vortex for approximately 10 seconds. Continue at step 5.5.

5.5. Place samples in a thermomixer and incubate at 56° C with shaking at 900 rpm for at least three hours (at least one hour for high template or reference samples). Samples may be incubated overnight; however, incubation times greater than 18 hours have been shown to decrease DNA yield.

NOTE: If NAO™ Basket/QIAGEN® Investigator Lyse and Spin baskets are being used to improve lysate recovery, then centrifuge samples for 1 minute at 10000xg.

OPTIONAL: Centrifuge time can be increased up to 3 minutes and speed increased up to maximum if residual lysate is observed in the baskets. Baskets and substrate may be discarded at this point.

5.6. Briefly centrifuge the samples to remove condensation from the inside of the lid.

5.7. 1 µL of carrier RNA (1 µg/µL) can be added at this point if, due to the concentration or condition of the biological material, it is determined by the analyst that carrier RNA may aid in the recovery of the DNA.

QIAcube® and QIAcube® Connect Workstation Setup

5.8. Wipe down the internal surface area of the workstation, shaker rack, and the waste drawer using 70% EtOH.

5.9. Fill applicable tip racks with 1000 µl filter tips. For the QIAcube®, both tip racks (1 and 2) are required. For the QIAcube® Connect, only tip racks 1 and 2 (not tip rack 3) are necessary.

5.10. Fill reagent bottles with current lot of Buffer AL, 100% EtOH, Buffer AW1, Buffer AW2, and TE^{-4}. Place the filled reagent bottles in the correct position in the reagent bottle rack as marked on the QIAcube®.

NOTE: Upon receipt of new Qiagen® Investigator kits and prior to quality control testing of the new kits, Buffers AW1 and AW2 will be prepared according to manufacturer specifications. When the buffers, EtOH, or TE are replenished on the instrument, record the lot # on the log.

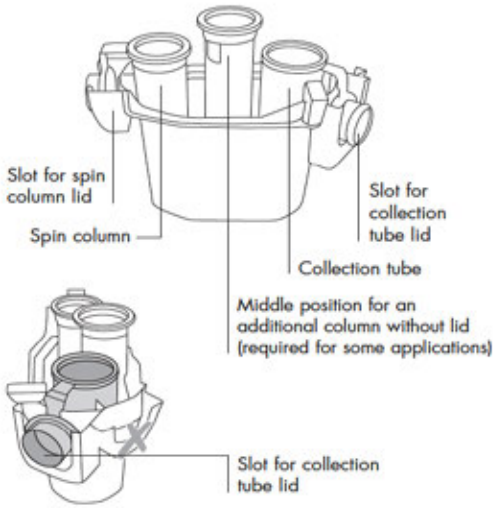
NOTE: Workstation setup will differ based on the number of samples being processed. For the QIAcube®, refer to the QIAcube® protocol sheet (Appendix A) for instructions on appropriate placement of loaded rotor adapters in centrifuge and sample tubes containing lysate into shaker rack. For the QIAcube® Connect, refer to the workstation screen for directions on placement of loaded rotor adapters in the centrifuge and sample tubes containing lysate into the shaker rack.

NOTE: The QIAcube® and QIAcube® Connect cannot process 1 sample or 11 samples in a single run due to balance requirements of the centrifuge.

5.11. Remove the shaker rack from the QIAcube® or QIAcube® Connect. Place labeled sample tubes containing lysate into the shaker rack with the lids of the samples placed in the slots on the edge.

5.12. Place appropriate number of QIAamp® MinElute™ columns and labeled 1.5 mL microcentrifuge elution (collection) tubes into rotor adapters. **NOTE: The QIAamp® MinElute™ column is placed in the rotor position with one corresponding lid holder with the lid in the slot and the microcentrifuge elution tube is placed in the rotor position with two corresponding lid holders with the lid placed in the lower left slot (Figure 1).**

Figure 1: Appropriate placement of QIAamp MinElute™ column and Elution tubes in rotor adapter.



5.13. Place loaded rotor adapters in centrifuge buckets. Ensure that the lips of the QIAamp® MinElute™ columns and labeled 1.5 mL microcentrifuge elution tubes are flush with the top of the rotor adapter. **NOTE: Rotor adapters only fit in the centrifuge buckets in one orientation to ensure correct instrument setup.**

5.14. Place the loaded shaker rack in the shaker. Ensure that the lips of the 2.0 mL microcentrifuge sample tubes are flush with the top of the shaker rack.

Performing the ATF Custom DNA Purification Run on QIAcube® or QIAcube® Connect

5.15. Power on the QIAcube® or QIAcube® Connect workstation and allow the instrument to perform start-up procedure.

5.15.1. For the QIAcube® Connect, first log into the workstation ([redacted]).

5.16. QIAcube®

5.16.1. From the home menu, press *DNA*.

5.16.2. From the "DNA" menu, use the down arrow to highlight option 2 "QIAamp DNA Investigator" and press *Select*.

5.16.3. The option titled "Surface and buccal swabs" will be highlighted. Press *Select*.
5.16.4. Press *Start* then follow the instructions on the screen to ensure the instrument is set-up properly and initiate the custom ATF DNA purification protocol (100 µl final extract volume).

5.17. QIAcube® Connect
5.17.1. From the home menu, press DNA. The option titled "QIAamp DNA Investigator" will be highlighted. Press *Next*.
5.17.2. The option titled "Surface and buccal swabs" will be highlighted. Press *Next*.
5.17.3. Select "Swab purificationmodified" and press *Next*.
5.17.4. The message "Modification of parameters is not possible for this protocol" will be displayed, press *Next*.
5.17.5. Select the number of samples. Press *Next.*
5.17.6. The minimum volumes for each buffer will be displayed. Press *Next*.
5.17.7. The minimum number of pipette tips required will be displayed. Press *Next*.
5.17.8. The rotor adaptor depiction is then displayed. Press *Next*.
5.17.9. Sample IDs are displayed (if the barcode scanner is used). Press *Next*.
5.17.10. Press *Start run*. The estimated time will be displayed if the number of samples being extracted has been run before.

5.18. Upon completion of the protocol, check the sample tubes on the shaker rack for any remaining lysate. Less than 50µl of lysate should be left in the original lysate tube. Volumes greater than this are an indication that the instrument may need calibration or something occurred during the extraction. If an excessive volume is observed, this lysate may be manually extracted and the DNA extract added to the associated sample or it can be preserved for a later determination to discard or finish the extraction manually based on the analyst's discretion. If excess lysate is manually extracted, an additional reagent blank shall be initiated and will be extracted with the excess lysate. This additional reagent blank will then be combined with the associated reagent blank. Carefully remove rotor adapters from the centrifuge and retain the labeled 1.5 mL microcentrifuge elution (collection) tubes containing the DNA extracts. Place caps on the elution tubes containing the DNA extracts. Discard the remaining rotor adapter and its contents.

5.19. The DNA extracts may be concentrated at this point or may proceed to quantification directly (generally, quantification is performed prior to concentration).

5.20. Discard the sample tubes from the shaker rack.

5.21. Place the appropriate lids on the reagent bottles.

5.22. Discard the used tips located in the waste drawer under the touch screen.

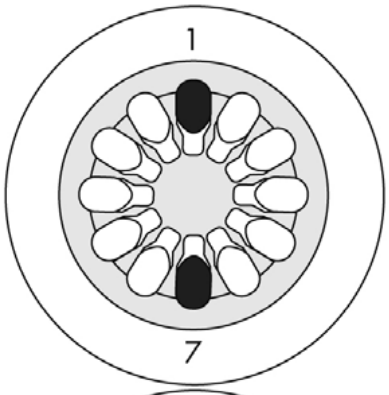
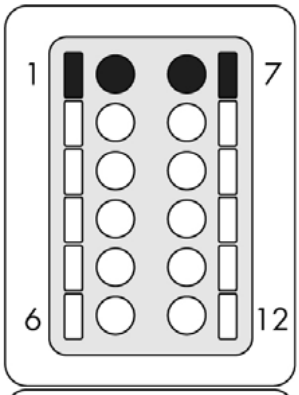
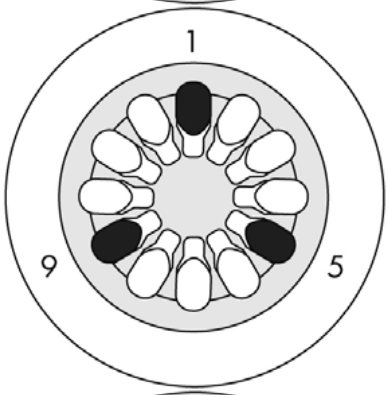
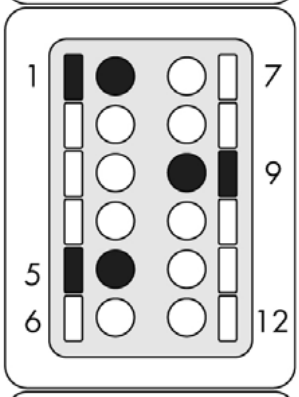
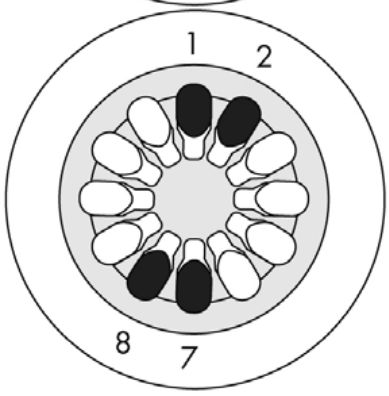
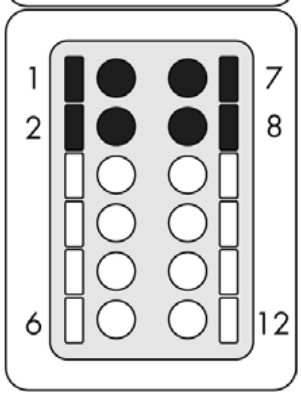
5.23. Discard any empty tip racks.

5.24. Wipe down the internal surface area of the workstation, shaker rack, and the waste drawer using 70% EtOH.

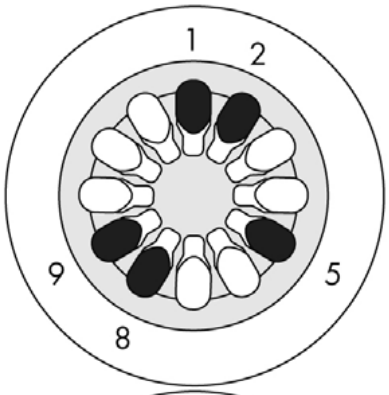
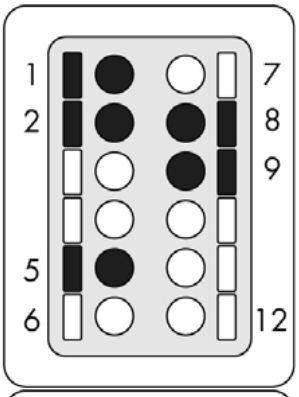
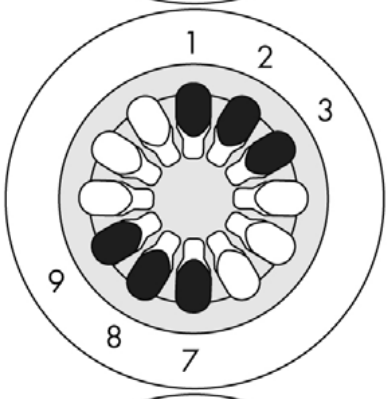
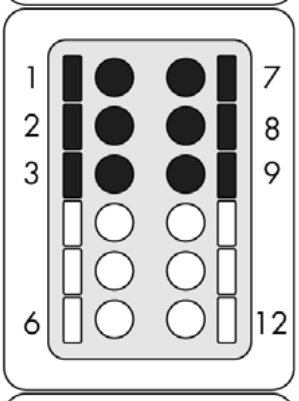
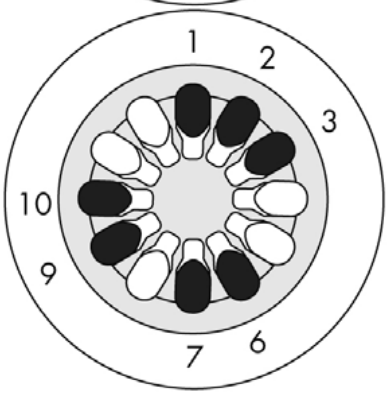
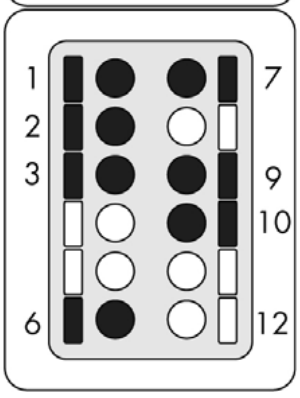
5.25. For the QIAcube® Connect, run the UV decontamination cycle if the workstation will not be in use immediately after the run is complete.

5.26. Complete the appropriate instrument run log to include run date and analyst's initials. Optionally, other information such as number of samples on run, run type (casework, research, etc.) can be added to the run log.

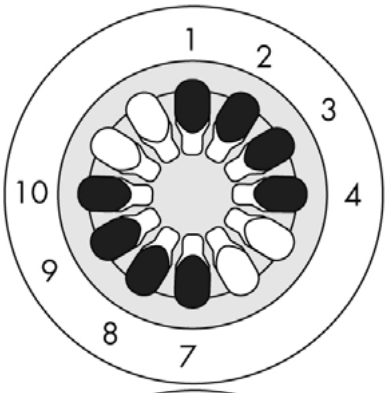
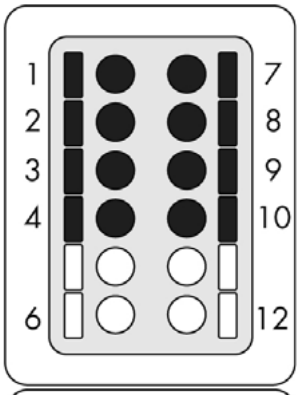
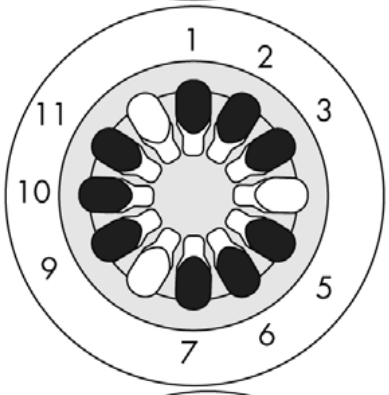
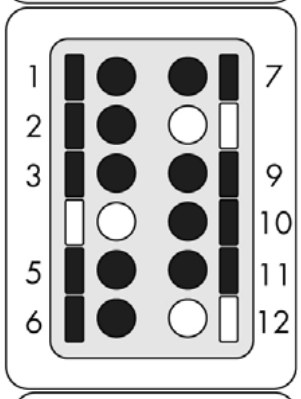
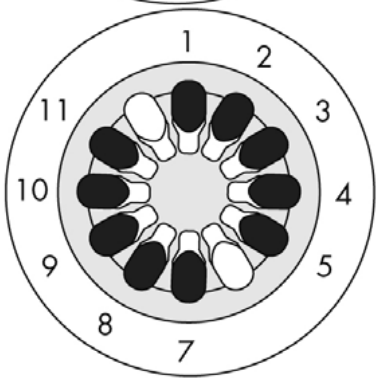
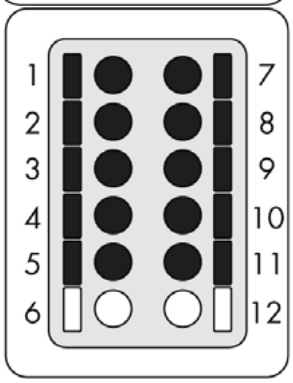
Appendix A: Loading the Centrifuge and Shaker Rack

# of Samples	Centrifuge Setup	Shaker Rack Setup
2 samples		
3 samples		
4 samples		

Appendix A: Loading the Centrifuge and Shaker Rack

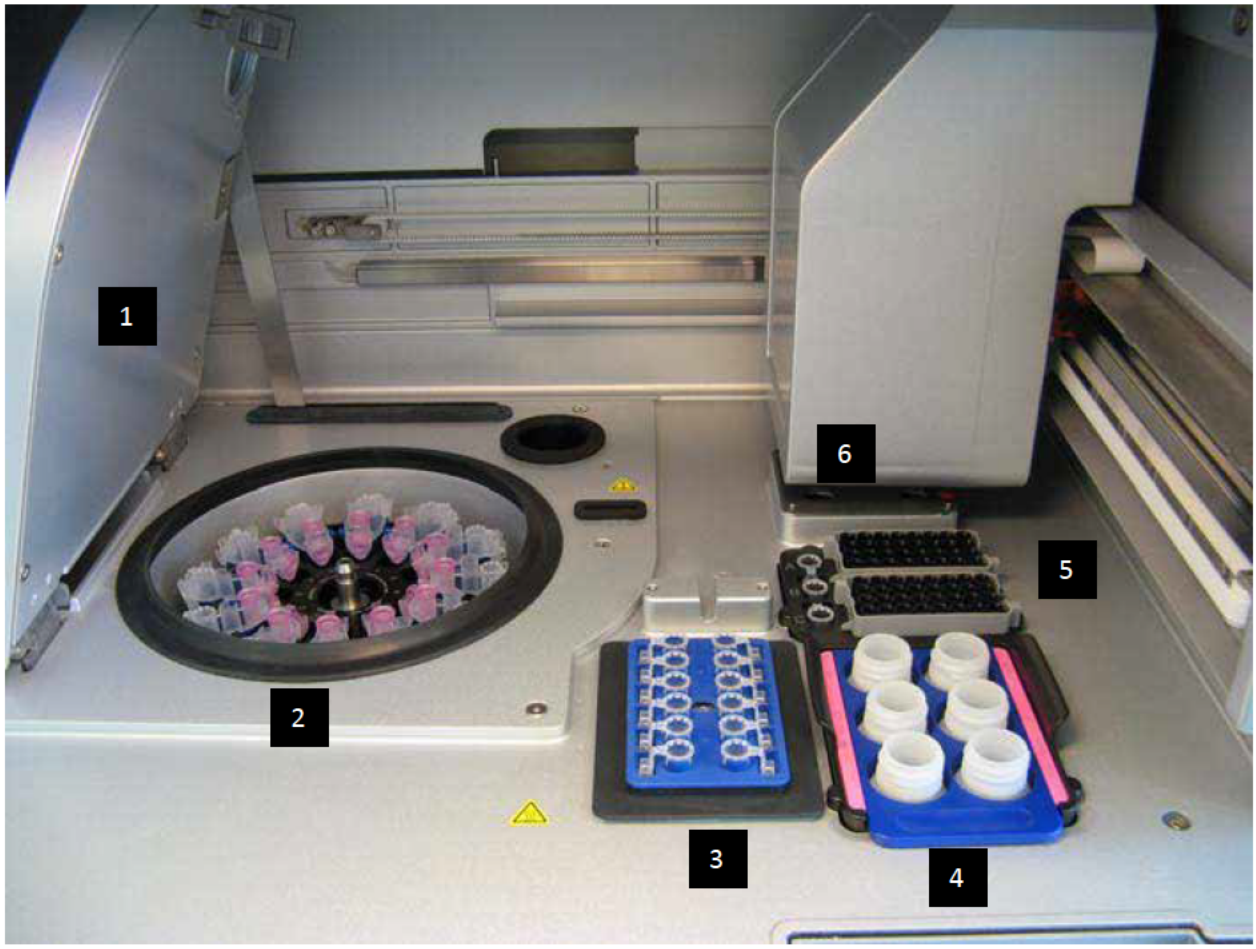
# of Samples	Centrifuge Setup	Shaker Rack Setup
5 samples		
6 samples		
7 samples		

Appendix A: Loading the Centrifuge and Shaker Rack

# of Samples	Centrifuge Setup	Shaker Rack Setup
8 samples		
9 samples		
10 samples		

Appendix B: Internal View

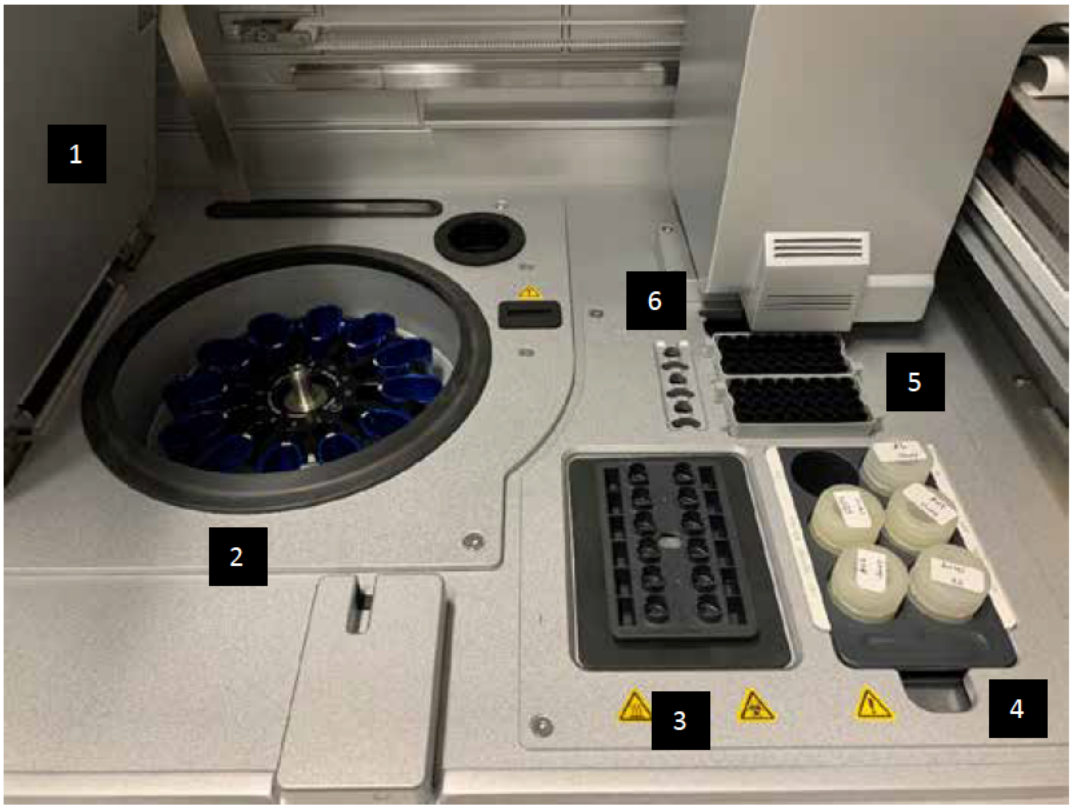
QIAcube®



Components of Note:

1. Centrifuge Lid
2. Centrifuge with Loaded Rotor Adapters
3. Shaker with Loaded Shaker Rack
4. Reagent Rack with Reagents Bottles
5. Tip Rack with 1000µl Filter Tips
6. Disposal Slot for tips

QIAcube® Connect



Components of Note:

1. Centrifuge Lid
2. Centrifuge and Rotor
3. Shaker with Shaker Rack
4. Reagent Rack with Reagents Bottles
5. Tip Racks #1 and #2 (used for the Investigator Protocol)
6. Tip Rack #3 (not used for the Investigator Protocol)

1. Scope

Outsourcing is the utilization of a qualified vendor laboratory to provide DNA analysis services to the Bureau of Alcohol, Tobacco, Firearms and Explosives (ATF) where the ATF Laboratory may take ownership of the DNA data. ATF assumes ownership of DNA data for entry into CODIS, when applicable. This procedure covers outsourcing of casework samples and ensures that a review is performed to verify the integrity of the DNA data received from the vendor laboratory.

2. References

2.1. The Genetic Information Nondiscrimination Act of 2008 (exception for laboratories conducting DNA analysis for law enforcement purposes) 42 USC 2000FF-1 § (b)(6)
2.2. FBI Quality Assurance Standards for Forensic DNA Testing Laboratories (current version)
2.3. FBI Quality Assurance Standards for DNA Databasing Laboratories (current version)
2.4. ATF-LS-FB19 CODIS Manual
2.5. ATF-LS-FB22 Staff DNA Index
2.6. ISO/IEC 17025 (current version)

3. Equipment

3.1. Not applicable.

4. Safety/Quality Assurance

4.1. Not applicable.

5. Procedure

The following procedures shall be used to evaluate the ability of the vendor laboratory to perform DNA analysis on ATF cases and evaluate DNA data developed for ownership by the ATF Laboratory and entry into the Combined DNA Index System (CODIS).

NOTE: The ATF Laboratory will not take possession of DNA data for any DNA analysis performed by a vendor laboratory through a contract that has not undergone this review procedure before the analysis has been performed, except in rare instances under the circumstances described in the QAS. If a contract exists that was not reviewed as per this protocol, a review of the contract, as detailed in this protocol shall be completed, and the contract amended as necessary. A new contract awarded date will be established after completion of the review process and any modifications as

applicable. DNA analysis performed by the vendor laboratory after the contract award date may be accepted by the ATF Laboratory.

5.1. Vendor Laboratory Accreditation and Quality Assurance Standards Compliance

5.1.1. The vendor laboratory must comply with current FBI Quality Assurance Standards (QAS) for Forensic DNA Testing Laboratories and be accredited to ISO/IEC 17025.

5.1.1.1. The ATF DNA Technical Leader shall review the vendor laboratory's most recent external QAS audit document including all responses and/or corrective actions for any findings.

5.1.1.2. Copies of the vendor laboratory's most recent external QAS audit and ISO/IEC 17025 accreditation, including the accreditation certificate and scope shall be maintained by the ATF Laboratory.

5.1.1.3. If the ATF Laboratory elects to use an on-site visit coordinated by a designated FBI employee, the vendor laboratory's accreditation and external audit documentation does not need to be retained separately.

5.2. Approval of Vendor Laboratory Technical Specifications

5.2.1. The ATF DNA Technical Leader shall approve the technical specifications of the outsourcing agreement with the vendor laboratory before it is awarded.

5.2.1.1. The date of documented approval of the technical specifications of the outsourcing agreement by the ATF DNA Technical Leader shall be maintained.

5.3. Vendor Laboratory Site Visits

5.3.1. The ATF Laboratory shall perform an initial on-site visit of the vendor laboratory to assess its ability to perform analysis on ATF cases, prior to beginning casework.

5.3.1.1. This visit may be conducted by the ATF DNA Technical Leader or a designated ATF employee who is currently or previously qualified in the technology, platform, and typing test kit used to generate the DNA data.

5.3.1.2. Alternatively, the ATF DNA Technical Leader shall evaluate and approve an on-site visit coordinated by a designated FBI employee.

5.3.1.3. The ATF Laboratory shall retain documentation of the internal on-site visit, or the review of the external visit, demonstrating the date the on-site visit was performed, a summary of the visit, and documentation of the personnel who performed the visit.

5.3.2. If the outsourcing agreement extends beyond one year, annual on-site visits shall be conducted. Each annual on-site visit shall occur once every calendar

year and be at least six (6) months and no more than eighteen (18) months apart.

5.3.2.1. In lieu of an actual on-site visit, the ATF DNA Technical Leader may accept documentation of an on-site visit by another NDIS laboratory using the same technology, analysis platform, and amplification kit, if it was conducted within the past twelve (12) months. Alternatively, the ATF DNA Technical Leader may accept an on-site visit conducted by a designated FBI employee.

5.4. Reviewing Data for Ownership

5.4.1. Only CODIS eligible or potentially CODIS eligible data developed by the vendor laboratory will be reviewed for ownership by the ATF Laboratory. This may include other data that would affect the validity of the CODIS eligible or potentially CODIS eligible data in some instances (e.g. reference profile used for conditioning).

5.4.2. Ownership review of data received from the vendor laboratory shall be conducted by the ATF Laboratory prior to official acceptance and upload of the DNA data to SDIS.

5.4.3. Ownership review shall be conducted by an ATF DNA examiner or technical reviewer who is qualified in the technology, platform, and typing test kit used to generate the data and participates in the ATF Laboratory's proficiency testing program.

5.4.4. The ownership review shall be documented.

5.4.5. Ownership review shall include the following elements:

5.4.5.1. All DNA types shall be reviewed to verify that they are supported by the raw or analyzed data (electropherograms).

5.4.5.2. All associated analytical controls, internal size standards, and allelic ladders shall be reviewed to verify that the expected results were obtained.

5.4.5.3. The vendor laboratory's final report (if provided) shall be reviewed to verify that the results and conclusions are supported by the DNA data.

5.4.5.4. Verification of DNA types, eligibility, and correct specimen category for CODIS entry by a current ATF Laboratory CODIS user.

5.4.6. Upon completion of the ownership review, a copy of the vendor laboratory report (if applicable) and data shall be maintained.

5.5. CODIS Evaluation and Entry of Outsourced Samples

5.5.1. After completion of ownership review, CODIS eligible DNA profiles will be further evaluated by a qualified ATF Laboratory DNA examiner for CODIS entry.

5.5.2. All CODIS eligible DNA profiles will be entered into CODIS and a CODIS notification will be issued to the investigating agent upon completion of a technical and administrative review.

5.5.3. A technical and administrative review of the CODIS packet, including the CODIS notification, CODIS paperwork, 95% calculations (if applicable), and agent communications, will be performed and documented prior to marking the DNA profiles for upload to SDIS or NDIS.

1. Scope

This protocol outlines the frequency and type of quality control checks that are to be conducted on critical reagents to ensure the high quality of work suitable for a forensic casework laboratory.

2. References

2.1. AmpFlSTR Identifiler PCR Amplification Kit, User's Manual. Ref J, 2012

2.2. Applied Biosystems™ GlobalFiler™ PCR Amplification Kit, User's Manual, Rev. E, 2016.

2.3. Applied Biosystems™ Quantifiler™ HP and Trio DNA Quantification Kits User Guide, publication 4485354, Rev. H, 2018.

2.4. Qiagen® QIAamp® DNA Investigator Handbook, 2012.

3. Equipment

See individual reagent sections in Appendix A.

4. Safety / Quality Assurance

See individual reagent sections in Appendix A.

5. Procedure

5.1. Documentation

5.1.1. All paperwork related to quality checks shall be kept in the *Reagent Quality Control Log* and/or electronically.

5.1.2. User manuals shall be stored with the reagent, in the *Reagent Quality Control Log*, and/or electronically.

5.2. Quality Checks

5.2.1. Unless otherwise stated, the quality control check will consist of the evaluation or a test for function listed in Appendix A.

5.2.2. A quality control check will be conducted on any critical reagent prior to its use in casework for:

5.2.2.1. New reagents

5.2.2.2. Reagents that have been stored greater than 8 hours outside of their recommended temperature range

5.2.3. No reagent will be used for casework unless it passes its designated quality control check.

5.3. Review

5.3.1. All worksheets and data associated with a quality control check will be generated and analyzed by a qualified casework analyst. A specialist or technician may perform the laboratory work for a quality control check, but in this case, a qualified analyst must take ownership prior to its review. A second qualified examiner will perform and document a review of the associated paperwork as well as approval or rejection for use in casework.

5.3.1.1. Approval

5.3.1.1.1. If the quality control check for a given lot of a reagent meets all requirements listed in Appendix A and the work is both technically and administratively sound, that reagent lot shall be approved for use in casework.

5.3.1.1.2. If approved, the cover sheet shall be initialed by the approver and reviewer. The packet, including all associated worksheets and data, will be stored in the appropriate log and/or electronically.

5.3.1.1.2.1. Electropherograms may be generated and stored electronically.

5.3.1.2. Rejection

5.3.1.2.1. If the quality control check for a given lot of a reagent does not meet the specific requirements listed in Appendix A, that reagent lot will not be used in casework until it passes.

5.3.1.2.2. The DNA Technical Leader will be notified.

5.3.1.2.3. Further tests will be performed at the discretion of the analyst/technical leader to include:

5.3.1.2.3.1. Additional laboratory evaluations and/or

5.3.1.2.3.2. Contact the manufacturer for replacement.

5.4. Reagents

5.4.1. See Appendix A for reagent specific quality control procedures for the following reagents (Critical Reagents are designated with an *):

5.4.1.1. Phenolphthalein (Kastle-Meyer) Kit

5.4.1.2. 200 Proof Ethanol*

5.4.1.3. Tris-EDTA^{-4} (TE^{-4})*

5.4.1.4. Qiagen® QIAamp® DNA Investigator Kit*

5.4.1.5. Proteinase K (ProK)*

5.4.1.6. Carrier RNA (cRNA)*

5.4.1.7. Dithiothreitol (DTT)*

5.4.1.8. Quantifiler™ HP Quantification Kit*

5.4.1.9. GlobalFiler™ Amplification Kit*

5.4.1.10. GeneScan™ 600 LIZ™ Size Standard v2.0*

5.4.1.11. Hi-Di™ Formamide*

5.4.1.12. Molecular Grade Water

5.4.1.13. BSA/Tri-Peptide (BTmix)*

APPENDIX A – Reagent Specific Quality Control Procedures

Reagent: **Phenolphthalein (Kastle-Meyer) Test Kit**	Manufacturer: Various

1. **Equipment**
   - 1.1. Disposable gloves
   - 1.2. Lab coat
   - 1.3. Swabs or filter paper

2. **Safety**
   - 2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   - 3.1. The expiration date will be set 12 months from the date of receipt.

4. **Procedure**
   - 4.1. Make a serial dilution using liquid whole blood and water at the following dilutions: neat, 1:100, 1:500, 1:1,000, 1:5,000, 1:10,000, 1:50,000, and 1:100,000.
   - 4.2. Spot 10 µL of each dilution on a swab or filter paper.
   - 4.3. Test a known non-human positive control (supplied in kit), a negative control, and the full dilution series from Section 4.1 using the reagents from the new kit.
   - 4.4. Record the results.
     - 4.4.1. A positive result is indicated by a pink color change within 5 seconds.
     - 4.4.2. A negative result is indicated by no color change within 5 seconds.

5. **Acceptable Results**
   - 5.1. The positive control must produce a positive result.
   - 5.2. The negative control must produce a negative result.
   - 5.3. At a minimum, the neat, 1:100, 1:500, and 1:1,000 dilutions must produce a positive result.

6. **Documentation**
   - 6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **Ethanol (200 Proof)**	Manufacturer: Various

1. **Equipment**
   1.1. Disposable gloves
   1.2. Lab coat
   1.3. Safety cabinet

2. **Safety**
   2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   3.1. The expiration date will be set 9 years from the date of receipt or the manufacturer's expiration date, whichever is sooner.

4. **Procedure**
   4.1. Extract at least one sample using 1-5 µL of known liquid blood or an oral swab from an individual with a known DNA profile and a reagent blank using the ATF Laboratory QIAmp® DNA Investigator protocol and the new lot of ethanol at all appropriate steps throughout the extraction process.
   NOTE: The 100% ethanol used to initially create the AW1 and AW2 buffers can be a different previously QC'd lot than the 100% ethanol used during the extraction protocol.
   4.2. Quantify, amplify, and genetically type these samples using the appropriate controls and the standard ATF Laboratory protocols for each step of the process.

5. **Acceptable Results**
   5.1. Correct DNA profiles must be obtained for the QC sample(s) and positive control.
   5.2. No additional peaks above the analytical threshold (AT) can be observed in the QC sample or positive control.
   5.3. The negative control and reagent blank should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. **Documentation**
   6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **Tris-EDTA^{-4} (TE^{-4})**	Manufacturer: Teknova

1. **Equipment**
   1.1. Disposable gloves
   1.2. Lab coat

2. **Safety**
   2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   3.1. The expiration date will be set 2 years from the date of receipt or the manufacturer's expiration date, whichever is sooner.

4. **Procedure**
   4.1. Where TE^{-4} is required during this process besides the initial three aliquots and the additional amplification negative control, a lot of TE^{-4} that has previously passed QC should be used.
   4.2. Separately extract three aliquots of 50 µL of molecular grade water along with a reagent blank using either ATF-LS-FB09 or ATF-LS-FB23 and the incubation time for evidence samples (i.e., 3 to 18 hours).
   4.3. Quantify then combine and concentrate the three QC samples into one.
   4.4. Amplify and genetically type the sample and reagent blank using the appropriate controls and ATF-LS-FB protocols for each step of the process. A second amplification negative control prepared using the lot of TE^{-4} currently undergoing QC should be included.

5. **Acceptable Results**
   5.1. The correct DNA profile must be obtained for the positive control. No additional peaks above the analytical threshold (AT) should be observed in the positive control.
   5.2. The QC sample, negative controls, and reagent blank should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. **Documentation**
   6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **QIAamp® DNA Investigator Kit**	Manufacturer: Qiagen®

1. **Equipment**
   - 1.1. Disposable gloves
   - 1.2. Lab coat
   - 1.3. 200 proof ethanol

2. **Safety**
   - 2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   - 3.1. The expiration date will be set 12 months from the date of receipt or the manufacturer's expiration date, whichever is sooner.

4. **Procedure**
   - 4.1. Optional: Prepare new cRNA:
     - 4.1.1. Add 310 µL of ATE to each cRNA tube.
       - 4.1.1.1. If multiple tubes from the same lot are reconstituted, combine and mix all tubes before proceeding to next step.
     - 4.1.2. Aliquot 30 µL of vortexed solution into capped 1.5 ml tubes.
     - 4.1.3. Spin down and place in freezer.
   - 4.2. Extract at least two samples using 1-5 µL of known liquid blood or an oral swab from an individual with a known DNA profile and three reagent blanks using the new lot of QIAmp® DNA Investigator Kit.
   - 4.3. Quantify, combine, and concentrate the three reagent blanks. Amplify and genetically type these samples using the appropriate controls and the standard ATF Laboratory protocols for each step of the process.

5. **Acceptable Results**
   - 5.1. Correct DNA profiles must be obtained for the QC samples and positive control.
   - 5.2. No additional peaks above the analytical threshold (AT) can be observed in the QC samples or positive control.
   - 5.3. The negative control and reagent blank should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. **Documentation**
   - 6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **Proteinase K**	Manufacturer: Various

1. **Equipment**
   1.1. Disposable gloves
   1.2. Lab coat

2. **Safety**
   2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   3.1. The manufacturer derived expiration date will be used.

4. **Procedure**
   4.1. Extract at least one sample using 1-5 µL of known liquid blood or an oral swab from an individual with a known DNA profile and a reagent blank using the new lot of proteinase K.
   4.2. Quantify, amplify, and genetically type these samples using the appropriate controls and standard ATF Laboratory protocols for each step of the process.

5. **Acceptable Results**
   5.1. Correct DNA profiles must be obtained for the QC sample(s) and positive control.
   5.2. No additional peaks above the analytical threshold (AT) can be observed in the QC sample or positive control.
   5.3. The negative control and reagent blank should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. **Documentation**
   6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **Carrier RNA (cRNA)**	Manufacturer: Qiagen®

1. **Equipment**
   1.1. Disposable gloves
   1.2. Lab coat

2. **Safety**
   2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   3.1. The expiration date will be set 12 months from the date of receipt of the associated QIAamp® DNA Investigator Kit or the expiration date of the TE^{-4}, whichever is shortest.

4. **Procedure**
   4.1. Prepare new cRNA:
      4.1.1. Add 310 µl of ATE in each cRNA tube.
         4.1.1.1. If multiple tubes from the same lot are re-constituted, combine and mix all tubes before proceeding to next step.
      4.1.2. Aliquot 30 µl of vortexed solution into capped 1.5 ml tubes.
      4.1.3. Spin down and place in freezer in a rack labeled with the identity of the reagent, lot number/expiration date, and initials of individual who prepared the reagent.
   4.2. Extract at least one sample using 1-5 µL of known liquid blood or an oral swab from an individual with a known DNA profile and a reagent blank using the procedure for the QIAmp® DNA Investigator Kit.
   4.3. Quantify, amplify, and genetically type these samples using the appropriate controls and the standard ATF Laboratory protocols for each step of the process.

5. **Acceptable results**
   5.1. Correct DNA profiles must be obtained for the QC sample(s) and positive control.
   5.2. No additional peaks above the analytical threshold (AT) can be observed in the QC sample or positive control.
   5.3. The negative control and reagent blank should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. **Documentation**
   6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **Dithiothreitol (DTT)**	Manufacturer: Invitrogen™

1. Equipment

1.1. Disposable gloves
1.2. Lab coat
1.3. Scale

2. Safety

2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. Expiration

1.1. The expiration date for the stock reagent will be set 2 years from the date of receipt or the manufacturer's expiration date, whichever is sooner.
1.2. The expiration date for the prepared reagent will be set 6 months from the date of preparation.

4. Procedure

4.1. Prepare new DTT:
  4.1.1. Measure 0.154 g of DTT powder using scale paper.
  4.1.2. Put DTT in 2 mL tube along with 1 mL molecular grade water and vortex until DTT is fully dissolved.
  4.1.3. Aliquot 100 µL of DTT solution into 1.5 mL tubes and label caps with "DTT."
  4.1.4. Store tubes in freezer in a rack labeled with the identity of the reagent, lot number/expiration date, and initials of individual who prepared the reagent.
4.2. Extract two freshly plucked hairs from an individual with a known DNA profile and a reagent blank using the ATF-LS-FB09 or ATF-LS-FB23 and the new lot of DTT.
4.3. Quantify, amplify, and genetically type these samples using the appropriate controls and the ATF-LS-FB protocols for each of the processes.

5. Acceptable Results

5.1. Correct DNA profiles must be obtained for the QC samples and positive control.
5.2. No additional peaks above the analytical threshold (AT) can be observed in the QC sample or positive control.
5.3. The negative control and reagent blank should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. Documentation

6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **Quantifiler™ HP Quantification Kit**	Manufacturer: Applied Biosystems™

1. Equipment

1.1. Disposable gloves
1.2. Lab coat
1.3. 7500 thermal cycler

2. Safety

2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. Expiration

3.1. The PCR Reaction Mix expires six months after the addition of the Quantifiler™ Automation Enhancement Kit buffer. Therefore, the PCR Reaction Mix will expire six months from the date of the addition or the manufacturer's expiration date of the kit, whichever is sooner.

4. Procedure

4.1. Using one of the components of the NIST SRM 2372, or a NIST SRM 2372 traceable sample, create the following dilutions for a standard curve:

1 - 50 ng/µl	4 - 0.05 ng/µl
2 - 5.0 ng/µl	5 - 0.005 ng/µl
3 - 0.5 ng/µl	

NOTE: If the NIST SRM 2372 component DNA concentration is between 45 ng/µL and 55 ng/µL, the analyst may proceed with the dilutions as if the concentration is 50 ng/µL.

4.2. Combine all of the standard tubes from the new lot of Quantifiler™ HP Human DNA Standard to create one pooled tube of Quantifiler™ HP Human DNA Standard. Using the pooled standard, create the following dilutions: 1:20, 1:10, and 1:2.
4.3. If the Quantifiler™ Automation Enhancement Kit buffer has not been added to the PCR Reaction Mix, add it at this time in accordance with the Quantifiler™ HP protocol.
4.4. Run the following in duplicate using the new lot of reagents included in the Quantifiler™ HP kit: the standard curve components, the dilutions of the pooled Quantifiler™ HP Human DNA Standard, and a dilution (e.g. neat, 1:10, etc.) of the NIST SRM 2372/NIST SRM 2732 traceable sample. A plate blank must be included in the run.

5. Acceptable Results

5.1. The plate blank value should be "undetected."
5.2. Evaluate the Standard Curve to determine that the quantification results are accurate and reliable by examining the slope and the R^2 values.

5.2.1. Small Autosomal Slope: The value shall be between -3.0 and -3.6. A slope of -3.3 indicates 100% amplification efficiency.

5.2.2. Large Autosomal Slope: The value shall be between -3.1 and -3.7. A slope of -3.4 indicates 100% amplification efficiency.

5.2.3. R^2: The value shall be greater than 0.98.

5.3. The NIST SRM 2372, or NIST SRM 2372 traceable sample should be within 20% of its established value.

6. Documentation

6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

6.2. The calculated concentration of the Quantifiler™ HP standard and its associated recommended dilution factor will be recorded in the *Reagent Quality Control Log* and/or electronically.

7. Dilution Calculation Examples

7.1. Pooled human DNA standard concentration / Target concentration of initial dilution (50 ng/µl) = Dilution factor.

7.2. Example: Actual concentration is 100 ng/µL, then 100 / 50 = 2.

7.2.1. Dilution would then be 10 µL of DNA Std + 10 µL of TE.

Reagent: **GlobalFiler™ PCR Amplification Kit**	Manufacturer: Applied Biosystems™

1. Equipment

1.1. Disposable gloves
1.2. Lab coat
1.3. Veriti™ thermal cycler
1.4. AB 3500xL Genetic Analyzer

2. Safety

2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. Expiration

3.1. The expiration date will be set 12 months from the date of receipt for the kit, or the manufacturer's expiration date, whichever is sooner.
3.2. Individual tubes will be given an expiration date of 6 months from the date they are thawed not to exceed the kit expiration date.

4. Procedure

4.1. Using the new kit, amplify the following samples using the GlobalFiler™ PCR Amplification Kit protocol:
  4.1.1. 5 replicates of the same QC sample (previously characterized and quantified standard sample)
  4.1.2. 2 replicates of the new GlobalFiler™ Control DNA 007.
  4.1.3. 5 replicates of the negative amplification control (15 µL TE.)
4.2. Type the above listed samples on the AB 3500 Genetic Analyzer using the allelic ladder from the new kit.
4.3. Calculate the average total RFU / locus for the five replicate amplifications using the new kit and the QC sample.
  4.3.1. Sum the RFU across all loci and divide by 24 for each sample (average total RFU / locus), then calculate the global average across all five replicate samples.
  4.3.2. Compare the global average total RFU / locus for the five replicates of the new kit to the global average total RFU / locus for the five replicates of the standard reference set (baseline sensitivity set).

5. Acceptable Results

5.1. Difference in average total RFU / locus must be within 20% between the new lot and the standard reference set.

5.2. Correct DNA profiles must be obtained for the QC and positive control samples.

5.3. No additional peaks above the analytical threshold (AT) can be observed in the QC or positive control samples.

5.4. The negative controls should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. Documentation

6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **GeneScan™ 600 LIZ™ Size Standard v2.0**	Manufacturer: Applied Biosystems™

1. **Equipment**
   1.1. Disposable gloves
   1.2. Lab coat
   1.3. 3500xL Genetic analyzer

2. **Safety**
   2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   3.1. The expiration date will be set 12 months from the date of receipt or the manufacturer's expiration date, whichever is sooner.

4. **Procedure**
   4.1. A separate 3500 injection will be set-up to include a positive control, negative control, and an allelic ladder using the new lot of GeneScan™ 600 LIZ™ Size Standard v2.0.

5. **Acceptable Results**
   5.1. The 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, and 460bp fragments are present and sized correctly.
   5.2. The positive control must type correctly with no additional peaks above the analytical threshold (AT).
   5.3. The negative control should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. **Documentation**
   6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **Hi-Di™ Formamide**	Manufacturer: Applied Biosystems™

1. **Equipment**
   1.1. Disposable gloves
   1.2. Lab coat
   1.3. 3500xL Genetic analyzer

2. **Safety**
   2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   3.1. The manufacturer derived expiration date will be used.
      3.1.1. Call Applied Biosystems™ (1-800-955-6288) and follow the prompts to speak to a customer service representative to get the expiration date for the lot number. You will need to provide the service representative the catalog and/or lot number.

4. **Procedure**
   4.1. A separate 3500 injection will be set-up to include a positive control, negative control, and an allelic ladder using the new lot of Hi-Di™ Formamide.

5. **Acceptable Results**
   5.1. The positive control must type correctly with no additional peaks above the analytical threshold (AT).
   5.2. The negative control should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. **Documentation**
   6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **Molecular Grade Water**	Manufacturer: Various

1. **Equipment**
   1.1. Disposable gloves
   1.2. Lab coat

2. **Safety**
   2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   3.1. The expiration date will be set 5 years from the date of receipt or the manufacturer's expiration date (if one exists), whichever is sooner.

4. **Procedure**
   4.1. Separately extract three aliquots of 50 µL of molecular grade water using either ATF-LS-FB09 or ATF-LS-FB23 and the incubation time for evidence samples (i.e., 3 to 18 hours).
   4.2. Quantify then combine and concentrate the three QC samples into one.
   4.3. Amplify and genetically type the concentrated sample using the appropriate controls and the ATF-LS-FB protocols for each step of the process.

5. **Acceptable Results**
   5.1. The correct DNA profile must be obtained for the positive control. No additional peaks above the analytical threshold (AT) should be observed in the positive control.
   5.2. The QC sample, negative control, and reagent blank should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded.

6. **Documentation**
   6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

Reagent: **BSA/Tri-Peptide Mix (BTmix)**	Manufacturer: Various

1. **Equipment**
   1.1. Disposable gloves
   1.2. Lab coat
   1.3. Scale

2. **Safety**
   2.1. Lab coat and disposable gloves must be worn at all times while performing this procedure.

3. **Expiration**
   3.1. The expiration date for the stock reagents Bovine Serum Albumin (BSA) and Gly-Gly-His tripeptide (GGH) will be set 2 years from the date of receipt or the manufacturer's expiration date (if provided), whichever is sooner.
   3.2. The expiration date for the prepared reagent will be set 6 months from the date of preparation or the nearest expiration date for the stock reagents, whichever is sooner.

4. **Procedure**
   4.1. Prepare 125 mg/mL GGH solution. The following weights and volumes may be scaled as needed:
      4.1.1. Clean and dry the outside of the tube containing solid GGH using 70% ethanol and Kim wipes.
      4.1.2. Add a volume of molecular grade water directly to the GGH tube sufficient to prepare 125 mg/mL GGH (i.e. 743 µL water for 100 mg GGH, 3714 µL water for 500 mg GGH). The final volume takes the GGH into account.
      4.1.3. Gently vortex the GGH solution and spin down or "flick" the tube to collect solution at the bottom.
      4.1.4. Transfer the GGH solution to a conical tube or 5 mL tube using a pipette. Take note of the volume transferred (100 mg GGH should yield approximately 800 µL solution, 500 mg GGH should yield approximately 4 mL solution.
   4.2. Prepare BTmix solution (62.5 mg/mL GGH and 2 mg/mL BSA). The following volumes may be scaled as needed:
      4.2.1. For 100mg GGH: Add 64 µL of 50 mg/mL BSA and 736 µL of molecular grade water to precursor GGH solution in the conical tube.
      4.2.2. For 500mg GGH: Add 320 µL of 50 mg/mL BSA and 3680 µL of molecular grade water to precursor GGH solution in the conical tube.
   4.3. Aliquot volumes (e.g., 300 µL) of BTmix solution into 1.5 mL tubes and label caps with "BT."

4.3.1. Place aliquots in freezer in a rack labeled with the identity of the reagent, lot number/expiration date, and initials of individual who prepared the reagent.

4.4. Extract at least two samples using known liquid blood or an oral swab from an individual with a known DNA profile and four reagent blanks. The reagent blanks will be combined and concentrated in pairs.

4.5. Process these samples through CE using the appropriate controls and the standard ATF Laboratory protocols for each step of the process

.

5. Acceptable Results

5.1. Correct DNA profiles must be obtained for the QC samples and positive control.

5.2. No additional peaks above the analytical threshold (AT) can be observed in the QC sample or positive control.

5.3. The negative control and reagent blank should not contain any true peaks over the AT or any pattern of peaks below the AT. Pull-up peaks from the LIZ™ size standard can be disregarded. One or two peaks above the AT and/or a limited number of peaks below the AT in one of the combined reagent blanks is acceptable if the other reagent blank does not demonstrate any sign of contamination with approval from the DNA Technical Leader. Possible contaminant detected in only one of the combined reagent blanks is an indication of a sample-specific issue and not contamination of the BTmix solution as a whole.

6. Documentation

6.1. Records will be maintained in the *Reagent Quality Control Log* and/or electronically.

1. Scope

This protocol outlines the frequency and type of maintenance, calibration, and/or verifications that are to be conducted to keep our instruments and equipment in good working order and suitable for use in forensic casework.

2. References

2.1. Applied Biosystems™ 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide, 2010.
2.2. Mettler Toledo® B-S Line of Balances AB-S, PB-S Operating Instructions, 2005.
2.3. Hermle Table Top Centrifuge Z 300 Instruction Manual, 2003.
2.4. Beckman Coulter® Microfuge® 18 Centrifuge Instruction Manual, 2009.
2.5. Fisher Scientific™ Isotemp® Plus and General Purpose Laboratory Freezers Instruction Manual, 2003.
2.6. Fisher Scientific™ Isotemp® Plus, Chromatography, and General Purpose Laboratory Refrigerators Instruction Manual, 2004.
2.7. Labnet International Digital Dry Bath Models D1100 and D1200 Instruction Manual, 2003.
2.8. AirClean® Systems Ductless PCR Workstation Operator's Manual, 2006.
2.9. Rainin® Pipet-Lite® Magnetic Assist Pipette User Manual, 2007.
2.10. Rainin® Pipet-Lite® XLS+™ Manual Pipettes with RFID Operating Instructions, 2014.
2.11. Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide, 2006.
2.12. Applied Biosystems™ Thermal Cycler Temperature Verification System for GeneAmp® PCR System 9700: Dual 384 Well block User's Manual, 2006.
2.13. Applied Biosystems™ GeneAmp® PCR System 9700 Base Module User's Manual, 2010.
2.14. Applied Biosystems™ GeneAmp® PCR System 9700 96-Well Sample Block Module User's Manual, 2010.
2.15. Eppendorf® Thermomixer® Compact / Thermomixer® Operating Manual, 2008.
2.16. Eppendorf® Thermomixer® Comfort / Thermomixer® R Operating Manual, 2007.
2.17. Eppendorf® Thermomixer® C Operating Manual, 2014.
2.18. Spectroline® Microprocessor-Controlled UV Crosslinkers Select™ XLE-1000 and Spectrolinker™ XL-1000 and XL-1500 Operator's Manual, 2007.
2.19. Millipore Milli-Q® Gradient and Milli-Q Gradient A10® User Manual, 2006.
2.20. Applied Biosystems™ Veriti™ Thermal Cycler User Guide, 2010.
2.21. ATF-LS-FB11 Quantifiler
2.22. ATF-LS-FB30 Globalfiler™ PCR Amplification Kit
2.23. ATF-LS-FB31 Applied Biosystems™ 3130 Genetic Analyzer
2.24. Applied Biosystems™ 3500/3500xl Genetic Analyzers User Guide, 2018.
2.25. ATF-LS-FB38 Applied Biosystems™ 3500xL Genetic Analyzer

3. Equipment

3.1 The following is a list of Critical Instruments:

a. Mechanical pipettes
b. NIST-traceable thermometers (used for calibrating)
c. Applied Biosystems™ 7500 Real-Time PCR System
d. Applied Biosystems™ GeneAmp® PCR System 9700
e. Applied Biosystems™ Veriti™ Thermal Cycler
f. Eppendorf® Thermomixer® / Heat Block
g. QIAcube
h. Applied Biosystems™ Temperature Verification Kit
i. Applied Biosystems™ 3500xL Genetic Analyzer

See individual equipment sections in Appendix A.

4. Safety / Quality Assurance

See individual safety sections in Appendix A.

5. Procedure

5.1. Documentation

5.1.1. All paperwork related to repairs, calibration, and maintenance of instruments shall be kept in the *Maintenance & Repair Log* for each room (lab) and/or electronically.

5.1.2. Performance check paperwork will be kept in the *Maintenance & Repair Log* and/or electronically.

5.1.3. User manuals, ordering/shipping paperwork, and/or warranty paperwork should be stored with the instruments, in the main instrument file, and/or electronically.

5.2. Performance Checks

5.2.1. Unless otherwise stated, the performance check will consist of the listed calibration or a test for function.

5.2.2. A performance check will be conducted on instruments and equipment prior to casework for:

5.2.2.1. New instruments and equipment (when appropriate)

5.2.2.2. Instruments and equipment that have undergone extensive repair and/or maintenance

5.2.3. No equipment or instrument will be used for casework unless it passes its scheduled checks within the stated interval.

5.3. Review

5.3.1. All worksheets and data associated with a calibration, verification, and/or performance check will be reviewed by a qualified casework analyst and either approved or rejected for use in casework. A second qualified individual will perform and document a review of the data if the calibration, verification, or performance check required requires extraction, quantification, or capillary electrophoresis.

5.3.1.1. Approval

5.3.1.1.1. If the calibration, verifications, and/or performance check for a given instrument or equipment meet all of the requirements listed in Appendix A and the work is both technically and administratively sound, that instrument or equipment shall be approved for use in casework.

5.3.1.1.2. If approved, the cover sheet shall be initialed by the approver and reviewer. The packet, including all associated worksheets and data, will be stored in the appropriate log and/or electronically.

5.3.1.1.2.1. Electropherograms may be generated and stored electronically.

5.3.1.2. Rejection

5.3.1.2.1. If the calibration, verifications, and/or performance check for a given instrument or equipment does not meet the specific requirements listed in Appendix A, that instrument/equipment will not be used for casework until it passes.

5.3.1.2.2. The DNA Technical Leader will be notified.

5.3.1.2.3. Further tests will be performed at the discretion of the analyst/DNA Technical Leader to include:

5.3.1.2.3.1. Additional laboratory evaluations and/or;

5.3.1.2.3.2. Contact the manufacturer for repair.

5.4. Maintenance

5.4.1. See Appendix A for instrumentation and/or equipment specific maintenance procedures.

5.5. Maintenance Schedules

Maintenance Required					
Weekly	**Bi weekly***	**Monthly**	**Quarterly**	**6 month**	**Yearly**
3130 and 3500xL Genetic Analyzers	3500xL Genetic Analyzers	3130 / 3500xL Genetic Analyzers	3130 / 3500xL Genetic Analyzers	7500 Real-Time PCR Systems	3130 / 3500xL Genetic Analyzers
Freezer (large), Isotemp Plus	UV Crosslinkers	QIAcube® Robotic WorkstatQion	7500 Real-Time PCR Systems	AirClean PCR Workstations	7500 Real-Time PCR Systems
Freezer (small), Isotemp			9700 Thermal Cyclers		9700 Thermal Cyclers
Heat Block, Accublock Digital			Veriti Thermal Cyclers		Veriti Thermal Cyclers
Milli-Q Water Purification System			QIAcube® Robotic Workstation		Centrifuges
Refrigerator (large), Isotemp Plus					Digital Balance
Refrigerator (small), General Purpose					Pipettes
Thermomixers					Temperature Verification Kit
QIAcube® Robotic Workstation					Thermometers
					STRmix Software
					QIAcube® Robotic Workstation

*- every two weeks

APPENDIX A – Instrument Specific Maintenance Procedures

Instrument: **Balance – Digital** *Mettler/Toledo®*	Model #: PB303-S/FACT

1. Equipment

1.1. Disposable gloves
1.2. Eye protection
1.3. Lab coat
1.4. Digital balance
1.5. Calibration weight set
1.6. Forceps

2. Safety

2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. Frequency

3.1. Performance will be verified using a weight set on an annual basis.

4. Procedure

4.1. Press and hold the *calibration button* to perform the internal calibration.
4.2. To check this calibration, measure each weight three times and record the measurements.
4.3. Calculate and record the average for each weight.
4.4. If any average is outside of the accepted tolerance of ± 4%, the balance will be repaired or replaced.

5. Documentation

5.1. Records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Centrifuge** *Hermle*	Model #: Z300

1. **Equipment**
   1.1. Disposable gloves
   1.2. Eye protection
   1.3. Lab coat
   1.4. Centrifuge
   1.5. NIST Certified Tachometer
   1.6. Reflective Tape

2. **Safety**
   2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   3.1. Verify the speed of the centrifuge with a NIST certified tachometer on an annual basis.

4. **Procedure**
   4.1. Check the calibration date on the certificate and make sure the NIST certified tachometer has not expired.
   4.2. Make sure the centrifuge is empty, and then add a piece of reflective tape to the top of the rotor cover so that it is visible through the clear viewing window in the centrifuge lid.
   4.3. Set the centrifuge for 500 RPM (low setting) and start. Wait approximately one minute for the centrifuge to get up to speed.
   4.4. Turn on the tachometer and hold it over the viewing window with the laser pointing down into the centrifuge.
   4.5. Examine the readings for a few seconds. Make sure the readings are within ± 10% of the RPM setting.
   4.6. If the actual speed varies by more than 10% of the set speed, the centrifuge will be repaired or replaced.
   4.7. Repeat sections 4.1 through 4.5 at 13500 RPM (high setting) for a tube centrifuge or 3500 RPM (high setting) for a plate centrifuge.

5. **Documentation**
   5.1. Records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Freezer (large) Isotemp® Plus -20° C** *Fisher Scientific™*	Model #: 13-986-142

1. **Equipment**
   - 1.1. Disposable gloves
   - 1.2. Eye protection
   - 1.3. Lab coat
   - 1.4. Freezer
   - 1.5. Thermometer

2. **Safety**
   - 2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   - 3.1. Check the temperature of the freezer weekly.

4. **Procedure**
   - 4.1. Note the temperature of the thermometer in the freezer.
   - 4.2. Record the temperature on the log sheet.
   - 4.3. If the actual temperature varies more than $\pm$ 2°C from the desired temperature, the freezer will be adjusted to achieve the desired temperature.
   - 4.4. If the freezer cannot hold temperature, it will be repaired or replaced.

   NOTE: If a problem with a thermometer is suspected, verify the performance of the thermometer against a NIST certified thermometer.

5. **Documentation**
   - 5.1. Current records will be maintained in proximity to the instrument.
   - 5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Freezer (small), Isotemp**® *Fisher Scientific*™	Model #: 13-986-154

1. **Equipment**
   - 1.1. Disposable gloves
   - 1.2. Eye protection
   - 1.3. Lab coat
   - 1.4. Freezer
   - 1.5. Thermometer

2. **Safety**
   - 2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   - 3.1. Check the temperature of the freezer weekly.

4. **Procedure**
   - 4.1. Note the temperature of the thermometer in the freezer.
   - 4.2. Record temperature on the log sheet.
   - 4.3. If the actual temperature varies more than $\pm$ 2°C from the desired temperature, the freezer will be adjusted to achieve the desired temperature.
   - 4.4. If the freezer cannot hold temperature, it will be repaired or replaced.

   NOTE: If a problem with a thermometer is suspected, verify the performance of the thermometer against a NIST certified thermometer.

5. **Documentation**
   - 5.1. Current records will be maintained in proximity to the instrument.
   - 5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Genetic Analyzer** *Applied Biosystems*™	Model #: 3130

1. Equipment

1.1. See applicable section in ATF-LS-FB31.

2. Safety

2.1. See applicable section in ATF-LS-FB31.

3. Frequency

3.1. Each Use

3.1.1. Check that the polymer has been on the instrument less than two (2) weeks.

3.1.2. Check that the 1X running buffer has been on the instrument less than 2 days.

3.1.3. Add the number of runs to the Run Count Log.

3.2. Weekly

3.2.1. Change polymer if it has been on the instrument more than two (2) weeks before running samples. Polymer older than two weeks can remain on the instrument as long as no casework samples are run using this polymer. To replace polymer, use the *Replenish Polymer Wizard*. Fill out Polymer Log for the instrument.

3.2.2. Restart computer and instrument.

3.3. Monthly

3.3.1. Run the *Water Wash Wizard*.

3.3.2. Clean the water trap with syringe.

3.3.3. Defragment the computer hard drive.

3.4. Quarterly

3.4.1. Check database and hard disk space.

3.4.2. Ensure the data files have been backed up.

3.5. Yearly

3.5.1. Scheduled preventative maintenance by vendor (Applied Biosystems).

3.6. As needed

3.6.1. A new spatial calibration, spectral calibration, and a performance check will be conducted after the installation of a new capillary array, yearly preventative maintenance, adjustment/replacement of optics/laser, or other extensive vendor maintenance/repair.

3.6.2. A new spectral calibration can be performed if excessive pull-up is noted.

4. Procedure

4.1. Refer to the *Applied Biosystems 3130 Genetic Analyzer Maintenance, Troubleshooting and Reference Guide* for directions on performing calibration and maintenance tasks.

4.2. Performance check procedure

4.2.1. Amplify and genetically type a set of five (5) QC Samples (e.g. NIST 2372) at 500 pg. A positive and negative control sample should also be included.

4.3. Minimum for passing performance check

4.3.1. Positive control must type correctly with an acceptable level of pull-up and no extra true peaks in samples. No true peaks should be observed in negative control sample.

4.3.2. The average RFU/locus for the 5 QC samples should be within 20% of the original NIST SRM baseline.

5. Documentation

5.1. QC records and performance checks will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Heat Block – Accublock Digital** *Labnet International*	Model #: D1100

1. **Equipment**
   1.1. Disposable gloves
   1.2. Eye protection
   1.3. Lab coat
   1.4. Heat Block
   1.5. Thermometer

2. **Safety**
   2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   3.1. Check the temperature of the heat block weekly.

4. **Procedure**
   4.1. Measure the temperature of the heat block using the temperature verification unit.
   4.2. Record temperature on the log sheet.
   4.3. If the actual temperature varies more than $\pm$ 2°C from the desired temperature, the heat block will be adjusted to achieve the desired temperature.
   4.4. If the heat block cannot hold temperature, it will be repaired or replaced.

5. **Documentation**
   5.1. Current records will be maintained in proximity to the instrument.
   5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **PCR Workstation – AirClean® 600** *AirClean® Systems*	Model #'s: AC648LFUVC and AC632LFUVC

1. **Equipment**
   - 1.1. Disposable gloves
   - 1.2. Eye protection
   - 1.3. Lab coat
   - 1.4. Pre-Filter(s)
   - 1.5. HEPA Filter(s)
   - 1.6. PCR Workstation

2. **Safety**
   - 2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   - 3.1. Replace pre-filter(s) every 6 months.
   - 3.2. Replace HEPA filter(s) every four (4) years assuming ~100 hours of use per year.

4. **Procedure**
   - 4.1. Refer to the *AirClean Systems Ductless PCR Workstation Operator's Manual* for instructions on replacing the pre-filters and HEPA filters.

5. **Documentation**
   - 5.1. Records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **QIAcube® Robotic Workstation,** *Qiagen®*	Model: QIAcube

1. Equipment

1.1. Disposable gloves
1.2. Eye protection
1.3. Lab coat
1.4. Sterile swabs
1.5. Sterile water
1.6. 70% ethanol or alcohol wipes
1.7. 10% bleach solution
1.8. QIAcube® Robotic Workstation containing the following:
  1.8.1. QIAcube® Reagent bottles
  1.8.2. QIAcube® Reagent rack with appropriate labeling strips
  1.8.3. QIAcube® Centrifuge rotor adapters
  1.8.4. QIAcube® 1000ul Filter tips
1.9. QIAamp® DNA Investigator Kit (Qiagen Catalog # 56504) containing the following:
  1.9.1. QIAamp® MinElute™ columns
  1.9.2. Collection Tubes
  1.9.3. Buffer ATL
  1.9.4. Buffer AL
  1.9.5. Buffer AW1
  1.9.6. Buffer AW2
  1.9.7. Buffer AE
  1.9.8. Carrier RNA
  1.9.9. Proteinase K (typically not used)
1.10. Invitrogen Proteinase K
1.11. 96-100% Ethanol (EtOH)
1.12. Pipettes
1.13. Disposable aerosol-resistant pipette tips
1.14. Microcentrifuge tubes
1.15. NAO™ Baskets/QIAGEN® Investigator Lyse and Spin Baskets
  (OPTIONAL: DNA IQ™ Spin Baskets)
1.16. Benchtop hood
1.17. TE^{-4} (10mM Tris-HCl , 0.1mM EDTA, pH 8.0)
1.18. Bench paper
1.19. Thermomixer
1.20. Centrifuge
1.21. Vortexer
1.22. Spectrolinker
1.23. Mineral oil

1.24. QIAcube® O-ring
1.25. QIAcube® Tip Adapter Ring Tool

2. Safety/Quality Assurance

2.1. Disposable gloves shall be worn when handling kit reagents and evidence.
2.2. Extraction steps performed outside of the QIAcube® robotic workstation should be performed in a hood to reduce the risk of contamination. Clean surfaces with 10% bleach solution, followed by 70% EtOH, before and after use. After exiting hood, turn on UV light (automatically set for 15 minutes of exposure).
2.3. Use aerosol-resistant pipette tips when transferring liquids containing DNA.
2.4. Change pipette tips after transferring any liquids potentially containing DNA.
2.5. Record the lot number and expiration date of each reagent used in notes. Do not use the reagents after the expiration date.
2.6. Initiate the appropriate number of reagent blanks as the final samples of the set of extractions.
2.7. Lab coat and eye protection must be worn at all times while performing this procedure.
2.8. Only tubes associated with one DNA extract shall be open at a time.
2.9. Exercise caution when opening tubes.
2.10. The laboratory bench surface shall be cleaned before use with 10% bleach solution or other sanitizing agent and may be followed by 70% ethanol.
2.11. The QIAcube® worktable shall be cleaned with 70% ethanol solution before and after use.

3. Frequency

3.1.1. Before and after each use
3.1.1.1. Wipe down the worktable and components before and after use using a lint-free cloth moistened with 70% ethanol.
3.1.1.2. Wipe down the waste drawer using a lint-free cloth moistened with 70% ethanol.
3.1.2. Weekly
3.1.2.1. Decontaminate reagent bottle rack and shaker rack using Spectrolinker.
3.1.3. Monthly
3.1.3.1. Wipe down the optical sensor, tip adapter, gripper unit, gripper, stabilizing rod, and spin column lid holder using a lint-free cloth moistened with water.
3.1.3.1.1. To gain access to the above mentioned components:
3.1.3.1.1.1. Turn on the QIAcube®.
3.1.3.1.1.2. Press “Tools” in the main menu.
3.1.3.1.1.3. Select “Maintenance” using ▲or ▼ then press “Select”.
3.1.3.1.1.4. Select “Cleaning position” using ▲or ▼ then press “Start”.
3.1.3.1.1.5. Follow the displayed instructions.
3.1.3.2. Perform Tightness Test

3.1.3.2.1. Fill half of the QIAcube® reagent bottle with 96-100% EtOH and place in position 1 of the reagent bottle rack.
3.1.3.2.2. Place one 2mL microcentrifuge tube in position 1 of the shaker rack
3.1.3.2.3. Turn on the QIAcube®.
3.1.3.2.4. Press “Tools” in the main menu.
3.1.3.2.5. Select “Maintenance” using ▲or ▼ then press “Select”.
3.1.3.2.6. Select “Tightness Test” using ▲or ▼ then press “Select”.
3.1.3.2.7. Select “1000µL tips” using ▲or ▼ then press “Select”.
3.1.3.2.8. Press “Start”.
3.1.3.2.9. Follow the displayed instructions.
3.1.3.2.10. After completing the Tightness Test, check the 2mL microcentrifuge tube for presences of liquid. If any liquid is present, replace the O-ring by following the QIAcube® Tip Adapter Ring Replacement procedure. Tightness Test must be performed after replacing the O-ring.

3.1.4. Quarterly
3.1.4.1. Clean the centrifuge and all centrifuge components.
3.1.4.1.1. Remove the rotor and buckets from the centrifuge.
3.1.4.1.2. Wipe down the inside of the centrifuge, rotor, and buckets using a lint-free cloth moistened with 70% EtOH.
3.1.4.1.3. Rinse the rotor and buckets with distilled water and allow to dry.
3.1.4.1.4. Decontaminate all components using Spectrolinker.
3.1.4.1.5. Apply thin film of mineral oil to the bucket mounts and rotor claws.
3.1.4.1.6. Re-install the rotor and buckets into the centrifuge.
3.1.4.1.7. Check the centrifuge gasket for damage or wear.

3.1.5. Yearly
3.1.5.1. Scheduled preventative maintenance by vendor (Qiagen®).

4. Procedure

4.1. Performance Check

4.1.1. A performance check will be conducted after yearly preventative maintenance or other extensive vendor maintenance/repair.
4.1.1.1. Use a known DNA amount (cell suspension, blood, etc.) targeting approximately 2ng of DNA in triplicate and a reagent blank using the QIAcube® robotic workstation. Minimum of two QIAcube® robotic workstation should be used for the performance check.
4.1.1.1.1. Set up three known samples as described above for each QIAcube® being evaluated along with one reagent blank. Add at least one known DNA sample to the total number. For example, if the performance check is being performed for two QIAcubes®, then

seven known samples should be prepared (3 for QIAcube® 1 + 3 for QIAcube® 2 + 1 extra).

4.1.1.1.2. After the lysis step, combine all known DNA sample lysates into a single 50 mL conical tube and vortex briefly.

4.1.1.1.3. Aliquot 400 µL of the combined lysate into three separate sample tubes along with a reagent blank for extraction on each QIAcube®.

NOTE: Upon completion of the QIAcube® robotic workstation protocol, verify the tubes containing the lysate are empty. Less 50 µl of lysate should remain in the tube. If greater than 50 µl remain, this is an indication that the workstation needs calibration and should be addressed. The workstation can be taken off-line immediately and a calibration requested or the extraction can be attempted a second time to ensure that the excessive volume of lysate remaining is reproducible.

4.1.1.2. Quantify and confirm the quantification measurements between QIAcube® robotic workstations. The measurements between the workstations should be performing similarly (within 20%).

4.1.1.3. After confirming the measurements are within range, concentrate, amplify, and perform capillary electrophoresis on each reagent blank

4.1.1.4. To pass the performance check:

4.1.1.4.1. The quantity for the small autosomal targets between QIAcube® robotic workstations should not differ by greater than 20%.

4.1.1.4.2. The negative control and reagent blank should not contain any true peaks above the analytical threshold or any pattern of peaks below the analytical threshold.

5. **Documentation**

5.1. Current records will be maintained in proximity to the instrument.

5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Pipettes - P10, P100, P1000** *Rainin*®	Model #'s: L-10, L-20, L-100, L-1000

1. **Equipment**
   1.1. Disposable gloves
   1.2. Eye protection
   1.3. Lab coat
   1.4. Pipettes

2. **Safety**
   2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   3.1. Pipettes will be calibrated annually by an appropriate vendor.
   3.2. The pipettes should be cleaned with 70% ethanol prior to being used for casework.
   NOTE: If a pipette is not performing as expected it may be pulled out of service at any time and sent for repair or replacement.

4. **Procedure**
   4.1. Contact vendor to schedule service and/or calibration.

5. **Documentation**
   5.1. Records will be maintained in the *Pipette Log* and/or electronically.

Instrument: **Refrigerator (large) Isotemp® Plus** *Fisher Scientific™*	Model #: 13-986-120

1. **Equipment**
   - 1.1. Disposable gloves
   - 1.2. Eye protection
   - 1.3. Lab coat
   - 1.4. Refrigerator
   - 1.5. Thermometer

2. **Safety**
   - 2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   - 3.1. Check the temperature of the refrigerator weekly.

4. **Procedure**
   - 4.1. Note the temperature of the thermometer in the refrigerator.
   - 4.2. Record temperature on the log sheet.
   - 4.3. If the actual temperature varies more than $\pm$ 2°C from the desired temperature, the refrigerator will be adjusted to achieve the desired temperature.
   - 4.4. If the refrigerator cannot hold temperature, it will be repaired or replaced.

   NOTE: If a problem with a thermometer is suspected, verify the performance of the thermometer against a NIST certified thermometer.

5. **Documentation**
   - 5.1. Current records will be maintained in proximity to the instrument.
   - 5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Refrigerator (small) - General Purpose** *Fisher Scientific™*	Model #: 97-920-1

1. **Equipment**
   1.1. Disposable gloves
   1.2. Eye protection
   1.3. Lab coat
   1.4. Refrigerator
   1.5. Thermometer

2. **Safety**
   2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   3.1. Check the temperature of the refrigerator weekly.

4. **Procedure**
   4.1. Note the temperature of the thermometer in the refrigerator.
   4.2. Record temperature on the log sheet.
   4.3. If the actual temperature varies more than $\pm$ 2°C from the desired temperature, the refrigerator will be adjusted to achieve the desired temperature.
   4.4. If the refrigerator cannot hold temperature, it will be repaired or replaced.
   NOTE: If a problem with a thermometer is suspected, verify the performance of the thermometer against a NIST certified thermometer.

5. **Documentation**
   5.1. Current records will be maintained in proximity to the instrument.
   5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Real-Time PCR System** *Applied Biosystems*™	Model #: 7500

1. Equipment

1.1. See applicable section in ATF-LS-FB36 Quantifiler™ HP.

2. Safety

2.1. See applicable section in ATF-LS-FB36 Quantifiler™ HP.

3. Frequency

3.1. 3 Months
- 3.1.1. Background calibration
- 3.1.2. Restart computer, check hard disk space, and organize data files

3.2. 6 Months
- 3.2.1. Region of interest (ROI) calibration
- 3.2.2. Optical calibration
- 3.2.3. Pure dye calibration (can be performed by Applied Biosystems)
- 3.2.4. Defragment the computer's hard drive

3.3. Yearly3500
- 3.3.1. Scheduled preventative maintenance by vendor (Applied Biosystems)

3.4. As Needed
- 3.4.1. Replace halogen lamp if decrease in performance is noted.
- 3.4.2. Clean the heating block if contamination is present.
- 3.4.3. Call Applied Biosystems for repairs.
- 3.4.4. A performance check will be conducted after ROI / optical calibration, yearly preventative maintenance, replacement of bulb, or other extensive vendor maintenance/repair.

4. Procedure

4.1. Refer to Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide for directions on performing calibration and maintenance tasks.

4.2. Performance check procedure
- 4.2.1. Run a plate with the appropriate standards for the quantification kit using a dilution from the NIST SRM 2372, a plate blank, and one NIST SRM 2372 quantification sample run in duplicate.

4.3. Minimum for passing performance check
- 4.3.1. The NIST SRM 2372 sample must have a quantification value within 15% of the published value (see NIST SRM 2372 paperwork).
- 4.3.2. The standard curve must meet appropriate requirements (See *the appropriate quantitation* protocol).
- 4.3.3. Plate blank shall have no detected value or be $< 1x10^{-3}$.

5. Documentation

5.1. Current QC records will be maintained in proximity to the instrument.

5.2. Older QC records and performance checks will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Temperature Verification Kit** *Applied Biosystems*™	Serial #: *06C40218* - Eutechs Precision Thermometer (Model: 4500) Serial #: *507911* - 0.2 mL SmartProbe

1. **Equipment**
   - 1.1. Disposable gloves
   - 1.2. Eye protection
   - 1.3. Lab coat
   - 1.4. Temperature Verification Kit

2. **Safety**
   - 2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   - 3.1. Send out to an appropriate vendor for calibration yearly.

4. **Procedure**
   - 4.1. Contact vendor to schedule service.

5. **Documentation**
   - 5.1. Records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Thermal Cycler – GeneAmp® PCR System** *Applied Biosystems™*	Model #: 9700

1. Equipment

1.1. See applicable section in ATF-LS-FB30 Globalfiler™ PCR Amplification Kit.

2. Safety

2.1. See applicable section in ATF-LS-FB30 Globalfiler™ PCR Amplification Kit.

3. Frequency

3.1. 3 Months

3.1.1. Perform temperature verification.

3.1.2. Perform non-conformity test.

3.1.3. Perform the rate test.

3.1.4. Perform the cycle test.

3.2. Yearly

3.2.1. Performance check.

3.3. As Needed

3.3.1. A performance check will be conducted following vendor maintenance.

4. Procedure

4.1. Refer to the *Applied Biosystems GeneAmp PCR System 9700 User's Manual* for directions on performing calibration tests.

4.2. Performance check procedure

4.2.1. Perform all of the above listed calibration tests.

4.2.2. Amplify and genetically type a positive control sample (0.5 ng) and a negative control sample.

4.3. Minimum for passing performance check.

4.3.1. All of the quarterly calibration tests must pass.

4.3.2. Positive control sample must type correctly, electropherogram characteristics (peak heights, inter and intra locus balances, etc.) should be comparable to expected results. No true peaks present in negative control sample.

5. Documentation

5.1. Records and performance checks will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Thermal Cycler** -- Veriti *Applied Biosystems*™	Model #: Veriti™

1. Equipment

1.1. See applicable section in ATF-LS-FB30 Globalfiler™ PCR Amplification Kit.

2. Safety

2.1. See applicable section in ATF-LS-FB30 Globalfiler™ PCR Amplification Kit.

3. Frequency

3.1. 3 Months

3.1.1. Perform temperature non-uniformity test.

3.1.2. Perform temperature verification test.

3.1.3. Perform heated cover verification test.

3.1.4. Perform cycle performance test.

3.2. Yearly

3.2.1. Performance check.

3.3. As Needed

3.3.1. A performance check will be conducted following vendor maintenance.

4. Procedure

4.1. Refer to the *Applied Biosystems Veriti Thermal Cycler User's Manual* for directions on performing calibration tests.

4.2. Performance check procedure

4.2.1. Perform all of the above listed calibration tests.

4.2.2. Amplify and genetically type a positive control sample (0.5 ng) and a negative control sample.

4.3. Minimum for passing performance check

4.3.1. All of the quarterly calibration tests must pass.

4.3.2. Positive control sample must type correctly, electropherogram characteristics (peak heights, inter and intra locus balances, etc.) should be comparable to expected results. No true peaks present in negative control sample.

5. Documentation

5.1. Records and performance checks will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **Thermometers** *VWR*	Model #: Various

1. **Equipment**
   1.1. Disposable gloves
   1.2. Eye protection
   1.3. Lab coat
   1.4. Thermometer
   1.5. NIST certified thermometer

2. **Safety**
   2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   3.1. Compare to a NIST certified thermometer yearly.

4. **Procedure**
   4.1. Check the calibration date on the certificate and make sure the NIST certified thermometer has not expired.
   4.2. Place the NIST certified thermometer and the laboratory thermometer(s) in an oven, refrigerator, or freezer based on the range of the thermometer.
   4.3. Let sit for at least 2 hours and then record the temperatures.
   4.4. Any laboratory thermometer that differs from the NIST certified thermometer by more than ±1°C will be replaced.

5. **Documentation**
   5.1. Records will be maintained in the *Thermometer Log* and/or electronically

Instrument: **Thermomixers®** *Eppendorf®*	Model #: Various

1. **Equipment**
   1.1. Disposable gloves
   1.2. Eye protection
   1.3. Lab coat
   1.4. Thermometer
   1.5. Thermomixer

2. **Safety**
   2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   3.1. Check the temperature of the thermomixer weekly.

4. **Procedure**
   4.1. Note the temperature of the thermometer in the thermomixer.
   4.2. Record temperature on the log sheet.
   4.3. If the actual temperature varies more than $\pm$ 2°C from the desired temperature, the thermomixer will be adjusted to achieve that desired temperature.
   4.4. If the thermomixer cannot hold temperature, it will be repaired or replaced.
   NOTE: If a problem with a thermometer is suspected, verify the performance of the thermometer against a NIST certified thermometer.

5. **Documentation**
   5.1. Current temperature records will be maintained in proximity to the instrument.
   5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically.

Instrument: **UV Crosslinker – Spectrolinker™** *Spectronics® Corporation*	Model #: XL-1500

1. **Equipment**
   - 1.1. Disposable gloves
   - 1.2. Eye protection
   - 1.3. Lab coat
   - 1.4. UV crosslinker
   - 1.5. Calculator
   - 1.6. Timer

2. **Safety**
   - 2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. **Frequency**
   - 3.1. Evaluate crosslinkers to ensure that the proper energy is being delivered bi-weekly (every two weeks).

4. **Procedure**
   - 4.1. Turn crosslinker on.
   - 4.2. Select "Energy" mode and enter "9999"; Hit “Start”.
   - 4.3. Time how long it takes the crosslinker to deliver this energy (essentially 1.0 J/cm^2).
     - 4.3.1. Calculate time for 2.0 J/cm^2 [i.e. 2*(time for 1.0 J/cm^2)].
       - 4.3.1.1. Use this time to crosslink solid items (e.g. tubes, racks, etc.).
     - 4.3.2. Calculate time for 6.0 J/cm^2 [i.e. 3*(time for 2.0 J/cm^2)].
       - 4.3.2.1. Use this time to crosslink liquid reagents (e.g. reagents in conical tubes).
   - 4.4. Visually verify through the front window that all UV bulbs are functioning.
   - 4.5. Record the observed and calculated energy delivery times, and if all bulbs are functioning.

5. **Documentation**
   - 5.1. Current records will be maintained in proximity to the instrument.
   - 5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically

Instrument: **Water Purification System** *Millipore*	Model #'s: Milli-Q® Gradient A10 Milli-Q® Gradient w/Bio Pak

1. Equipment

1.1. Disposable gloves
1.2. Eye protection
1.3. Lab coat
1.4. Water purification system

2. Safety

2.1. Lab coat, eye protection, and disposable gloves must be worn at all times while performing this procedure.

3. Frequency

3.1. Weekly
  3.1.1. Check the resistance (MΩ) of the water. The higher the resistance (MΩ reading), the fewer ions are present to carry the electrical charge.
3.2. As Needed
  3.2.1. Replace the cartridges and filters when indicated by the water system, and record lot numbers on the *Filter Log sheet*.

4. Procedure

4.1. Turn on the water system, and let it run for approximately 5 seconds.
4.2. Record the resistance in megaohms (MΩ) on the weekly record sheet.
4.3. If the reading is below 17 MΩ, call vendor for service.
4.4. Refer to the *Millipore Milli-Q® Gradient and Milli-Q Gradient A10® User Manual* for directions on replacing the cartridges and filters.

5. Documentation

5.1. Current records will be maintained in proximity to the instrument.
5.2. Older records will be maintained in the *Maintenance & Repair Log* and/or electronically

Instrument: **STRmix™ Software**	Version #'s: Current

1. **Equipment**
   1.1. Computer loaded with STRmix software

2. **Safety**
   2.1. NA

3. **Frequency**
   3.1. Analyze with STRmix the profiles generated from NIST SRM 2391 A through D as part of the annual processing of the NIST sample set.

4. **Procedure**
   4.1. Run STRmix deconvolution and generate an LR for each sample.
      4.1.1. Components A through C are single source samples.
      4.1.2. Component D is a mixture of components A and C (do not condition the LR on either contributor).
   4.2. The point LR for components A through C should be the same as the previous year.
   4.3. Component D mixture proportions should be within 70-80% for the major and 20-30% for the minor.

5. **Documentation**
   5.1. Records will be maintained electronically.

Instrument: **Genetic Analyzer** *Applied Biosystems*™	Model #: 3500xL

1. Equipment

1.1. See applicable section in ATF-LS-FB38.

2. Safety

2.1. See applicable section in ATF-LS-FB38.

3. Frequency

3.1. Each Use

3.1.1. Check that the polymer, anode buffer, and cathode buffer have been on the instrument no more than two (2) weeks, and are at appropriate levels for use.

3.1.2. Check for bubbles in the pump block and channels.

3.1.3. Ensure the pump block is in the pushed-back position.

3.1.4. Check for leaks around the buffer pin valve, check valve, and array locking lever. Clean any dried residue.

3.1.5. Add the number of runs to the Run Log.

3.2. Weekly

3.2.1. Restart the computer and instrument, following *Shutting Down the Instrument* steps (5.43 through 5.45) and *Preparing the Instrument* steps (5.1 through 5.4) in ATF-LS-FB38.

3.3. Biweekly

3.3.1. Run the *Wash Pump And Channels* wizard with a new conditioning reagent pouch prior to each polymer change. This may occur biweekly, or more frequently if a polymer pouch reaches the maximum number of injections.

3.3.2. Change polymer, anode buffer, and cathode buffer. Reagents older than two weeks can remain on the instrument as long as no casework samples are run.

3.3.2.1. To replace polymer, use the *Replenish Polymer* wizard.

3.4. Monthly

3.4.1. Empty the oven condensation reservoir.

3.4.2. Flush the water trap with DI water.

3.4.3. Defragment the computer hard drive.

3.5. Quarterly

3.5.1. Check database and hard disk space. Archive or purge records if needed.

3.5.2. Back up data files.

3.6. Yearly

3.6.1. Scheduled preventative maintenance by vendor (Applied Biosystems).

3.7. As needed

3.7.1. A new spatial calibration, spectral calibration, and a performance check will be conducted after the installation of a new capillary array, yearly preventative

maintenance, adjustment/replacement of optics/laser, or other extensive vendor maintenance/repair.

3.7.2. A new spectral calibration can be performed if excessive pull-up is noted.

3.7.3. Run the *Bubbles Remove* wizard as needed when bubbles are observed.

4. Procedure

4.1. Refer to the *Applied Biosystems 3500/3500xL Genetic Analyzer User Guide* for directions on performing calibration and maintenance tasks.

4.2. Performance check procedure

4.2.1. Amplify three NIST SRM 2372 samples (same component in triplicate) targeting 500 pg of template DNA. A positive and negative control sample should also be included.

4.2.2. When setting up the 3500 plate, combine the three sample amplicons into a single tube. Create a master mix of amplified product, LIZ, and formamide in the appropriate proportions for at least 24 wells (e.g. 26 µl of product, 10.4µl of LIZ, and 249.6 µl of formamide).

4.2.3. Aliquot 11 µl of the master mix into wells A01 to H03 (24 wells). Load the amplification positive control, negative amplification control, allelic ladders in the column D of the plate.

4.2.4. Analyze the samples, removing stutter and artifact peaks, after the 3500 run has been completed. Calculate the average RFU per allele or locus for each capillary.

4.3. Minimum for passing performance check

4.3.1. Positive control must type correctly with an acceptable level of pull-up and no extra true peaks in samples. No true peaks should be observed in negative control sample.

4.3.2. The average RFU/allele or locus for each capillary should be within 30% of the original NIST SRM baseline.

5. Documentation

5.1. QC records and performance checks will be maintained in the *Maintenance & Repair Log* and/or electronically.

1. Scope

This protocol is to be used to amplify DNA extracted from known reference samples or biological material found on items of evidence at the following loci:

21 autosomal STR loci (D3S1358, vWA, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D12S391, and D2S1338)
1 Y-STR locus (DYS391)
1 insertion/deletion polymorphic marker on the Y chromosome (Y indel)
Amelogenin (sex determining marker)

The Applied Biosystems™ (AB) GlobalFiler™ PCR Amplification Kit utilizes the polymerase chain reaction (PCR) to make copies of specific regions of the extracted DNA samples. These regions allow the analyst to characterize the source of the DNA. The GlobalFiler™ PCR Amplification Kit allows for the simultaneous amplification of 20 autosomal tetranucleotide repeat regions (loci), 1 autosomal trinucleotide repeat region (D22S1045), 1 Y-STR, 1 insertion/deletion polymorphic marker (indel) on the Y chromosome, and the sex marker Amelogenin in one reaction tube. Amplification is performed in a thermal cycler. These 21 autosomal STR loci represent the expanded set of 20 Combined DNA Index System (CODIS) core loci plus SE33.

The GlobalFiler™ PCR Amplification Kit contains all reagents needed for amplification, which includes: primer sets specific for the various loci, PCR reaction buffer, enzyme, and the positive control DNA 007. Allelic ladders are also included in the kit; however, they are not needed during amplification. The primer sets contained within each kit consist of both unlabeled primers and those that are labeled with one of five distinctive fluorescent dyes. The incorporation of fluorescent dyes during the amplification process allows for the subsequent detection, characterization, and sizing of the fragments on the AB 3130 Genetic Analyzer. The use of multicolor dyes permits the analysis of loci with overlapping size ranges.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

2.1. Applied Biosystems™ GlobalFiler™ PCR Amplification Kit User's Manual, Revision E, July 2016.
2.2. V. Bogasa, M. Carvalho, F. Corte-Real, M.J. Porto, Testing the behavior of GlobalFiler® PCR amplification kit with degraded and/or inhibited biological samples, Forensic Sc. Int. Genet. Suppl. Ser. 5 (2015) e21–e23.

2.3. D. Hares, Expanding the CODIS core loci in the United States, Forensic Sci. Int. Genet. 6 (2012) e52–e54.
2.4. D. Hares, Selection and implementation of expanded CODIS core loci in the United States, Forensic Sc. Int. Genet. 17 (2015) 33–34.
2.5. J.M. Butler, Commonly used short tandem repeat markers and commercial kits, biology of STRs, and forensic issues, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, pp 85-180.
2.6. A. Edwards, A. Civitello, H. Hammond, C.T. Caskey, DNA typing and genetic mapping with trimeric and tetrameric tandem repeats, Am. J. Hum. Genet. 49 (1991) 746–756.
2.7. H.A. Hammond, L. Jin, Y. Zhong, C.T. Caskey, R. Chakraborty, Evaluation of 13 short tandem repeat loci for use in personal identification applications, Am. J. Hum. Genet. 55 (1994) 175–189.
2.8. B. Budowle, T.R. Moretti, K.M. Keys, B.W. Koons, J.B. Smerick, Validation studies of the CTT STR multiplex system, J. Forensic Sci. 42 (1997) 701–707.
2.9. C.J. Fregeau, R.M. Fourney, DNA typing with fluorescently tagged short tandem repeats: a sensitive and accurate approach to human identification, Biotechniques 15 (1993) 100–119.
2.10. P.S. Walsh, N.J. Fildes, R. Reynolds, Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA, Nucleic Acids Res. 24 (1996) 2807–2812.
2.11. R.K. Saiki, T.L. Bugawan, G.T. Horn, K.B. Mullis, H.A. Erlich, Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes, Nature 324 (1986) 163-166.
2.12. R.K. Saiki, D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, H.A. Erlich, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, Science 239 (1988) 487–491.

3. Equipment

3.1. Disposable gloves
3.2. Lab coat
3.3. Eye protection
3.4. Benchtop hood
3.5. TE^{-4} (10mM Tris-HCl , 0.1mM EDTA, pH 8.0)
3.6. AB GlobalFiler™ PCR Amplification Kit
  3.6.1. GlobalFiler™ Primer Set
  3.6.2. GlobalFiler™ Master Mix
  3.6.3. DNA Control 007
3.7. GeneAmp® PCR System 9700 or Veriti® 96-well Thermal Cycler
3.8. 0.2 mL thin-walled PCR tubes
3.9. Microcentrifuge tubes

3.10. 10% bleach solution and/or 70% ethanol
3.11. Tube racks
3.12. Pipette
3.13. Disposable pipette tips
3.14. Vortexer
3.15. Centrifuge

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling reagents and DNA extracts.
4.2. Change gloves frequently.
4.3. Lab coat and eye protection must be worn at all times while performing this procedure.
4.4. PCR setup for unknown samples shall be performed separately from PCR setup for known samples.
4.5. Samples thought to contain lower levels of DNA will be handled before those thought to contain large amounts of DNA.
4.6. Sample setup must be performed in a PCR setup hood. Clean surfaces with 10% bleach solution and/or 70% ethanol prior to use. After exiting hood, turn on UV light.
4.7. Exercise caution when opening tubes.
4.8. Use aerosol-resistant pipette tips when transferring liquids containing DNA.
4.9. Change pipette tips after transferring any liquids potentially containing DNA.
4.10. Only tubes associated with one sample shall be open at a time.
4.11. The amount of reagent blank amplified must equal or exceed the amount amplified for its associated DNA extracts.
4.12. Tubes containing DNA extract shall not be opened in benchtop hood prior to aliquoting master mix into 0.2 mL tubes.
4.13. A positive amplification control (Control DNA 007) must be initiated.
4.14. A negative amplification control must be initiated. Add 15 µL TE^{-4} to the negative control tube last, after all DNA samples have been added to the other tubes. This tube functions as a negative control for the PCR setup.
4.15. Record the lot number of each reagent used. Do not use the reagents after the expiration date.
4.16. Store the DNA amplification reagents in a refrigerator or freezer separate from the DNA extracts and evidence.
4.17. Once the PCR reagents have been thawed, do not refreeze the reagents.
4.18. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. Turn on the thermal cycler.
5.2. If DNA extracts have been frozen, allow them to thaw.
5.3. Dilutions can be prepared at any time prior to adding DNA extracts and/or dilutions to 0.2 mL PCR tubes. If preparing dilutions in PCR setup hood, do not prepare them until after master mix has been aliquoted to 0.2 mL PCR tubes.
5.4. In the PCR setup hood, appropriately label 0.2 mL PCR tubes and 1.5 mL master mix tube(s). Place the 0.2 mL PCR tubes in a clean tray dedicated for PCR setup.
5.5. Determine amount of DNA extract and TE^{-4} required for each sample and control based on quantification results. Optimal target amount of template DNA per sample and positive control is approximately 0.5 ng.
NOTE: If necessary, lesser or greater quantities of DNA may be used to obtain the desired result. Factors such as degradation, inhibition, and mixture ratio may affect this decision.
5.6. Aliquot required TE^{-4} into 0.2 mL PCR tubes and/or 1.5 mL dilution tubes (if necessary). The final reaction volume in each PCR tube is 25 µL (10 µL master mix and 15 µL sample).
5.7. Vortex and briefly spin DNA extracts.
5.8. Add appropriate volume of DNA extracts to 1.5 mL dilution tubes (if necessary).
5.9. Vortex and briefly spin dilution tubes (if necessary).
5.10. Determine total number of sample and control tubes.
5.11. Vortex and briefly spin the amplification reagents (GlobalFiler™ Primer Set and GlobalFiler™ Master Mix).
5.12. Add appropriate volumes of reagents to 1.5 mL tube(s) to create the PCR master mix. The following volumes are 'per sample.' Include additional reactions to the total number of samples to account for loss of reagent during transfers. Multiply the volumes by the number of samples to be amplified including controls.

Globalfiler™ Component	Volume (µL)
GlobalFiler™ Master Mix	7.5
GlobalFiler™ Primer Set	2.5

5.13. Vortex and briefly spin master mix.
5.14. Aliquot 10 µL master mix to each sample and control 0.2 mL tube.
5.15. Add appropriate volume of DNA extracts or dilutions, and reagent blank(s) to 0.2 mL PCR tubes. Add a total of 15 µL DNA extract/TE^{-4} to each PCR tube for a final volume of 25 µL.
5.16. Vortex and briefly spin Control DNA 007 positive control DNA.
5.17. Add appropriate volume of Control DNA 007 to positive control 0.2 mL tube.
5.18. Add 15 µL TE^{-4} to negative control 0.2 mL tube.

5.19. Clean the bench top hood with 10% bleach and/or 70% ethanol. Turn on UV light when leaving bench top hood.
5.20. Briefly spin 0.2 mL tubes in centrifuge.
5.21. Place tubes in thermal cycler.
5.22. Ensure that all 0.2 mL tube caps are tightly sealed.
5.23. Close thermal cycler cover.
5.24. Select GlobalFiler™ protocol ("GF-28") and press start. If using the GeneAmp® PCR System 9700, verify the "Max" ramping mode has been selected. If you are using the Veriti™ Thermal Cycler, select the 100% ramping rate. Verify that thermal cycling conditions are as follows:

Initial Incubation Step	Hold	95°C	1 minute
Denature	Cycle (28 cycles)	94°C	10 seconds
Anneal/Extend		59°C	90 seconds
Final Extension Step	Hold	60°C	10 minutes
Final Hold	Hold	4°C	Up to 24 hours

5.25. When the amplification is complete (approximately 80 minutes), samples can be stored in the refrigerator or freezer according to the following chart:

Two weeks or less	2-8°C
More than two weeks	-15 to -25°C

6. Supplemental Information

Dyes Used in Globalfiler™ PCR Amplification Kit

Dye	Color	Label
6-FAM™	Blue	Samples, allelic ladders, and controls
VIC™	Green	
NED™	Yellow	
TAZ™	Red	
SID™	Purple	
LIZ™	Orange	GeneScan™ 600 LIZ™ Size Standard v2.0

Table source: Applied Biosystems™ GlobalFiler™ PCR Amplification Kit User's Manual, Revision E, July 2016

Description of Loci Amplified with the Globalfiler™ PCR Amplfication Kit

Locus designation	Chromosome location	Alleles included in Allelic Ladder	Dye label	DNA Control 007
D3S1358	3p21.31	9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20	6-FAM™	15, 16
vWA	12p13.31	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24		14, 16
D16S539	16q24.1	5, 8, 9, 10, 11, 12,13, 14, 15		9, 10
CSF1PO	5q33.3-34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		11, 12
TPOX	2p23-2per	5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15		8, 8
Y indel	Yq11.221	1, 2	VIC™	2
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y		X, Y
D8S1179	8q24.13	5, 6, 7, 8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19		12, 13
D21S11	21q11.2-q21	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38		28, 31
D18S51	18q21.33	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27		12, 15
DYS391	Yq11.21	7, 8, 9, 10, 11, 12, 13		11
D2S441	2p14	8, 9, 10, 11, 11.3, 12, 13, 14, 15, 16, 17	NED™	14, 15
D19S433	19q12	6, 7, 8, 9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18.2, 19.2		14, 15
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3		7, 9.3
FGA	4q28	13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2		24, 26
D22S1045	22q12.3	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	TAZ™	11, 16
D5S818	5q21-31	7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18		11, 11
D13S317	13q22-31	5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16		11, 11
D7S820	7q11.21-22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		7, 12
SE33	6q14	4.2, 6.3, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 20.2, 21, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35, 35.2, 36, 37		17, 25.2
D10S1248	10q26.3	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	SID™	12, 15
D1S1656	1q42.2	9, 10, 11, 12, 13, 14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18.3, 19.3, 20.3	SID™	13, 16
D12S391	12p13.2	14, 15, 16, 17, 18, 19, 19.3, 20, 21, 22, 23, 24, 25, 26, 27		18, 19
D2S1338	2q35	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28		20, 23

Table source: Applied Biosystems™ GlobalFiler™ PCR Amplification Kit User's Manual, Revision E, July 2016

Original CODIS Core 13 Loci with additional Expanded Core Loci

Locus
CSF1PO
D3S1358
D5S818
D7S820
D8S1179
D13S317
D16S539
D18S51
D21S11
FGA
TH01
TPOX
vWA
D1S1656
D2S441
D2S1338
D10S1248
D12S391
D19S433
D22S1045

Red is for original CODIS Core 13 Loci.
Blue is for new additional CODIS Core Loci.

Table Source: D. Hares, Selection and implementation of expanded CODIS core loci in the United States, Forensic Sci. Int. Genet. 17 (2015) 33–34.

1. Scope

This protocol is used to analyze the amplified DNA product on the Applied Biosystems™ (AB) 3130 Genetic Analyzer. The DNA fragments amplified with the AB GlobalFiler™ PCR Amplification Kit are separated according to size by capillary electrophoresis using the AB 3130 Genetic Analyzer, a multi-capillary instrument. An internal lane standard (ILS) is injected along with the amplified DNA fragments. The ILS fragments are then used to determine the base pair size for each amplified DNA fragment. The use of an ILS provides consistency in sizing between samples.

The amplified fragments are detected by laser excitation and the subsequent emission spectra are captured by a charge-coupled device (CCD) camera that displays the signals as peaks. The resulting data is graphically displayed as colored peaks noted by height in relative fluorescent units and time (scan number). This display is called an electropherogram.

The reference allelic ladders for the short tandem repeat (STR) loci, indel marker, and Amelogenin are also subjected to capillary electrophoresis. These allelic ladders contain the more common alleles in the general population for each locus. Using the ladders, the alleles present in known and questioned DNA specimens may be determined.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from the references cited below.

2.1. Applied Biosystems™ GlobalFiler™ PCR Amplification Kit User Guide, Revision E, July 2016.
2.2. Applied Biosystems™ 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide, 2004.
2.3. Applied Biosystems™ 3130/3130xl Genetic Analyzers Getting Started Guide, 2004.
2.4. Applied Biosystems™ 3130/3130xl Genetic Analyzers Using Data Collection v4 User Bulletin, Revision A, May 2012.
2.5. J.M. Butler, DNA separation methods, DNA detection methods, instrumentation for STR typing, and STR genotyping issues, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 313-388.
2.6. J.M. Butler, B.R. McCord, J.M. Jung, R.O. Allen, Rapid analysis of the short tandem repeat HUMTH01 by capillary electrophoresis, Biotechniques 17 (1994) 1062-1070.
2.7. M.M. Holland, L.A. Turni, S.A. Del Rio, M.A. Marino, R.S. Lofts, D.L. Fisher, J. Ross, J.W. Schumm, P.E. Williams, Typing human DNA using capillary electrophoresis: comparison of slab gel and capillary formats, in: W. Bär, A. Fiori, U. Rossi (Eds.), Advances in Forensic Haemogenetics, volume 5, Springer, Berlin, 1994, pp. 156-159.

2.8. T.R. Moretti, A.L. Baumstark, D.A. Defenbaugh, K.M. Keys, A.L. Brown, B. Budowle, Validation of STR typing by capillary electrophoresis, J. Forensic Sci. 46 (2001) 661-676.
2.9. J.M. Butler, E. Buel, F. Crivellente, B.R. McCord, Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis, Electrophoresis 25 (2004) 1397-1412.
2.10. A.R. Isenberg, R.O. Allen, K.M. Keys, J.B. Smerick, B. Budowle, B.R. McCord, Analysis of two multiplexed short tandem repeat systems using capillary electrophoresis with multiwavelength fluorescence detection, Electrophoresis 19 (1998) 94-100.
2.11. McCord, B.R., Budowle, B., Isenberg, A.R., and Allen, R.O. (1997) Capillary electrophoresis for the automated analysis of multiplexed STRs using multiwavelength fluorescence detection. Seventh International Symposium on Human Identification (1996). pp.116-122.

3. Equipment

3.1. Disposable gloves
3.2. Lab coat
3.3. Eye protection
3.4. Deionized water
3.5. Computer with data collection software
3.6. AB 3130 Genetic analyzer
3.7. AB 10X Buffer with EDTA (or other validated buffer such as Amresco A.C.E.™ Sequencing Buffer)
3.8. AB Performance-optimized polymer 4 (POP4)
3.9. AB Hi-Di™ Formamide
3.10. AB GlobalFiler™ allelic ladder
3.11. AB GS600 LIZ™ sizing standard, v2.0
3.12. MicroAmp™ Optical Reaction Plate
3.13. MicroAmp™ Splash Free Support Base
3.14. AB 96-well Plate Base
3.15. AB 96-well Plate Retainer
3.16. Buffer/Water/Waste Reservoirs
3.17. Plate and Reservoir septa
3.18. 10% bleach solution
3.19. 70% ethanol
3.20. 1.5 mL microcentrifuge tubes
3.21. 50 mL conical tubes
3.22. Pipette
3.23. Disposable aerosol-resistant pipette tips
3.24. Centrifuge
3.25. Heat block
3.26. Cold block

3.27. Vortexer

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling reagents and amplified DNA.
4.2. Lab coat and eye protection must be worn at all times while performing this procedure.
4.3. Sample setup must be performed on bench paper, tissue wipes, or laboratory bench surfaces cleaned with 10% bleach solution followed by 70% ethanol.
4.4. Exercise caution when opening tubes.
4.5. Only one tube of amplified DNA shall be open at a time.
4.6. Use aerosol-resistant pipette tips when transferring liquids containing DNA.
4.7. Change pipette tips after transferring any liquids potentially containing DNA.
4.8. Record the lot number of each reagent used. Do not use the reagents after the expiration date.
4.9. Check the AB 3130 Instrument Log to ensure that 1X buffer and water in the buffer, water, and waste reservoirs have not been on the instrument for more than two days. Replace reservoir septa when refilling reservoirs.
NOTE: When refilling reservoirs, the septa may be rotated 180 degrees and reused one time since only four capillaries are being used.
4.10. Check the AB 3130 Instrument Log to ensure that POP4 polymer has not been on the instrument for more than two weeks.
4.11. Clean plate base and retainer with water between runs.
4.12. Use new plate septa with each set of runs.
4.13. At least one positive amplification control (Control DNA 007) must be included with each sample run.
4.14. It is good practice to include multiple wells with allelic ladder and positive control to account for variations in electrophoresis conditions.
4.15. Buffer jar, reservoirs, plate base, and plate retainer shall be cleaned with tap water followed by deionized water. Do not clean with bleach.
4.16. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and should be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.
4.17. The spatial calibration determines the position on the CCD camera where the signal emitted by each capillary is expected to fall. A new spatial calibration is required when the capillary array is replaced, temporarily removed from the instrument detection block, or the instrument is moved.
4.18. The spectral calibration accounts for the overlapping emission spectra of the six dyes detected by the instrument. If optical components of the instrument (laser, CCD camera, capillary array) have been realigned/replaced, a new spectral calibration is required. Additionally, if there is a noticeable degradation in spectral separation (increased pull-up and/or pull-down peaks) in the electropherograms, a new spectral calibration may be needed.

4.19. For instructions on performing spatial and spectral calibrations, refer to *Applied Biosystems™ 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide*.

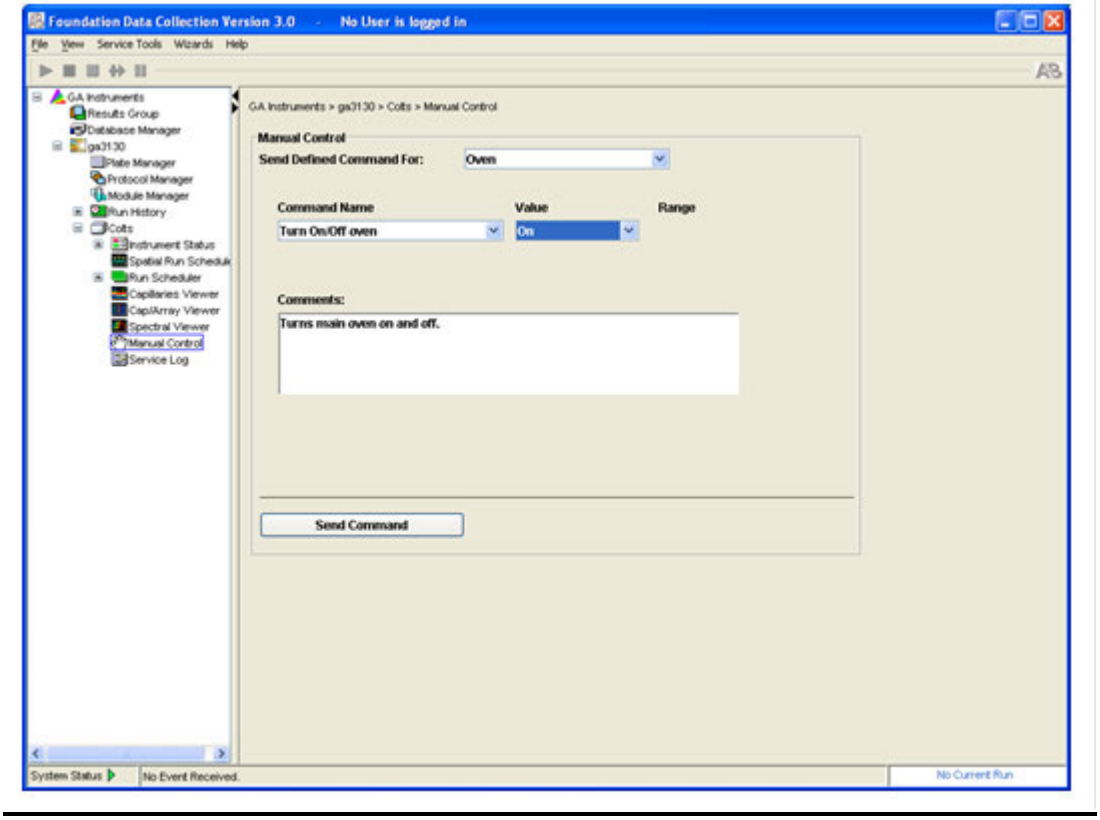
5. Procedure

5.1. Remove appropriate number of tubes of formamide from freezer and allow them to thaw completely.

Preparing the Instrument

5.2. Turn on computer workstation and log in.
5.3. Turn on instrument and wait for green status light to come on and stop flashing.
5.4. Launch Data Collection software. Wait for all four status indicator lights in *Service Console* window to turn green (if lights do not turn green, refer to *Applied Biosystems™ 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide*).
5.5. Check quality control chart for instrument, and if necessary, press "Tray" button on the front of the instrument and wait for green status light to stop flashing prior to opening instrument doors and performing necessary maintenance tasks. These tasks include, but are not limited to, installing or replacing the capillary array, replenishing or changing polymer, preparing buffer, and filling reservoirs. When possible, follow wizards contained in data collection software (see *Applied Biosystems™ 3130/3130xl Genetic Analyzers Getting Started Guide* or *Applied Biosystems™ 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide* for specific instructions).
5.6. Check levels and time-on-instrument of buffer in buffer jar and buffer and water in reservoirs. Ensure that reservoir septa fit snugly on the reservoirs.
5.7. Check levels and time-on-instrument of polymer. If polymer must be added or replaced, ensure that new polymer is warmed to room temperature before using. Use the *Polymer Replenish Wizard* to replenish polymer.
5.8. Check for bubbles in the pump block, lower polymer block, interconnect tube, polymer supply tube, and channels. Very small bubbles on top of the array port may be disregarded. Use the *Bubble Remove Wizard* to remove bubbles. If *Bubble Remove Wizard* is used, ensure that sufficient polymer remains for completing the scheduled runs.
5.9. Check for leaks around the array knob, the interconnecting tube nuts, and the polymer supply tube nut. Clean dried polymer from nuts and/or knob, if possible.
5.10. Shut instrument doors and wait for tray to complete homing procedure.
5.11. In the tree pane of the Data Collection software, click *GA Instruments > ga3130 > instrument name > Manual Control*.
5.12. In the *Send Defined Command For* drop-down menu, select "Oven." In the *Command Name* dropdown menu, select "Temperature Set," type "60" in the *Value* field, and click *Send Command*.

5.13. In the *Command Name* drop-down menu, select "Turn On/Off," select "On," then click *Send Command* (see image below).
NOTE: The oven will turn itself off after 45 minutes if the run has not been started.



5.14. In the *Command Name* drop-down menu, select "Select Oven Temperature," select 60° C, and then click *Send Command.*

Preparing the Samples

5.15. Determine plate configuration including placement of samples, allelic ladders, and controls. Four wells in a column (rows A through D or E through H) are injected simultaneously. For each injection, ensure that all four wells contain either a sample, allelic ladder, control or formamide blank.
5.16. Prepare formamide / GS600 LIZ v2.0 sizing standard master mix (9.8 µL formamide and 0.2 µL GS600 LIZ v2.0 sizing standard per sample, adding several extra samples to ensure adequate volume). The volume of GS600 LIZ v2.0 sizing standard can be reduced to prevent spectral pull-up. Any remaining formamide shall be discarded.
5.17. Vortex and briefly spin tube of master mix.
5.18. Dispense 10 µL master mix to each well of 96-well optical reaction plate that will contain sample, allelic ladder, or control.

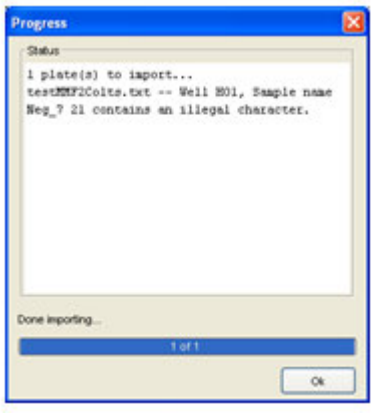
5.19. Add 1 µL of amplified product or allelic ladder to the appropriate wells.
5.20. Cover plate with new plate septa.
5.21. Briefly spin plate on plate centrifuge.
5.22. Place plate in heat block at ~95˚C for 3 minutes.
5.23. Place plate in frozen cold block for 3 minutes.
5.24. Place optical reaction plate in plate base, ensuring that it is correctly aligned.
5.25. Snap Plate Retainer onto optical reaction plate / plate base assembly, ensuring that the holes in the septa align with those in the plate retainer.
5.26. Align and place plate assembly securely on the plate deck of the instrument. Note the correct orientation of the notch on the bottom of the plate.

Creating a Plate Record, Linking the Plate, and Starting the Instrument

5.27. In the tree pane of the Data Collection software, click *GA Instruments* > *ga3130* > *Plate Manager*.
5.28. To create a new plate:
    5.28.1. Select *New*.
    5.28.2. In the *New Plate Dialog* window, type in the "plate name." The plate name shall include at a minimum the date, analyst's initials, and instrument name.
    5.28.3. For the *Application* drop-down menu, select "GeneMapper-instrument name."
    5.28.4. For the *Plate Type* drop-down menu, select "96-Well."
    5.28.5. Type the analyst's initials in the *Owner and Operator* fields, then click *Ok*. The *Plate Editor* opens.
    5.28.6. In the *Plate Editor* window, type in the sample name.
    5.28.7. Select the appropriate sample type in the *Sample Type* drop-down menu.
    5.28.8. Select "HID_GLOBALFILER" in the *Results Group* drop-down menu.
    5.28.9. Select "HID_GLOBALFILER" in the *Instrument Protocol* drop down menu. **NOTE: Only select the *Size Standard, Panel, Analysis Method, Results Group,* and *Instrument Protocol* for the first sample. Typing *Alt-D* will fill down the information for four samples. Alternatively, highlight a number of wells and type *Ctrl-D*. This will fill down the information for any highlighted wells.**
5.29. To duplicate an existing plate:
    5.29.1. Click *Find All*.
    5.29.2. Highlight the plate you want to duplicate and click the *Duplicate* button at the bottom of the screen.
    5.29.3. In the *Duplicate Plate* dialog window, type in the new "plate name." The plate name shall include at a minimum the date, analyst's initials, and instrument name.
    5.29.4. Type the analyst's initials in the *Owner and Operator* fields, then click *Ok*.
5.30. To import a plate:
    5.30.1. Click *Import*.
    5.30.2. In the *Open* window, find the saved plate that will be imported, highlight the file name, and click the *Open* button.

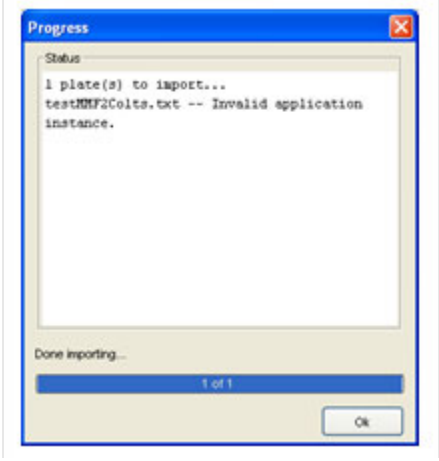
5.30.3. A window will appear with the status of the import. For a successful import, click *Ok*.

5.30.4. Importing problems occur when incorrect characters are used in sample names. The following screen will appear if this is the case:



5.30.5. Click *Ok* and check the sample name of the well listed in the error message. Attempt to import the file again after making corrections. The following characters are not allowed to be used in creating sample names: \ / : " < > ? ' | SPACE.

5.30.6. Importing problems occur when a template created for one 3130 is imported to a different 3130. The following screen will appear if this is the case:



5.30.7. Click *Ok*. Check to make sure that the correct template is being used for the desired 3130. If it is the correct 3130, check/change the application instance in the import file. Attempt to import the file again on the correct 3130.

5.31. The run module and conditions are as follows:

Run Module	HIDFragmentAnalysis36 POP4 1
Injection Conditions	3 kV/5 sec
Run Conditions	15 kv/1500 sec
Dye Set	J6

NOTE: Injection conditions may vary to normalize sensitivity

5.32. By default, the priority setting for each sample is 100. The priority setting determines the order in which the injections are analyzed by the instrument. Lowering the priority setting (1-99) will result in the injections being processed before those with higher priority settings.

5.33. To inject the same sample(s) multiple times, select *Edit* > *Add Sample Run* from the menu at the top of the screen. This will create additional results group and instrument protocol columns for all of the samples in the run. Be sure to select the appropriate results group and instrument protocol settings for only the set of four samples that contains the sample(s) that needs to be injected multiple times. Leave the settings blank for those samples that will be injected only once.

5.34. Click *Ok* to save and close plate record.

5.35. In the tree pane of the data collection software, click *GA Instruments* > *ga3130* > *instrument name* > *Run Scheduler*.

5.36. Highlight the plate name and click on the yellow plate diagram on the right hand side of the screen to "link" the plate. The plate diagram will turn green once it has been linked. A run cannot be started until a plate has been linked.

5.37. In the tree pane of the data collection software, click *GA Instruments* > *ga3130* > *instrument name* > *Run Scheduler* > *Run View*. Verify that all the necessary injections have been scheduled. When a particular injection is highlighted, the corresponding wells to be injected will be highlighted in the plate diagram.

5.38. After verifying that all settings are correct, click the green triangle icon in the toolbar. Click *Ok* on the pop-up screen to start the run.

Shutting Down the Instrument

5.39. In the *Service Console* window, click the *Stop All* button. Wait for all four status indicator lights to turn red. At this point, you can close the *Service Console* window by clicking on the "x" in the upper right hand corner.
NOTE: Do not close the *Service Console* window without first waiting for all four status indicator lights to turn red.

5.40. Press the power button on the 3130 Genetic Analyzer.

5.41. Shut down the computer.

1. Scope

This protocol is to be used to analyze the raw data generated by the Applied Biosystems™ (AB) 3130 or 3500 Genetic Analyzer. The data collected by the AB 3130 or 3500 Genetic Analyzer for each sample is displayed graphically as an electropherogram. These samples include questioned and known specimens, positive and negative controls, and allelic ladders. The intensity, color, and scan number of each peak detected for the sample are recorded in the electropherogram. The intensity of the peak corresponds to the amount of amplified DNA present; the scan number corresponds to the size of the fragment. AB GeneMapper® ID-X is an automated genotyping software program that converts the information contained in the electropherograms ("raw data") to a more readily usable format ("analyzed data").

The size of each fragment of amplified DNA is determined by comparison to the internal lane standard, GS600 v2.0, containing 35 fragments that range in length between 20 to 600 base pairs, are labeled with the LIZ™ dye, and are displayed as orange peaks in the electropherogram. GeneMapper® ID-X generates allelic designations from the sized data by comparison to the allelic ladder. In general, the GeneMapper® ID-X software recognizes the first allele of the ladder and creates approximately one base pair bins (+/- 0.5 base pair) around each allele in the ladder. Sample peaks are labeled by comparison of their size to the size of the ladder bins. The ladders are composed of the more common alleles in the general population. They also contain virtual bins for some less common alleles. If a sample peak falls into a bin or a virtual bin, an allelic designation will be assigned to the peak. However, if the peak is not represented by either a bin or a virtual bin created by the ladder, the GeneMapper® ID-X software will designate this peak as *OL* for "off-ladder" allele.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from the references cited below.

2.1. Applied Biosystems™ GeneMapper® ID-X Software Version 1.2 Reference Guide, 2009.
2.2. Applied Biosystems™ GlobalFiler™ PCR Amplification Kit User Guide, revision E, July 2016.
2.3. J.M. Butler, DNA separation methods, DNA detection methods, instrumentation for STR typing, and STR genotyping issues, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 313-388.
2.4. S.B. Klein, J.M. Wallin, M.R. Buoncristiani, Addressing the ambient temperature variation effects on sizing precision of AmpFlSTR® Profiler Plus™ alleles detected on the ABI Prism® 310 Genetic Analyzer, Forensic Sci. Comm. 5 (2003), available at:

https://archives.fbi.gov/archives/about-us/lab/forensic-science-communications/fsc/jan2003/klein.htm.

3. Equipment

3.1. Computer with GeneMapper® ID-X software

4. Safety/Quality Assurance

4.1. The positive control (Control DNA 007) for each amplification set-up must type correctly in order to use the associated samples. Refer to *ATF-LS-FB33 GlobalFiler™ STR Interpretation and STRmix™ Analysis Guidelines*for a discussion of positive controls.

4.2. Negative controls (negative amplification controls and reagent blanks) must be run and analyzed along with the associated samples. Refer to *ATF-LS-FB33 GlobalFiler™ STR Interpretation and STRmix™ Analysis Guidelines* for a discussion of negative controls.

4.3. At least one positive control, allelic ladder, and one negative control must be included in each GeneMapper® ID-X project.

4.4. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience, and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. Turn on the computer station and login.

5.2. If not performing data analysis on a computer directly attached to a 3130 or 3500 Genetic Analyzer, connect to that computer via the network and copy the run folder of interest to your computer.

5.3. Launch the GeneMapper® ID-X software and login.

5.4. Select *File >Add Samples to Project* from the menu. Find the run folder of interest, highlight that folder (or the sample files in the folder) and click the *Add to List* button. When all samples have been selected for the project, click the *Add* button.

5.5. In the tree pane of the GeneMapper® ID-X window, highlight the samples. Select *View > Raw Data* from the menu. Viewing the raw data may be useful in determining the start and stop points for the analysis range, the overall quality of the injection and electrophoresis, and whether or not artifacts are present.

5.6. Select *View > Samples* from the menu. Under the Samples tab, make sure that the correct *Sample Type* (sample, negative control, positive control, or allelic ladder), *Analysis Method* (GlobalFiler Evidence or GlobalFiler Ref*), Panel* (GlobalFiler_v1),

and *Size Standard* (GS600_LIZ_(60-460)) are selected. See Appendix A for further information.

5.7. Select *Analysis* > *Analyze* from the menu or click on the green triangle icon. A *Save Project* window will pop up. Type in the name of the project. The project name shall include at a minimum the date, the analyst's initials, and the instrument name. Be sure to save your project frequently when working with it.

5.8. After analyzing the data, look at the *Sizing Quality (SQ)* column at the far right hand side of the GeneMapper® ID-X window. If the icon in this column is yellow or red for any samples, verify that all of the GS600 v2.0 peaks for that sample fall within the range of analysis.

5.8.1. In the tree pane, select the sample that has a yellow/red SQ icon.

5.8.2. Select *View>Raw Data* from the menu.

5.8.3. Place the pointer at the 60 base pair (bp) peak and 460 bp peak, and note the number listed at each data point.

5.8.4. Select *View>Samples* from the menu.

5.8.5. Select *Analysis>Analysis Method Editor* from the menu (or use the bell curve icon).

5.8.6. Make sure that the *Start Point* number is less than the *Data Point* number for the 60 bp peak.

5.8.7. Make sure that the *Stop Point* number is greater than the *Data Point* number for the 460 bp peak.

5.8.8. If necessary, adjust the range of analysis in the *Analysis Method Editor* to encompass all peaks from 60 bp to 460 bp.

5.8.9. Alternatively, a different analysis method can be chosen that encompasses the necessary range. If there has been a significant migration shift due to a variation in the temperature of the room or for other reasons, it may be necessary to break up the run into separate analyses.

NOTE: All samples in a project shall be analyzed with the same method.

To choose a different analysis method:

5.8.9.1. Select *View>Samples* from the menu.

5.8.9.2. For the first sample, choose a method in the *Analysis Method* drop-down menu.

5.8.9.3. Click on the column heading *Analysis Method* to highlight the whole column.

5.8.9.4. Type *Ctrl-D* to copy down the analysis method to all samples.

5.8.9.5. Select *Analysis* > *Analyze* from the menu or click on the green triangle icon to re-analyze the samples.

5.9. Select *Analysis>Display Plots* from the menu. Choose the appropriate plot from the *Plot Setting* drop-down menu.

5.10. Verify that all of the peaks in the GS600 v2.0 size standard have been correctly assigned for each sample.

NOTE: Each project created from one run must contain, at a minimum, one allelic ladder, a correctly typed positive control, and a negative control in addition to the samples of interest. It may be necessary to re-inject samples along with an allelic ladder, positive control, and a negative control if these criteria cannot be met.

5.11. Ensure that the peaks of the allelic ladder are labeled with the correct allele designations. For guidance, see *ATF-LS-FB33 GlobalFiler™ STR Interpretation and STRmix™ Analysis Guidelines*.

5.12. While in the *Samples Plot* window, view each sample and examine the peak labels. It may be necessary to edit peak labels to account for things such as stutter, spectral pull-up, or spikes. For guidance, see *ATF-LS-FB33 GlobalFiler™ STR Interpretation and STRmix™ Analysis Guidelines*. In general, stutter peaks shall be removed from reference samples and shall not be removed from evidence samples.

5.13. To edit allele calls, peak labels must be displayed. In the *Samples Plot* window, select *View>Labels>Vertical Labels* to display peak labels. To edit a peak label, select the peak and then right-click the mouse.

5.14. The peak label options are described below. Choose the appropriate option by clicking on it.

If the peak is already labeled…	If the peak is not labeled…
Delete the allele call.	Add an allele call.
Rename the allele.	View the history.
View the history.	

5.15. If renaming or adding an allele call, choose the allele call or enter a custom name.

5.16. After making the necessary changes, enter an allele edit comment and click *Ok*. The following are common allele edit comments:

Stutter	Used for stutter peaks
Spike	Used for spikes
Artifact	Used for non-specific artifact peaks
Pull-up	Used for pull-up peaks
Minus A	Used for minus A peaks
Matrix	Used for raised baseline

NOTE: All allele edits (computer-generated or by hand) must be displayed on the electropherogram.

5.17. Print the analyzed data to be placed in each case file. See Appendix A for specifics on printing plots. In the *Samples Plot* window, select *File>Print*. The following data must be printed:

5.17.1. The GS600 v2.0 Size Standard Plot for all of the associated samples and controls within the project

5.17.2. The electropherogram for each case sample

5.17.3. The electropherograms for all allelic ladders, positive controls, and negative controls (reagent blanks and negative amplification controls) associated with the case samples (The electropherograms for unused wells, formamide blanks, do not need to be printed.)

5.17.4. The raw data for all negative amplification controls and reagent blanks

NOTE: If multiple cases are in the same GeneMapper® ID-X project, the data for the associated allelic ladders, positive controls, negative controls, GS600 v2.0 plots, and raw data will have to be printed for each case.

5.18. Print a table showing the list of samples within the project. In the GeneMapper® ID-X window, select the appropriate table from the *Table Setting* drop-down menu. Select *File>Print* from the menu. On the *Page Setup* tab in the *Print* screen, select the *Landscape* button. See Appendix A for specifics on printing the table.

5.19. Run information, including instrument settings, and data collection settings, will be printed for each case.

5.19.1. Highlight a positive control sample in the project and select *View>Sample Info* from the menu.

5.19.2. Print the sample info.

Appendix A

Panels and Bins Setup

Before using GeneMapper® ID-X for the first time, refer to the *Applied Biosystems™ GlobalFiler™ PCR Amplification Kit User Guide, Chapter 4* to set up the panels and bins.

Creating the Analysis Method

Select *Tools>GeneMapper® ID-X Manager* from the menu.

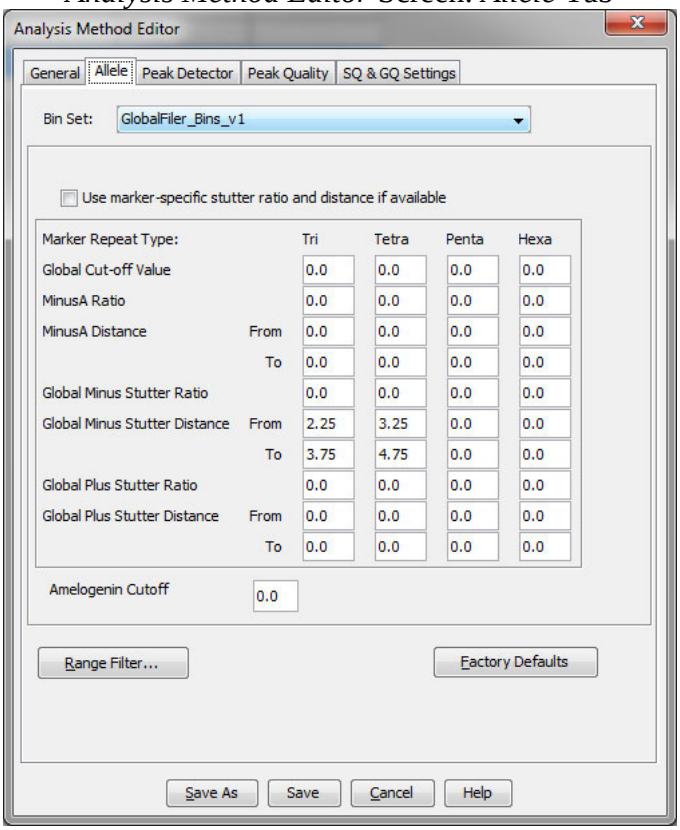
Select the *Analysis Methods* tab and click *New***.**

For *Select Analysis Type*, choose *HID***.**

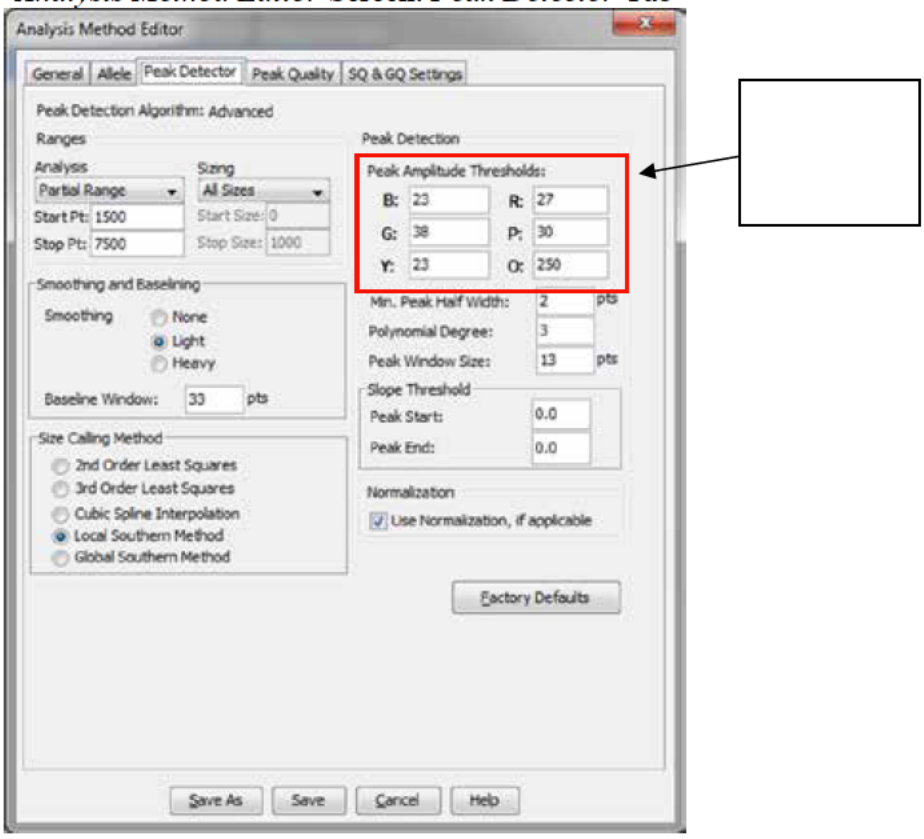
On the *Analysis Method Editor* screen, select the *General* tab and type in the method name.

On the *Analysis Method Editor* screen, for the *Allele* and *Peak Detector* tabs, make the following selections:

Analysis Method Editor Screen: *Allele* Tab

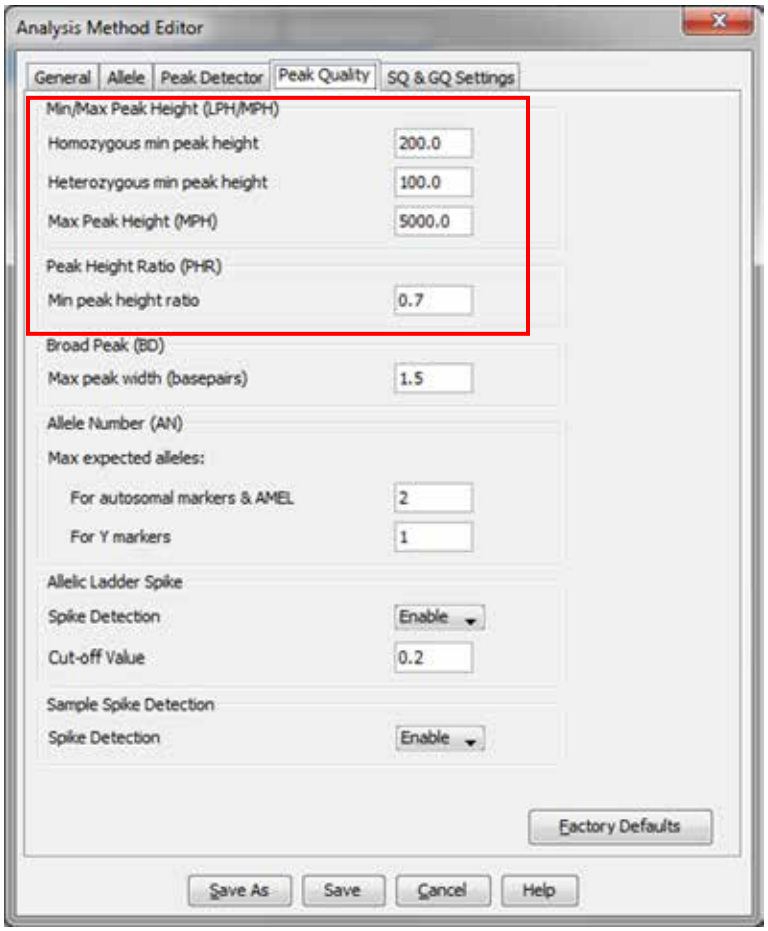


Analysis Method Editor Screen: *Peak Detector* Tab

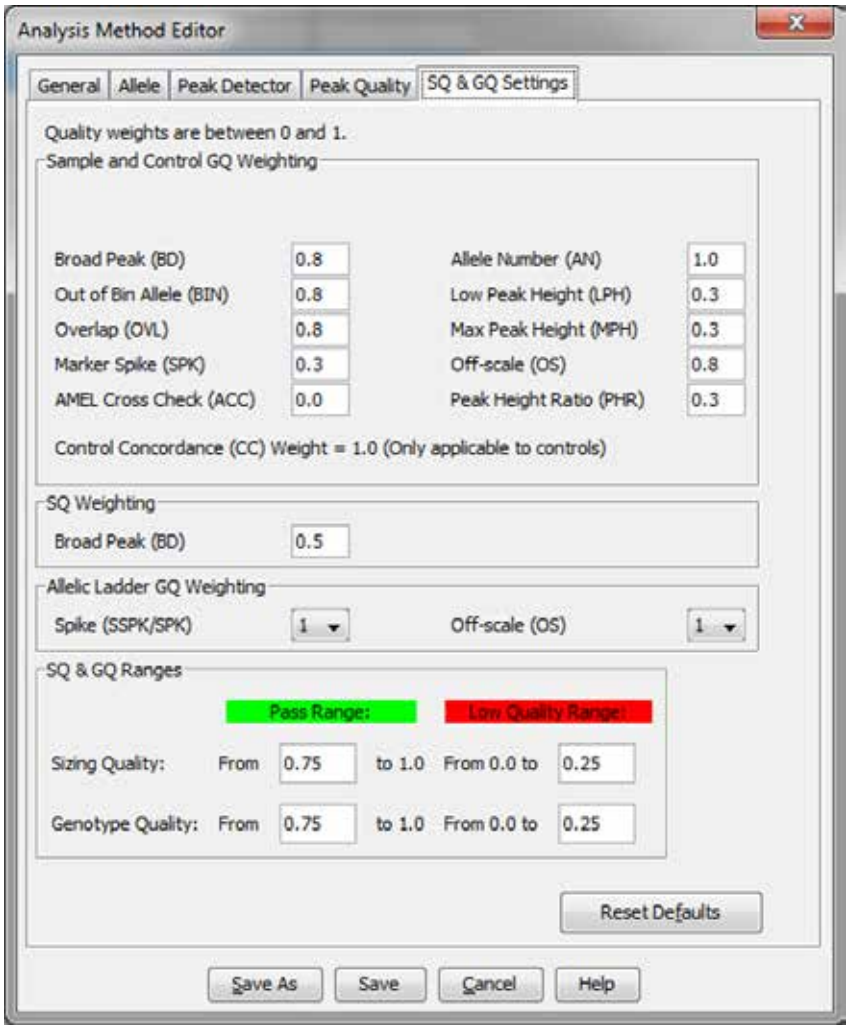


B: 23 rfu
G: 38 rfu
Y: 23 rfu
R: 27 rfu
P: 30 rfu
O: 250 rfu

For the *Peak Quality* tab, use the settings below. The settings in the box are not used since the data will be analyzed using STRmix to aid in the interpretation.



The default values for the *SQ & GQ Settings* tab can be used (shown below). These tools will not be used during interpretation.



Creating a Plot

The analyst may want to create several different plots to use during the analysis of samples. The following plots and plot settings are to be used when printing electropherograms for the case file.

Printing Samples Plot

Select *Tools>GeneMapper Manager*.

Choose the *Plot Settings* tab and click *New*.

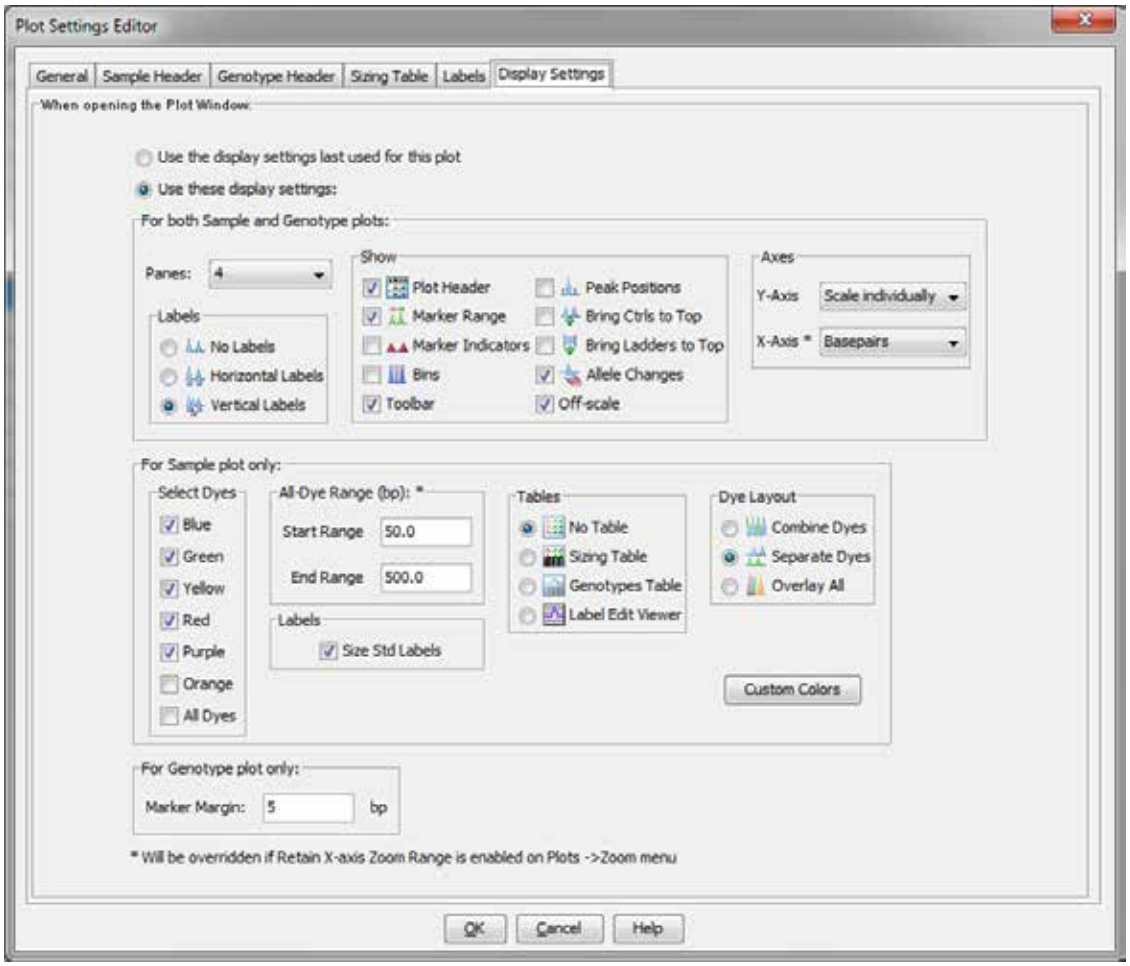
For the *General* tab, type in the name of the plot.

For the *Sample Header* tab, select only the *Sample File* and *Panel* boxes.

For the *Labels* tab, choose the following from the drop down menus:

Label 1	Allele Call
Label 2	Height
Label 3	Size
Label 4	AE Comment
Size	7 (or greater)

Use the default settings for the *Genotype Header* and *Sizing Table* tabs.
For the *Display Settings* tab, make the following selections:



Click *Ok* to close the *Plot Settings Editor*. Click *Done* to close *GeneMapper Manager*.

Printing Allelic Ladders Plot

Create a plot as described in the "Printing Samples Plot" section with the following exceptions:

- For the *Labels* tab, choose the following from the drop down menus:

Label 1	Allele Call
Label 2	None
Label 3	None
Label 4	None

Printing GS600 v2.0 LIZ Size Standard Plot

Create a plot as described in the "Printing Samples Plot" section with the following exceptions:

- For the *Labels* tab, choose the following from the drop down menus:

Label 1	Size
Label 2	None
Label 3	None
Label 4	None

- For the *Display Settings* tab, make the following changes:
  - Choose only the orange color
  - Deselect the *Show Off-scale* box

Printing Negative and Reagent Blank Plots

Create a plot as described in the "Printing Samples Plot" section with the following exceptions:

- For the *Display Settings* tab, make the following changes:
  - On the *Y-Axis* drop down menu, select "Scale to…" and then type in "100" as the value.
  - Deselect the *Show Off-scale* box.

Creating a Table

The following Table will be printed for each GeneMapper® ID-X project.

Project Samples Table

Select *Tools>GeneMapper Manager*.

Choose the *Table Settings* tab and click *New*.

For the *General* tab, type in the name of the table.

For the *Samples* tab, select only the following boxes:

- Sample File

- Sample Type
- Analysis Method
- Panel
- Size Standard
- Run Name

Use the default settings under the *Genotypes* tab.

Click *Ok* to close the *Plot Settings Editor*. Click *Done* to close *GeneMapper Manager*.

Contents

1. Scope 2
2. References 2
3. Equipment 4
4. Safety/Quality Assurance 5
5. Procedure 5
5.1 General DNA Profile Interpretation Steps 5
5.2 Evaluation of Controls 6
Verification of Internal Size Standard (GS-600 v2.0) 6
Verification of the GlobalFiler™ Allelic Ladder 6
Evaluation of Negative Controls 7
Evaluation of Amplification Positive Controls (Control DNA 007) 9
Single Injection/Capillary Events 10
5.3 Evaluation of Sample Profiles 11
Analytical Threshold (AT) 11
Stochastic Threshold 11
Off-Scale Peaks 12
Artifacts 13
Heterozygous Balance (Hb) 18
Preliminary Evaluation of Sample DNA Profiles 21
5.4 STRmix™ Analysis 25
Positive Control 25
Conditioning 25
Running STRmix 26
Reviewing STRmix™ Outputs 28
Comparisons and LR Calculations 32
Additional Guidelines on Interpretation and Comparison: 35
APPENDIX 36

1. Scope

The interpretation guidelines are intended to be a general guide for the evaluation and statistical analysis of the typing results for DNA amplified with the GlobalFiler™ PCR Amplification Kit and analyzed with the ABI 3130 Genetic Analyzer and GeneMapper® ID-X analysis software. The statistical analysis is performed, when necessary, using STRmix™ probabilistic genotyping software. The guidelines will ensure consistency in the evaluation of typing results and calculation of the statistical weight of the evidence between DNA analysts. These guidelines cannot encompass the entire range of samples or circumstances that will be encountered in forensic casework and exceptions may be made based on the analyst's training and experience. Significant deviations from the guidelines must be approved by the DNA Technical Leader. The guidelines are based on manufacturer's recommendations, manufacturer's user's manuals, internal validation studies, interpretation guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDAM), Department of Justice Uniform Language for Testimony and Reports, and the scientific literature.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

2.1. Applied Biosystems GeneMapper® *ID-X* Software Version 1.3 User Bulletin, revision A, October 2011.
2.2. Applied Biosystems GeneMapper® *ID-X* Software Version 1.0.1/1.1 Reference Guide, revision B, April 2009.
2.3. GlobalFiler™ PCR Amplification Kit User's Manual, revision E, July 2016.
2.4. Internal validation studies
2.5. STRmix™ Operation Manual
2.6. STRmix™ V2.4 User's Manual, October 2015.
2.7. ACME lab STRmix™ SOP, Institute of Environmental Science and Research Limited, 14 December 2015, obtained via the STRmix™ support website.
2.8. Oregon State Police GlobalFiler™: Interpretation and Reference Profile Comparison protocol.
2.9. J.M. Butler, DNA separation methods, DNA detection methods, and Instrumentation for STR typing, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 313-372.
2.10. J.M. Butler, Commonly used short tandem repeat markers and commercial kits, Biology of STRs, and Forensic issues, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 85–180.

2.11. A. Edwards, A. Civitello, H. Hammond, C.T. Caskey, DNA typing and genetic mapping with trimeric and tetrameric tandem repeats, Am. J. Hum. Genet. 49 (1991) 746–756.
2.12. H.A. Hammond, L. Jin, Y. Zhong, C.T. Caskey, R. Chakraborty, Evaluation of 13 short tandem repeat loci for use in personal identification applications, Am. J. Hum. Genet. 55 (1994) 175–189.
2.13. E. S. Mansfield, J. M. Robertson, M. Vainer, A. R. Isenberg, R. R. Frazier, K. Ferguson, S. Chow, D.W. Harris, D.L. Barker, P.D. Gill, B. Budowle, B.R. McCord, Analysis of multiplexed short tandem repeat (STR) systems using capillary array electrophoresis, Electrophoresis 19 (1998) 101-107.
2.14. P.S. Walsh, N.J. Fildes, R. Reynolds, Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA, Nucleic Acids Res. 24 (1996) 2807-2812.
2.15. B. Leclair, C.J. Fregeau, K.L. Bowen, R.M. Fourney, Systematic analysis of stutter percentages and allele peak height and peak area ratios for heterozygous STR loci for forensic casework and database samples, J. Forensic Sci. 49 (2004) 968-980.
2.16. T.M. Clayton, J.P. Whitaker, R. Sparkes, P. Gill, Analysis and interpretation of mixed forensic stains using DNA STR profiling, Forensic Sci. Int. 91 (1998) 55-70.
2.17. P.M. Schneider, R. Fimmers, W. Keil, G. Molsberger, Patzelt, W. Pflug, T. Rothamel, H. Schmitter, B. Brinkmann, The German Stain Commission: recommendations for the interpretation of mixed stains, Int. J. Legal Med. 123 (2009) 1-5.
2.18. J. Bright, J. Turkington, J. Buckleton, Examination of variability in mixed DNA profile parameters for the Identifiler multiplex, Forensic Sci. Int. Genet. 4 (2010) 111-114.
2.19. P. Gill, C.H. Brenner, J.S. Buckleton, A. Carracedo, M. Krawczak, W.R. Mayer, N. Morling, M. Prinz, P.M. Schneider, B.S. Weir, DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures, Forensic Sci. Int. Genet. 160 (2006) 90-101.
2.20. Scientific Working Group on DNA Analysis Methods, Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories, 2017.
2.21. B. Budowle, A.J. Onorato, T.F. Callaghan, A.D. Manna, A.M. Gross, R.A. Guerrieri, J.C. Luttman, D.L. McClure, Mixture interpretation: Defining the relevant features for guidelines for the assessment of mixed DNA profiles in forensic casework, J. Forensic Sci. 54 (2009) 810-821.
2.22. J. Bright, D. Taylor, C. McGovern, S. Cooper, L. Russell, D. Abamo, J. Buckleton, Developmental validation of STRmix™, expert software for the

interpretation of forensic DNA profiles, Forensic Sci. Int. Genet. 23 (2016) 226-239.

2.23. D. Hares, Expanding the CODIS core loci in the United States, Letter to the Editor; Forensic Sci. Int. Genet. 6 (2012) e52-e54.

2.24. D. Hares, Selection and implementation of expanded CODIS core loci in the United States, Letter to the Editor; Forensic Sci. Int. Genet. 17 (2015) 33-34.

2.25. V. Bogasa, M. Carvalho, F. Corte-Realc, M.J. Portoa, Testing the behavior of GlobalFiler PCR amplification kit with degraded and/or inhibited biological samples, Forensic Sci. Int. Genet. Suppl. Ser. 5 (2015) e21-e23.

2.26. D. Taylor, J. Bright, C. McGoven, C. Hefford, T. Kalafut, J. Buckleton, Validating multiplexes for use in conjunction with modern interpretation strategies, Forensic Sci. Int. Genet. 20 (2016) 6-19.

2.27. J. Bright, D. Taylor, J. Curran, J. Buckleton, Developing allelic and stutter peak height models for a continuous method of DNA interpretation, Forensic Sci. Int. Genet. 7 (2013) 296–304.

2.28. C. Brookes, J. Bright, S. Harbison, J. Buckleton, Characterising stutter in forensic STR multiplexes, Forensic Sci. Int. Genet. 6 (2012) 58-63.

2.29. H. Kelly, J. Bright, J. Curran, J. Buckleton, Modelling heterozygote balance in forensic DNA profiles, Forensic Sci. Int. Genet. 6 (2012) 729-734.

2.30. H. Haned, L. Pene, J. Lobry, A. Dufour, D. Pontier, Estimating the number of contributors to forensic DNA mixtures: Does maximum likelihood perform better than maximum allele count? J. Forensic Sci. 56 (2001) 23-28.

2.31. J. Buckleton, J. Bright, D. Taylor, I. Evett, T. Hicks, G. Jackson, J. Curran, Helping formulate propositions in forensic DNA analysis, Science and Justice 54 (2014) 258-261.

2.32. D. Taylor, J. Buckleton, I. Evett, Testing likelihood ratios produced from complex DNA profiles, Forensic Sci. Int. Genet. 16 (2015) 165-171.

2.33. Department of Justice Uniform Language for Testimony and Reports for Forensic Autosomal DNA Examinations Using Probabilistic Genotyping Systems, adopted 9/18/18, effective 3/18/19.

2.34. T. Moretti, R. Just, S. Kehl, L. Willis, J. Buckleton, J. Bright, D. Taylor, A. Onorato, International validation of STRmix™ for the interpretation of single source and mixed DNA profiles, Forensic Sci. Int. Genet. 29 (2017) 126-144.

2.35. Bille T, Weitz S, Buckleton JS, Bright J-Anne, Interpreting a major component from a mixed DNA profile with an unknown number of minor contributors, Forensic Sci Int: Genet (2019), https://doi.org/10.1016/j.fsigen.2019.02.017.

3. Equipment

3.1. Computer with GeneMapper® ID-X analysis software

3.2. STRmix™ v2.4 software

4. Safety/Quality Assurance

4.1. Safety: Not applicable

4.2. Quality Assurance: See Procedure section

5. Procedure

5.1 General DNA Profile Interpretation Steps

Evaluation of standards, allelic ladders, and controls.

Evaluation of evidence sample DNA profiles.

Analysis and/or deconvolution of DNA profiles using STRmix™ software.

Analysis of known DNA sample profiles.

Comparing known DNA profiles to evidentiary DNA profiles.

Calculation of the statistical weight (likelihood ratio) when an individual cannot be excluded as a possible contributor to an evidentiary DNA profile, except when noted below.

All profiles suitable for comparison purposes and meeting the minimum qualifications to be entered into CODIS will be compared to the Staff Index, Profiles Generated Index, and LDIS database prior to being reported as a way of detecting possible contamination. See *CODIS Manual* for instructions on entering and searching profiles in CODIS.

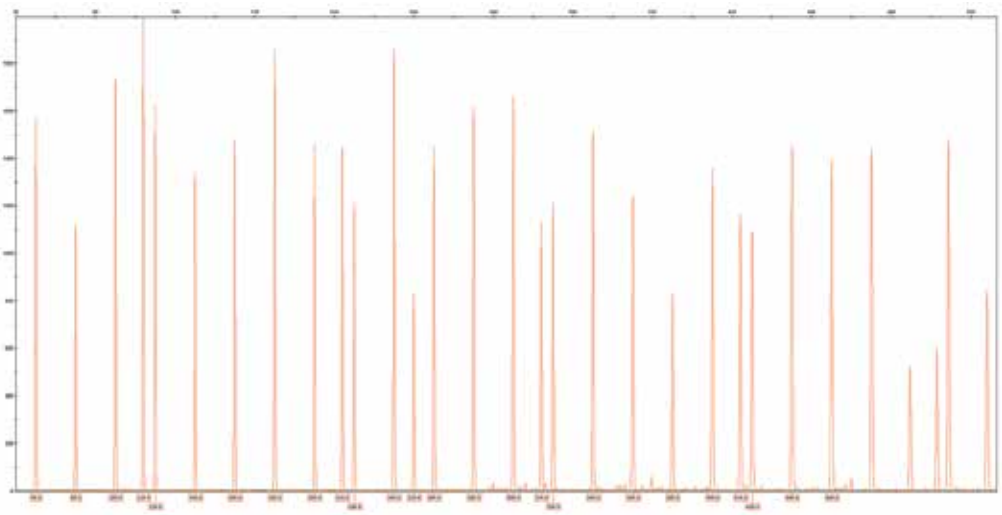
The results of the DNA analysis are then detailed in a laboratory report.

5.2 Evaluation of Controls

Verification of Internal Size Standard (GS-600 v2.0)
The Internal Size Standard (GS-600 v2.0, LIZ dye labeled) consists of 36 peaks of known base pair (bp) size ranging from 20bp to 600bp.

All Internal Size Standard peaks ranging from 60 to 460 base pairs must be present, have the expected peak morphology and peak balance across the range of sizing, and be labeled correctly, with peak heights greater than 250 relative fluorescent units (RFU) for the associated profile to be acceptable.

Extraneous peaks observed in the Internal Size Standard can be disregarded as long as they are not incorrectly labeled as peaks of the Internal Size Standard.



Typical Internal Size Standard (GS-600 v2.0)

Internal Size Standard (GS-600 v2.0) peaks necessary for the analysis of ABI GlobalFiler™ amplified products	
Base Pair (bp) Sizes	60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, and 460

Verification of the GlobalFiler™ Allelic Ladder
The presence of all alleles of the allelic ladder must be verified. Allelic ladders missing one or more alleles or containing allele peaks that are marked off-ladder are not valid.

Sample profiles must be associated with one or more valid allelic ladders to be considered for interpretation.

The Sample Type of allelic ladders that are found not to be valid may be changed from "Allelic Ladder" to "Sample." If this is done, the project must be re-analyzed.

Evaluation of Negative Controls

Reagent Blank

The reagent blank is a negative control initiated at the extraction step and is processed in parallel with the associated evidentiary sample(s). It consists of all the reagents used during the extraction. The reagent blank is used to detect DNA contamination from the reagents, disposables, or environment that may affect the evidentiary samples. Contamination detected in the reagent blank may be systemic in nature or a randomly occurring event. If contamination is detected, an effort should be made to identify the source to prevent future contamination events.

Amplification Negative Control

The amplification negative control is a negative control initiated at the amplification step and consists of all reagents used in the amplification, except TE^{-4} is used in place of template DNA.

An acceptable negative control will contain no peaks above the analytical threshold.

A negative control containing a peak above the analytical threshold between 60 and 460bp which is believed to be an artifact such as a spike or spectral pull-up from the internal size standard shall be re-injected. If, upon re-injection, no peaks above the analytical threshold are present, the negative control is acceptable. Artifacts outside the 60 to 460bp window are not significant and do not necessitate re-analysis of the control. If the issue is determined to be sample-specific (e.g. a spike) and not systemic, the associated samples do not need to be re-analyzed as long as there was at least one valid negative control in the run. If the issue is determined to be potentially systemic, the associated samples must be re-analyzed (re-plated).

If at least one non-artifact peak above the analytical threshold or a recognizable pattern of peaks below the threshold is confirmed to be present through re-injection (re-plated) in the negative control, this will be considered a contamination event (see further below for further guidance on the detection of contamination). If the non-artifact peak or recognizable pattern of peaks below the threshold are not present in the re-injection, all associated samples must be re-analyzed (re-plated) unless the issue is determined to be sample-specific and there was at least one valid negative control in the original run.

A printout of the negative control raw data demonstrating the presence of the primer peaks will be included with the case material along with a printout of the electropherogram with a maximum Y-axis of 100 RFU.

Re-analysis of a Reagent Blank

Based on the results of the other controls (e.g. amplification blank, positive control, etc.) associated with the evidentiary samples, the re-analysis of a reagent blank may initially consist of re-injection, re-amplification, or re-extraction of all associated samples.

All re-injections must contain a valid positive control, negative control, and ladder at a minimum, unless the re-injection(s) is added to a run in progress.

If it is not possible to re-extract a sample associated with a contaminated reagent blank and if the sample results differ from adventitious DNA observed in the control sample, the original typing results will be reported following the standard protocol.

- A note must be added to the report describing the contamination event if the interpretation of the evidentiary profile is affected (see *Report Wording Protocol*).
- If the DNA profile obtained from the evidence sample is consistent with the contaminating profile in the reagent blank, then the evidentiary sample is not suitable for comparison purposes.
- In the event of low-level contamination/drop-in, if a peak in an evidentiary profile cannot be distinguished from a contaminating peak, the locus should be evaluated to determine if the evidentiary locus/profile is potentially affected by the contamination. If not, the evidentiary locus/profile can be used for comparison purposes. If the evidentiary locus/profile is potentially affected by the contamination, the locus shall be ignored for statistical purposes, but may be used for exclusionary purposes with approval by the DNA Technical Leader.

If a reagent blank associated with a set of samples to be re-amplified cannot be re-amplified due to limited volume, and it was determined to be valid from a previous analysis (at the same level of sensitivity), the samples associated with the reagent blank can still be analyzed.

Re-analysis of a Negative Amplification Control

The re-analysis of a negative amplification control may consist of re-injection, or re-amplification of all associated samples with a new amplification negative control.

All re-injections must contain a valid positive control, negative control, and ladder at a minimum, unless the re-injection(s) is added to a run in progress.

If it is not possible to re-amplify an evidentiary sample and if the sample results differ from adventitious DNA observed in the control sample, the original typing results will be reported following the standard protocol.

- A note must be added to the report describing the contamination event if the interpretation of the evidentiary profile is affected (see *Report Wording Protocol*).
- If the DNA profile obtained from the evidence sample is consistent with the contaminating profile in the control sample, then the evidentiary sample is not suitable for comparison purposes.
- In the event of low-level contamination/drop-in, if a peak in an evidentiary profile cannot be distinguished from a contaminating peak, the locus should be evaluated to determine if the evidentiary locus/profile is potentially affected by the contamination. If not, the evidentiary locus/profile can be used for comparison purposes. If the evidentiary locus/profile is potentially affected by the contamination, the locus shall be ignored for statistical purposes, but may be used for exclusionary purposes with approval by the DNA Technical Leader.

Evaluation of Amplification Positive Controls (Control DNA 007)

The amplification positive control is initiated at the amplification step and is processed in parallel with the evidentiary samples. It consists of all the reagents used in the amplification reaction and template DNA of a known profile (Control DNA 007). The amplification positive control monitors the amplification reaction and ensures the reliability of the results of the associated evidentiary samples.

The typing results for the positive control should match the expected profile below.

Locus	Genotype	Locus	Genotype
D3S1358	15,16	D19S433	14,15
vWA	14,16	TH01	7,9.3
D16S539	9,10	FGA	24,26
CSF1PO	11,12	D22S1045	11,16
TPOX	8, 8	D5S818	11,11
Y Indel	2	D13S317	11,11
Amelogenin	X,Y	D7S820	7,12
D8S1179	12,13	SE33	17,25.2
D21S11	28,31	D10S1248	12,15
D18S51	12,15	D1S1656	13,16
DYS391	11	D12S391	18,19
D2S441	14,15	D2S1338	20,23

Expected typing results for Control DNA 007

If the allelic peak heights are greater than the relevant thresholds, match the expected profile, and generally fall within the expected heterozygous balance, the positive control is valid. On rare occasions, the heterozygote balance may fall outside expectations. The positive control is still valid in this situation. If multiple loci demonstrate greater than expected imbalance, this may be an indication of an issue with amplification or capillary electrophoresis.

If the typing results do not match the expected profile (incorrect profile, additional alleles due to possible contamination or partial/no results), the positive control is not valid and all evidentiary samples associated with the positive control shall be re-analyzed. If there is a valid positive control within the run, the associated samples may not have to be re-analyzed. Contamination in a positive control should be evaluated to determine if it is a sample-specific issue or possibly a systemic issue. If it is possibly a systemic issue, the associated samples shall be re-analyzed, where possible.

The re-analysis may consist of re-injection or re-amplification.

If it is not possible to re-amplify an evidentiary sample associated with a positive control sample displaying contamination that cannot be resolved by re-injection or re-plating and if the results differ from the adventitious DNA observed in the control sample, the original typing results will be reported following the standard protocol.

If it is not possible to re-amplify an evidentiary sample associated with a positive control sample displaying a partial profile or no profile and the issue cannot be resolved by re-injection or re-plating, the original typing results will be reported following the standard protocol if the analyst can reasonably assume the amplification of the associated samples was not affected.

- A note must be added to the report describing the issue if the interpretation of the evidentiary profile is affected (see *Report Wording Protocol*).
- If the DNA profile obtained from the evidence sample is consistent with a contaminating profile in the control sample, then the evidentiary sample is not suitable for comparison purposes.

Single Injection/Capillary Events

In general, if a single capillary or single injection event occurs that results in a poor injection (as evidenced by the ILS peaks) the sample or samples specifically affected may be re-injected; the entire run does not have to be re-injected and analyzed. Each run must have a valid positive control, allelic ladder, and negative control if the samples are set-up as a new analysis. If additional injections are added at the end of the current analysis (prior to the end of all the injections associated with the plate), no additional positive control sample, allelic ladder, or negative control samples must be added. Alternatively, samples can be re-injected from the original plate without re-plating within 24 hours of

the initiation of the first injection, but must include a positive control, negative control, and a ladder.

5.3 Evaluation of Sample Profiles

Analytical Threshold (AT)
The Analytical Threshold is the RFU value that, when exceeded by peaks that display the expected peak morphology, allows those peaks to be considered "real" products of amplification.

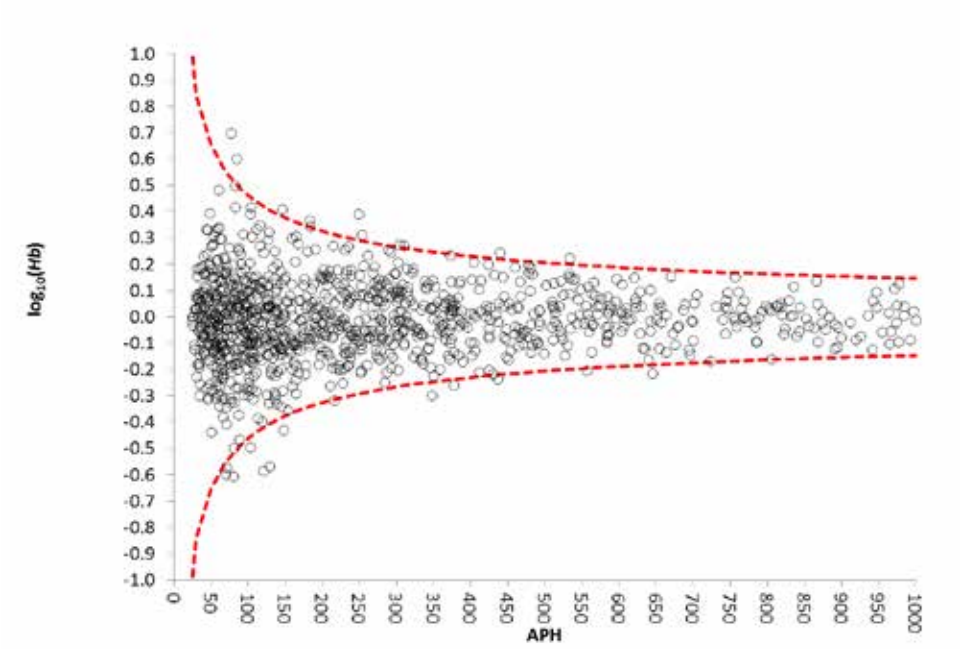
Analytical Thresholds are dye-specific:

Dye Channel	Analytical Threshold
Blue (6-FAM™)	23
Green (VIC™)	38
Yellow (NED™)	23
Red (TAZ™)	27
Purple (SID™)	30
Orange (LIZ™)	250

Stochastic Threshold
Due to the inherent nature of PCR amplification, the expected heterozygous balance will decrease as the levels of template DNA decrease. The two dashed red lines in the graph below represent the 95% confidence interval for the heterozygous balance data generated during validation. At higher levels of template DNA (average peak height of 1000 RFU) the expected heterozygous balance ranges from approximately 70% to 140%. At lower levels of template DNA (average peak height of 150 RFU) the expected heterozygous balances range from approximately 40% to 250%. Dramatic heterozygous peak height imbalance at the lower levels of template DNA may result in allele drop-out.

While a Stochastic Threshold has not been set due to the use of STRmix™ software to aid in interpretation, the graph below can be used to estimate the expected heterozygous balance when evaluating a DNA profile.

Graph: Expected Heterozygous Balance



Expected Heterozygous Balance: Data generated during the validation of GlobalFiler™ PCR Amplification Kit and STRmix™ software. The dashed red lines represent the 95% confidence interval.

Off-Scale Peaks

An Off-Scale Peak is a peak that exceeds the linear dynamic range of the instrument. Off-Scale Peaks may result in raised baselines and/or excessive "pull-up" in one or more colors.

Analyzed data from Off-Scale Peaks should not be used for quantitative comparisons since the true quantity of signal is not known. For example, the use of Off-Scale Peaks can result in disproportionately high stutter calculations.

The Saturation level has been set at 7000 RFU. DNA profiles containing peaks greater than 7000 RFU should not be analyzed using STRmix™.

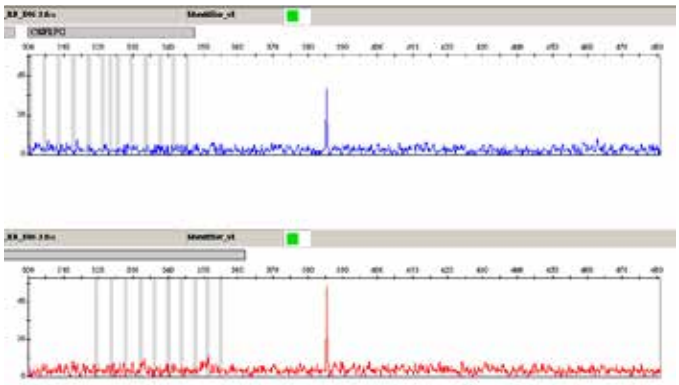
Sample profiles containing Off-Scale data may be re-analyzed at the discretion of the analyst. Re-analysis may consist of adding less amplified product to the ABI 3130 set-up tray, diluting the amplified product in formamide prior to adding it to the 3130 set-up tray, or re-amplifying the sample. Re-amplification is preferred and may be done at the analyst's discretion.

Artifacts

Spikes

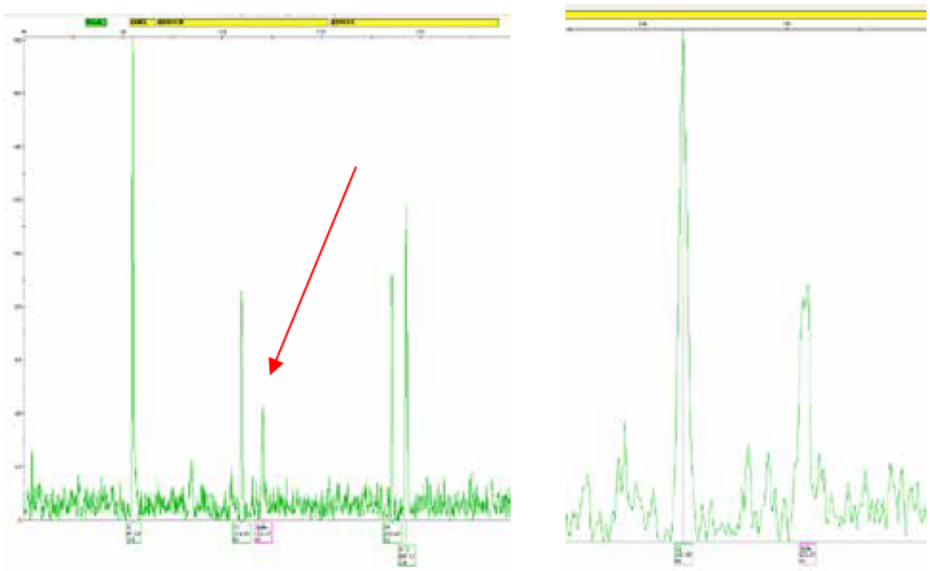
A spike is defined as a non-specific, non-reproducible, and non-allelic peak that is typically observed in one or more colors and is an artifact of the electrophoresis and signal detection. A spike does not typically display the same peak morphology as an allelic peak with respect to peak width, starting slope and ending slope.

If a spike is present in the analytical range (60 – 460 bp), the spike may be confirmed to be an artifact by re-injection if it interferes with interpretation. Upon re-injection, the same spike should not be present.



Example of a spike exhibiting the typical characteristics (narrow peak width, detected in multiple channels, similar peak height in different channels).

Occasionally, a peak may be labeled as a spike. Upon closer examination, the peak will have the expected allelic peak morphology and fall within an allelic bin. However, the top of the peak will be indented (not rounded). This is due to spectral pull-down (a peak in another dye channel is negatively affecting the signal of the current channel). If the peak is determined to be an allele, the label can be edited to reflect the appropriate allele call.



Example of allelic peak initially labeled as "spike".

Non-Template Nucleotide Addition

Some polymerases have the inherent property of non-template directed nucleotide addition. This nucleotide addition is reverse primer dependent. In extreme conditions (such as the addition of excessive template DNA), a portion of the amplified product will not contain the additional nucleotide, resulting in a fragment one base pair shorter than the expected allele. Since the nucleotide typically added is adenine (A), the peak representing amplified product in which the non-template directed nucleotide addition did not occur is oftentimes referred to as the –A peak. This should not be confused with true alleles that are one base shorter than full repeat alleles (e.g. D1S1656 15.3/16).

Stutter

Stutter peaks are an artifact of the Polymerase Chain Reaction when amplifying repeat regions of DNA. The primary peak is representative of the actual repeat number contained in the template DNA. The stutter peak is an artifact usually one repeat shorter in length than the primary peak (reverse stutter). For tetrameric repeats, the reverse stutter peak is n-4 base pairs where n is the base pair length of the primary peak. Forward stutter (n+4) may also be detected, but typically at a lower level than the reverse stutter. Forward stutter and n-2 stutter are more prevalent at some loci, such as SE33.

Stutter peaks can be characterized both by their size and by their peak height proportion when compared to the associated primary peak, expressed as the percentage of the stutter peak height compared to the primary peak. The average stutter ratios for each locus will vary and have been experimentally defined at the ATF Laboratory. These values are the

values used by STRmix. The values were calculated using one of three methods: the linear regression (allele X slope + intercept); the longest uninterrupted sequence; or the average per allele. For general evaluation purposes, the table below lists the maximum stutter percentages expected for each locus.

Locus [1]	% Stutter
CSF1PO	8.77
D10S1248	11.46
D12S391	13.66
D13S317	9.19
D16S539	9.48
D18S51	12.42
D19S433	9.97
D1S1656	12.21
D1S1656 (−2 nt)	2.45
D21S11	10.45
D22S1045	16.26
D22S1045 (+3 nt)	6.69
D2S1338	11.73
D2S441	8.10
D3S1358	10.98
D5S818	9.16
D7S820	8.32
D8S1179	9.60
DYS391	7.43
FGA	11.55
SE33	14.49
SE33 (−2 nt)	3.97
TH01	4.45
TPOX	5.55
vWA	10.73

[1] These percentages are used as stutter filters in GlobalFiler_stutter.txt. GlobalFiler PCR Amplification Kit User Guide, rev E, July 2016.

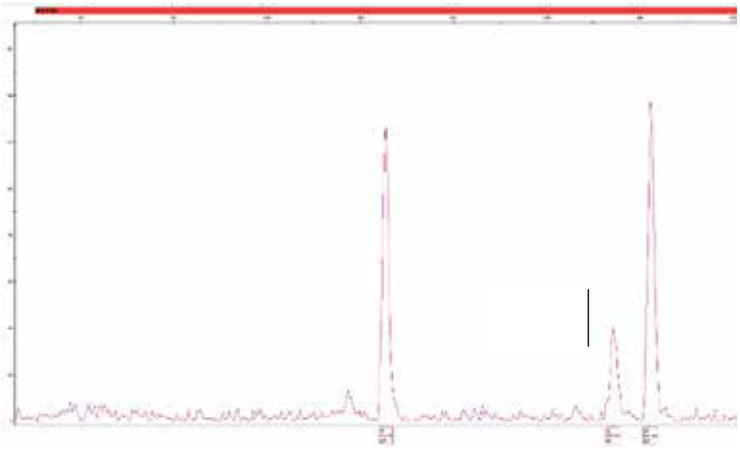
Under some circumstances, stutter peaks may exceed the maximum expected stutter percentages. These peaks may be considered stutter at the analyst's discretion based on the specific circumstances.

- Stutter peak calculations associated with off-scale allele peaks will not be reflective of the true percentage.
- Stutter peaks between two alleles that differ by two repeats (e.g. 15 and 17 allelic peaks with a stutter peak at the 16 position) may exceed the maximum expected

stutter percentages due to the n-4 contribution from the 17 allele and the n+4 contribution from the 15 allele.

- Stutter peak percentages may increase at lower levels of input template DNA.

Minor peaks cannot be absolutely identified as stutter peaks even though they meet both the size and stutter peak percentage criteria. If there is no other indication of a mixture in a profile, minor peaks meeting the stutter peak criteria can be attributed to stutter, however, these peaks should be left in for STRmix™ analysis. If evidence of a mixture is observed in a profile, minor peaks falling within the stutter peak criteria should be taken into consideration during the interpretation.



Example of increased stutter at D2S1338.

Matrix Failure (Spectral Pull-Up)

Matrix Failure, also known as spectral pull-up, results in peaks from one color "bleeding" into another color. The wavelength ranges emitted by the fluorescent tags overlap. The software is designed to use a matrix algorithm to separate the fluorescent signal given off by each of the fluorescent tags. This separation is not always complete, resulting in minor peaks being detected in the adjacent spectral colors. These pull-up peak heights are typically less than 5% of the peak height of the source peak. The pull-up peak is usually sized within ±0.25 base pairs of the source peak.

If pull-up peaks are observed in a sample, the sample may be re-analyzed at the analyst's discretion based on the specific circumstances. It may be necessary to perform a new Spectral Calibration prior to re-analysis if spectral pull-up is excessive.

If pull-up peaks are present in a mixed DNA profile, it may not be possible to distinguish a possible pull-up peak from a minor contributor allele. If a peak is observed at a locus that falls within the expected parameters of a pull-up peak and is at a level similar to one

or more minor contributor peaks, the *Ignore Locus* function in STRmix™ may be used for this locus at the analyst's discretion.

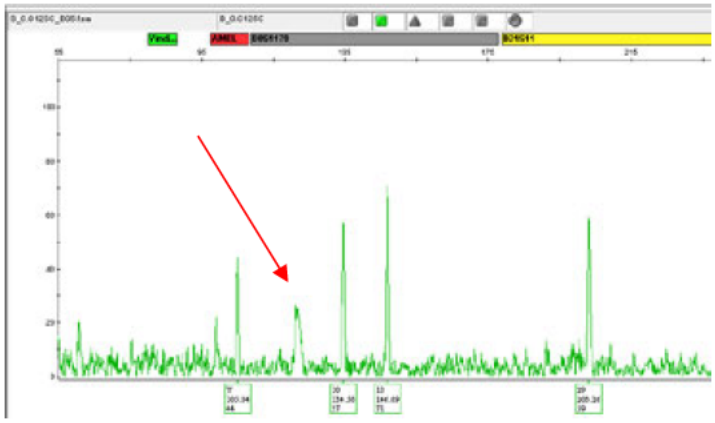
Raised Baseline

Raised baseline may be observed in samples containing excess template DNA or due to instrument failure. The raised baseline appears as non-specific elevation along the x-axis of the electropherogram.

If excess raised baseline is observed in a sample, the sample may be re-analyzed at the analyst's discretion.

Other Artifacts

Other peaks that do not represent genetic data from the template DNA may be observed in the sample electropherogram. These peaks or series of peaks may result from dissociated fluorescent dyes or other causes due to the instrument, capillary electrophoresis, or amplification kit. For example, a series of peaks is routinely observed between 115 and 135bp in the green dye (VIC™) channel.



Example of known, recurring artifact in the green (VIC™) channel.

Off-Ladder Peaks

Most common alleles are represented by the allelic ladder or virtual bins set by the software. Occasionally, non-artifact peaks will be observed outside of the allele bins and will be labeled as "OL." Off-ladder alleles may result from temperature fluctuations in the instrument's environment during the course of the electrophoresis. The change in temperature may cause a migration shift of allelic peaks that consequently fall outside the ±0.5 base pair bins even though the alleles are represented in the ladder. Micro-variant and rare variant alleles may also be observed that fall between alleles within a locus or the range of alleles between two loci.

Micro-variant and rare variant alleles that are off-ladder shall be re-injected to confirm the sizing unless the DNA profile is determined to be not suitable for comparison purposes.

Off-ladder (OL) allelic peaks observed between alleles within a locus should be labeled as a variant of the smaller flanking allele. If the OL peak is approximately one base pair longer than the smaller flanking allele (X), the OL peak will be designated as X.1. If the OL peak is approximately two base pairs longer than the smaller flanking allele, it will be designated as X.2. If the OL peak is approximately three base pairs longer than the smaller flanking allele, it will be designated as X.3. For example, an OL peak falling between the 8 and 9 alleles that is approximately two base pairs longer than the 8 allele would be designated as an 8.2.

An OL allelic peak that falls between the allele range of two loci first should be associated with one of the loci and then be labeled as described below.

> To associate the OL peak with one of the flanking loci, the alleles present in the flanking loci along with the base pair sizes of the alleles need to be considered.
> If one of the flanking loci contains two alleles and the other locus contains one, the OL peak will be associated with the locus that contains one.
> If both loci contain one allele, the base pair size of the OL peak should be compared to the base pair sizes of the flanking allelic ladders. If the OL peak is a "perfect repeat" of one of the allelic ladders, it will be associated with that locus. If it is not a "perfect repeat" of either flanking allelic ladder, then both loci may only be used for exclusionary purposes.
> If the OL peak has been associated with a locus, it will then be designated as "<" or ">" the first or last allele of the associated allelic ladder at that locus.

Some OL peaks may be the result of the presence of non-human DNA or other artifacts of the PCR. In these circumstances, the analysis of these samples will be evaluated on a case to case basis.

Heterozygous Balance (Hb)

Expected Variation in Heterozygous Balance

It is not unusual for PCR to produce imbalanced peak heights for heterozygous loci. As the amount of template DNA decreases, the imbalance may become more dramatic. The Expected Heterozygous Balance graph from above displays the log Hb derived from data generated during the internal validation. The data points represent the average peak height at the locus vs the log Hb for the heterozygous pair. The balance is calculated as a ratio of the smaller allele (repeat number) as compared to the larger allele (repeat number). The dashed red-lines represent the 95% confidence interval. At higher levels of

template DNA, for example an average peak height of 1000 RFU, the expected heterozygous balance ranges from approximately 72% to 140%. At lower levels of template DNA, for example an average peak height of 150 RFU, the expected heterozygous balances range from approximately 42% to 238%. Dramatic heterozygous peak height imbalance at the lower levels of template DNA may result in allele drop-out.

Expected Hb based on the average peak height (APH) can be calculated by:

$$\text{Log Hb} = \pm\sqrt{2} \times 1.96 \times \sqrt{\frac{c^2}{\text{APH}}}$$

Where c^2 (2.760) is the 95th percentile of the gamma distribution for alleles from Model Maker in STRmix™.

For heterozygous loci with alleles separated by relatively few repeats, the imbalance is not significantly affected by allele size. Hence, the imbalance will affect the larger allele as much as the smaller allele. Therefore, if a sample is amplified multiple times using the same template quantity, the imbalance may vary and may favor the larger allele one time and the smaller allele another time.

For heterozygous loci with alleles separated by a larger number of repeats (such as the locus FGA), the imbalance may be greater than expected and may favor the smaller allele.

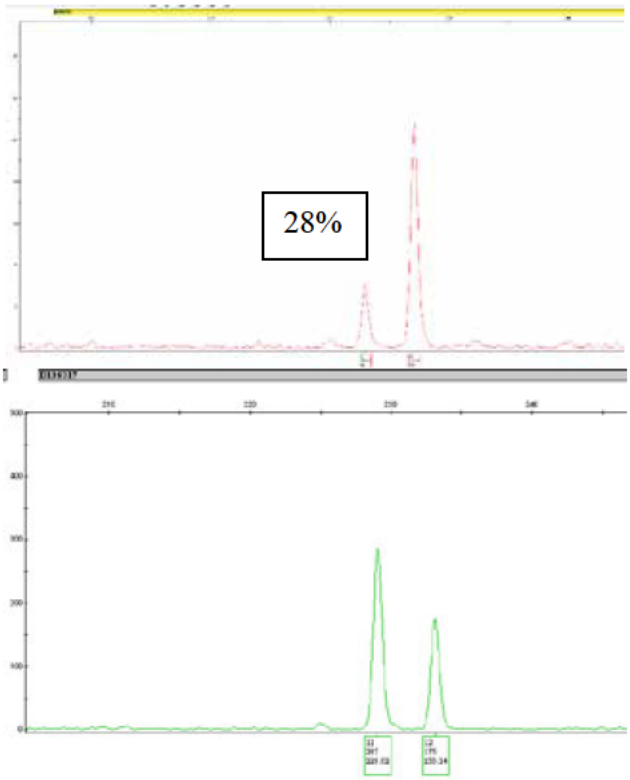
Peak Height Imbalance due to Primer Site Mutation

A sequence mutation in the primer region may have one of three effects. If the mutation causes significant destabilization, the allele will not be amplified and therefore not detected (null allele). If the mutation does not affect the stability of the primer binding, no effect will be observed. If, however, the mutation causes some destabilization of the primer binding but not to the extent to prevent amplification, the resulting peak balance at the locus may be outside the expected range. This form of imbalance is reproducible.

If a similar imbalance is observed in an evidence sample and a reference sample, this is an indication that a primer binding site mutation is the cause. After the initial deconvolution, the use of the *Ignore Locus* function in STRmix™ may be justified in this instance.

If the peak height imbalance falls outside the expected range at a single locus in an assumed single source profile, several causes should be considered:

- Expected Hb is based on a 95% confidence interval, therefore it is predicted that some values will fall outside of expectations.
- Hb has greater variation at low levels of template DNA.
- The Hb may be affected by a primer binding site mutation.
- A second, low-level contributor may be present.



Example of Heterozygous Balance (Hb) outside the expected range at D13S317. The top electropherogram was generated by amplification with the GlobalFiler™ PCR Amplification Kit. The bottom electropherogram was generated by amplification with the Identifiler™ PCR Amplification Kit.

Expected Peak Height Balance in Mixed DNA Profiles

The Expected Heterozygous Balance graph shown previously displays the expected Hb for single source samples. If a DNA profile is the result of DNA from multiple contributors, the additive effects of allele sharing must be considered.

Composite Profiles

In some instances, it may be useful to re-inject or re-amplify a sample to obtain interpretable data for additional loci. Combining data from multiple injections/amplifications resulting from a single DNA extract is called a composite

profile. For example, if a sample is severely degraded, the initial amplification using 1ng of template DNA may have produced interpretable data at a few of the smaller molecular weight loci. However, when the same DNA extract was re-amplified using 2ng of template DNA, additional interpretable data was obtained from a few of the higher molecular weight loci. The smaller molecular weight loci in the second amplification are now not interpretable due to saturation.

Composite profiles and replicate analysis with STRmix™ are not validated for use at this time.

Preliminary Evaluation of Sample DNA Profiles

The evidence profile shall be evaluated prior to the analysis of reference samples, where feasible. This will not be possible when additional evidence has been submitted after previous DNA analyses, including the analysis of references. However, it is imperative that the evidence profile is evaluated independently of any reference profiles.

The entire DNA profile should be evaluated to determine if the profile is suitable for comparison purposes and to determine the assumed number of contributors.

At a minimum, a DNA profile must have sufficient data to be able to reasonably determine the number of contributors for the profile to be considered suitable for comparison purposes.

If the DNA profile is the result of four or more contributors, the mixed DNA profile is not suitable for comparison purposes except as described below in the "Discerning a Major Component" section.

Possible allelic peaks below the Analytical Threshold may be considered when evaluating a DNA profile.

Number of Contributors

The assumed number of contributors must be determined for DNA profiles to be suitable for comparison purposes.

Peaks below the Analytical Threshold may be used to aid in the determination of the number of contributors, as it affects subsequent interpretation.

In general, the maximum number of detected alleles can be used to determine the number of contributors (see below). In addition, Hb expectations can be considered during this determination.

The presence of multiple alleles at the Y indel and/or DYS391 loci should be taken into account when determining the number of contributors to a sample.

If the apparent number of contributors is 1, 2, or 3, then the assumed number of contributors shall be documented as the number.

If the apparent number of contributors is greater than three or cannot be reasonably determined (e.g. due to limited results), the assumed number of contributors shall be documented as "at least X" where X is the minimum number of contributors. In both of these cases, the DNA profile is not suitable for comparison purposes, except in cases where a major component can be discerned.

Largest # of alleles at a locus	Minimum # of contributors
2	1
3	2
4	2
5	3
6	3
7	4
8	4
9	5
10	5

The interpretation of an apparent single source profile with allelic peaks significantly above the Analytical Threshold (e.g. 1000 RFU) will not be affected by one or more possible peaks below the analytical threshold.

The interpretation of a low-level apparent single or multiple source profile may be affected by the presence of possible allelic peaks just below the analytical threshold.

Mixtures containing four or more contributors shall be considered not suitable for comparison purposes due to the complexity of the mixture (except as described below in the "Discerning a Major Component" section).

Mixtures where the number of contributors cannot be reasonably determined, but have less than four contributors, shall be considered not suitable for comparison purposes due to limited results (except as described below in the "Discerning a Major Component" section).

Discerning a Major Component

To perform a Deconvolution using STRmix™, a number of contributors has to be assumed. In some mixed DNA profiles, the components of the mixture do not have equal contributions. It may not be possible to determine the total number of contributors to a mixture as the contribution from lower level components decreases. In these instances, the data for the higher-level components may still be valid and suitable for comparison and STRmix™ analysis. A major component of a mixture can be discerned under the following conditions:

The major component contains no more than three contributors.
There is no indication that the mixture contains more than a total of five contributors.
The lowest contributor to the major component has a Mixture Proportion of at least 20%, as determined by STRmix™.
The lowest contributor to the major component has a ratio of template amounts of at least 3:1 compared to the next highest component, as determined by STRmix™.
Each of the contributors to the major component has a Template Amount of at least 100 RFU, as determined by STRmix™.

If a major component is discerned, comparisons can only be made to those contributors. The minor component of the mixture shall be deemed not suitable for comparison purposes due to the complexity of the profile. The LR will only be reported for a comparison to a reference sample if the individual is determined by STRmix™ to fall in one of the positions of the Contributor Order that is part of the major component. For example, contributors 1 and 2 in the Contributor Order in the STRmix™ report of an assumed four person mixture can be discerned from the remaining two contributors where the number of minor contributors is somewhat ambiguous. No comparisons or LRs can be reported for individuals that are determined by STRmix™ to best fit in the third or fourth position of the Contributor Order. In these instances, the individual would be excluded as a contributor to the major component.

If a major component can be discerned and there is ambiguity with respect to the total number of contributors, the apparent number of contributors should be used for the STRmix™ analysis. In general, as the number of contributors increases, the LR trends to a value of 1. This means that the LR for true contributors will decrease and the LR for non-contributors will increase as the number of contributors increases. While there is a risk of a false exclusion for true contributors if the number of contributors is incorrectly low, this is less of a risk when a major component is being discerned from a mixture. These effects are typically absorbed by the minor component of the mixture that is not suitable for comparison purposes.

Single Source

A DNA profile may be considered to be from a single source under the following conditions:

No more than two alleles are present at each locus (excluding stutter and other explainable artifacts).

- Tri-allele patterns are not common but can be observed. If three alleles are observed at a single locus and all other loci display no more than two alleles, the possibility of a tri-allele pattern should be considered.

However, it is also possible that the third allele is the product of a second low-level contributor only detected at a single locus or increased stutter.

Loci containing two allelic peaks meet the expected Hb. Hb outside expectations at a single locus is not always by itself indicative of a mixture.

Typically, peaks below the Analytical Threshold can be ignored. However, numerous peaks detected just below the Analytical Threshold with expected allelic peak morphology and falling within allelic bins may be considered when interpreting a low-level sample. While these peaks cannot be considered true allelic peaks, their presence may be an indication of additional contributors that could influence the interpretation of low-level samples.

A sufficient number of alleles and loci are detected from which a conclusion as to the number of contributors can be reliably drawn. This determination will be made based on the analyst's training and experience.

- For example, a partial mixed DNA profile in which only three loci have one or two alleles above the analytical threshold may not be sufficient to reliably determine the number of contributors.
- Information specific to the case and the item of evidence should be taken into account when making this determination. For example, a small bloodstain collected from a sidewalk may be assumed to be from a single contributor. A swab from a doorknob in a public building will most likely contain DNA contributions from multiple individuals.

The presence of male DNA in a sample is established by a "Y" allele at Amelogenin and/or typing results at the Y indel or DYS391 locus. If it is determined that the mixture consists of two individuals and two alleles are present at DYS391 (excluding stutter) and/or the Y indel, then the analyst can conclude that the mixture contains two males. If it is determined that the mixture consists of three individuals and three alleles are present at DYS391 (excluding stutter), then the analyst can conclude that mixture contains three males. If the number of alleles present at DYS391 and the Y indel are less than the total number of individuals assumed to be in the mixture, then the analyst can only conclude that at least X (where X corresponds to the number of males indicated by male-specific loci) males are present in the mixture.

Documenting Sample Evaluation

The assumed number of contributors, presence of a male contributor (if applicable) and whether or not the DNA profile is suitable for comparison purposes will be documented on the electropherogram for evidentiary and reference samples. If the DNA profile is determined to be not suitable for comparison purposes, the reason shall be documented (e.g. due to limited results or due to the complexity of the mixture, see above). If a major component is discerned, the interpretation shall be documented on the electropherogram along with the components that are suitable and not suitable for comparison purposes.

Preparing for STRmix™ Evaluation

If a DNA profile has no more than two alleles at all loci, the analyst may proceed with the STRmix™ analysis under the assumption of a single contributor.

Exception: three alleles at one of the smaller molecular weight loci, and a significant peak height difference between the two "major" peaks and the single "minor" peak (at least 10x).

- The third allele will not be removed from the electropherogram, but will need to be removed from the STRmix™ input table. This will be recorded on the electropherogram.

If the profile has been determined to be suitable for comparison purposes, then a STRmix™ input table (text file) containing the sample name, marker, allele 1 – 10, size 1 – 10, and height 1 – 10 shall be exported for STRmix™ analysis.

In some instances, it may be necessary to increase the number of alleles, etc.

5.4 STRmix™ Analysis

All DNA profiles (single source, 2-person mixtures, 3-person mixtures, and up to a 3-person major component with no more than a total of five contributors) that have been determined to be suitable for comparison purposes shall initially be analyzed/deconvoluted with STRmix™ without a reference.

Positive Control

Before STRmix™ is used to analyze an evidentiary DNA profile that has been determined to be suitable for comparison, the DNA profile for the Control DNA 007 will be analyzed using STRmix™ and an LR calculated to ensure the software is functioning properly. The point LR from the current analysis will be compared to the known point LR for the Control DNA 007. If STRmix™ is functioning as expected, the values will be 3.64E28 for African American, 2.91E27 for Caucasian, and 2.77E27 for Hispanic populations. If the values are not identical, the analyst and possibly the DNA Technical Leader should investigate why and correct the issue.

Conditioning

Conditioning is a means by which an individual can be assumed to be a contributor to the mixture. This assumption should be reasonable for both hypotheses that will be considered in the later likelihood ratio.

Conditioning is appropriate in the following circumstances:

If the sample is an intimate sample, the STRmix™ deconvolution may be conditioned upon the donor of the sample.

If the sample is non-intimate, the STRmix™ deconvolution may be conditioned upon a non-probative contributor who may be reasonably assumed to have contributed DNA to the item (e.g. conditioning a mixture from a steering wheel on the vehicle's owner).

In the event of staff-to-sample contamination, the deconvolution may be conditioned upon the contaminating profile.

Initially, the deconvolution of the profile shall be performed in STRmix™ without any assumed contributors. Following the deconvolution, a *LR from Previous* should be run against the conditioning candidate.

If the likelihood ratio supports inclusion, the deconvolution may be rerun with conditioning. This new, conditioned, deconvolution will be used for subsequent comparisons.

If the likelihood ratio does not support inclusion, the non-conditioned deconvolution will be used for subsequent comparisons.

On a case-by-case basis, a mixture may be conditioned on a potentially probative contributor purely for the purposes of determining a profile for CODIS entry.

This may only be performed after a deconvolution of the mixture without conditioning was attempted and no CODIS-suitable profiles were obtained.

A likelihood ratio supporting inclusion of the individual upon whom the mixture will be conditioned must have been obtained prior to running the mixture with conditioning.

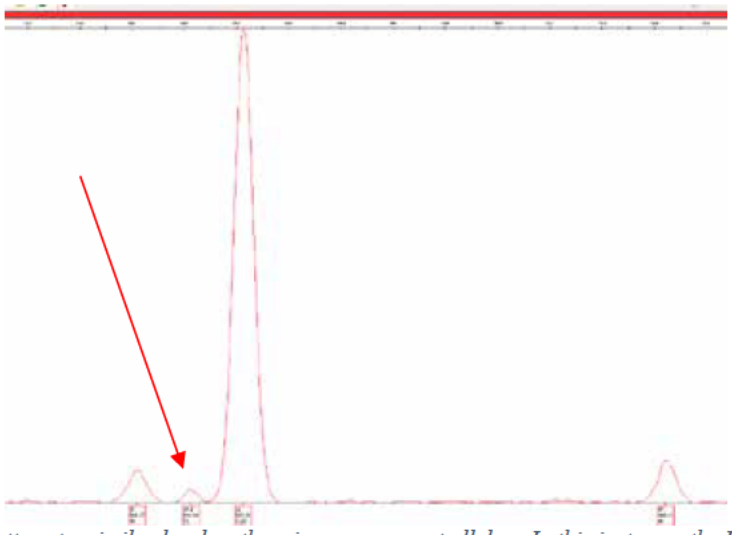
For example, two profiles are detected on a firearm. A single source profile is obtained from smudging on the slide (Male #1). A mixture of two individuals is obtained from the grips consistent with Male #1 and a second unknown individual. The mixed DNA profile may be conditioned on the Male #1 profile for the purposes of obtaining a CODIS-eligible profile for the second contributor.

Running STRmix™

The initial STRmix™ deconvolution will be run independent of any reference profiles. Comparisons to references will be achieved after deconvolution via the *LR from Previous* option.

1. Select *Start Analysis*.
2. Enter the case number into the *Case Number* field.
3. The *Sample ID* should be "Ex" followed by the exhibit number, an optional descriptor, and "Decon" (e.g. Ex1Q1-Decon or Ex. 1Q1-Grips-Decon).
4. The *Comments* box may be used to enter notes at the discretion of the analyst.
5. Enter the number of assumed contributors.
6. The DNA kit used is GlobalFiler.

7. Ignore Locus:
    a. STRmix™ cannot account for triallelic patterns. If a triallelic pattern (or other chromosomal abnormality such as a somatic mutation or null allele) is observed, the *Ignore Locus* function should be used.
    b. If an allelic peak is potentially the result of spectral pull-up and is at the same level as minor component alleles at a locus, the *Ignore Locus* function may be used.
    c. If a potential non-human peak is present that may interfere with the interpretation at a locus, the *Ignore Locus* function may be used.
    d. If an unresolved peak is detected at a locus (e.g. 15.3,16), the *Ignore Locus* function may be used.
    e. Some loci, especially SE33, exhibit n-2 bp and n+2 bp stutter. In single source DNA profiles and mixed DNA profiles where each of the components can be clearly distinguished from this stutter, the stutter peaks can be removed. If a component of a mixed DNA profile is similar in height to the n-2 bp and/or n+2 bp stutter peaks, the *Ignore Locus* function should be used for this locus.



Example of n-2 bp stutter at a similar level as the minor component alleles. In this instance, the Ignore Locus function should be used for the SE33 locus.

8. The number of MCMC and burn-in accepts should be left at the default of 500,000 and 100,000, respectively. These values may be raised as needed during troubleshooting of instrument diagnostics (see below).
9. In general, run settings should not be adjusted (see Appendix for screenshots of settings).
    a. In some instances, it may be necessary to adjust a run setting. In these cases, the change and justification shall be documented.
    b. The default settings will not be changed.

10. Select *Confirm* to proceed.
11. Select the evidence profile.
    a. If appropriate, reference profiles may be added for the purposes of conditioning. If conditioning, ensure that the reference is included in both the Hp and the Hd columns.
12. Select *Confirm Settings*.
13. Default population settings should be used (see Appendix for screenshots of settings).
14. Select *Start* to initiate sample deconvolution.
15. After each deconvolution, an advanced report should be run using the default settings.

The above steps may be achieved individually or in batch mode, which queues up the deconvolution of multiple profiles. See the STRmix™ Operation Manual for specific instructions on the use of batch mode. If batch mode is used, the advanced reports are automatically generated.

Reviewing STRmix™ Outputs

Review the run diagnostics listed below. With the exception of effective sample size, no single run diagnostic alone is demonstrative of a problem with the STRmix™ deconvolution. When multiple diagnostics are affected (e.g. very low acceptance rate with very high Gelman-Rubin value), that may be indicative of a problem with the STRmix™ deconvolution and warrant further action.

Total Iterations (Acceptance Rate)

The total iterations value is the number of post burn-in iterations run by the MCMC in order to reach the target of 400,000 accepts.

The acceptance rate is 400,000/total iterations. An acceptance rate of 1 in thousands or millions may indicate that the analysis needs to be re-run with additional iterations.

Effective Sample Size

The effective sample size is a value derived from the MCMC. During the MCMC, each iteration is correlated to the one before. The effective sample size is the number of samples from the MCMC posterior distribution that are considered independent from each other (non-correlated).

The smaller the effective sample size is compared to the number of total iterations, the greater the potential for a large difference in genotype weights from another MCMC

analysis of the same profile. The effective sample size is taken into account during the likelihood ratio calculation when considering the MCMC variability.

A "NaN" (not a number) value for this diagnostic is almost always indicative of a problem with the input file, usually a missing stutter peak.

Unless a "NaN" result is returned, this value alone is not indicative of a problem with the STRmix™ deconvolution.

Average log (likelihood)

This value is the average of the log probability values created at each of the post burn-in MCMC accepts

The larger this value, the better STRmix™ has been able to model the data.

A low or negative value may suggest that STRmix™ has not been able to describe the data well, given the information provided. Reasons for this include:

The profile is very low-level and there is little data making up the likelihood.

- The log (likelihood) is the sum of the log (likelihood) for each allele in the profile. Therefore, DNA profiles with relatively few alleles will inherently have a smaller average log (likelihood).

The incorrect number of contributors was entered and therefore forced stochastic events in the STRmix™ run as a result (e.g. large peak height imbalances or variation in contributor ratios across the profile).

Data has been removed that was real, particularly stutter peaks, and must now be described by dropout.

Artifact peaks have been left in the STRmix™ input and must be accounted for by drop-in.

Gelman-Rubin Convergence Diagnostic

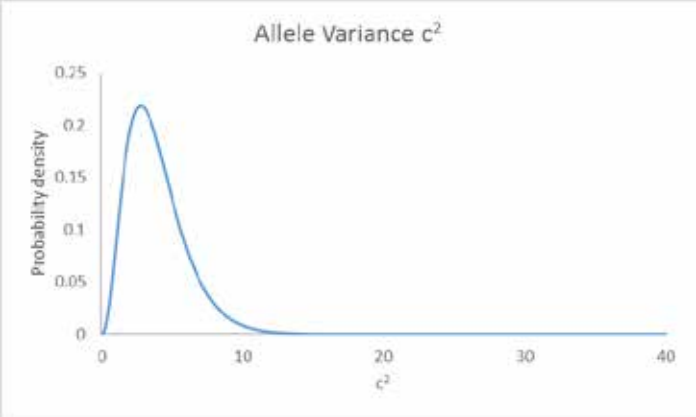
This diagnostic is a measure of how well the eight STRmix™ chains carrying out the MCMC have converged on a final profile deconvolution. For fully converged chains, this value is 1.0.

If this value is greater than 1.2, it is possible that the analysis did not run for long enough (e.g. the sample may need to be re-run with additional accepts in both burn-in and overall). A value greater than 1.2 does not automatically necessitate additional analysis, but does warrant further inspection. If the other diagnostics are acceptable and the genotype weightings, mixture proportions, and LRs meet qualitative expectations, then no further analysis is necessary.

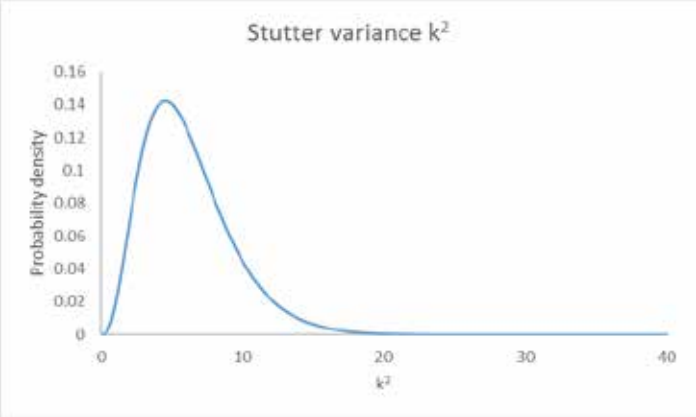
Allele Variance and Stutter Variance Constants

Both of these values are the average value for the allele variance and stutter variance constants across the entire post burn-in MCMC accepts. These values can be indicative of the level of stochastic variation in peak heights that is present in the profile.

The allele variance mode is 2.760, with a gamma distribution curve shown below:



The stutter variance mode is 4.544, with a gamma distribution curve shown below:



If the allele or stutter variance values are significantly higher than the mode, this may indicate that the DNA profile itself is of poor quality or that the incorrect number of contributors was assumed.

Used in conjunction with the average (log) likelihood, a large allele or stutter variance value can indicate poor amplification.

If the sample is simply low level, the deconvolution should yield a low average (log) likelihood and a variance constant close to the mode.

If some data has been inappropriately omitted, artifacts left in, or the profile was otherwise misinterpreted, the deconvolution should yield a low average (log) likelihood and high allele and/or stutter variances relative to the mode.

Genotype Weights and Mixture Proportions

In addition to the run diagnostics, the genotype weights and mixture proportions should be reviewed to ensure that the deconvolution conforms to the analyst's qualitative expectations of approximate contributor ratios and likely genotype pairs based upon their assessment of the data.

On rare occasions, the genotype weights will not conform to the analyst's qualitative expectations at a locus. This may be due to:

An unusually high stutter peak

An unresolved minor component peak in instances where the minor peak has a one base pair difference to a major component peak (e.g. major 15.3 allele and a minor 16 allele)

Other uncommon circumstances

In these instances, the deconvolution may be re-run using the *Ignore Locus* function for the affected locus.

Re-running a Deconvolution

Once the run diagnostics, contributor weights, and mixture proportions have been reviewed, the analyst may determine it is necessary to re-run the deconvolution. Reasons for a repeat deconvolution must be clearly documented on the first page of the advanced report or as a case note and retained in the case file.

Discrete contributor profiles will be found in the "SUMMARY >=99%" portion of the advanced report. This summary lists the genotype(s) that are found in the top 99% of the weights as determined by STRmix™ and may be listed as fully resolved (e.g. 17,17 or 17,18), partially-resolved (e.g. 17,0), or non-resolved (0,0).

Deconvolution may be re-run with an edited input file, with a greater number of MCMC accepts, with a change to the number of contributors, or by the introduction of user-informed mixture proportion priors (Mx priors). Mx priors are a means by which an analyst can provide STRmix™ with a starting-point for contributor ratios in circumstances when a mixture may contain relatives or peaks below the analytical threshold indicate a trace contributor. See the STRmix™ Operation Manual for specific instruction on the use of the Mx prior functionality.

The use of an increased number of MCMC accepts or Mx priors requires documented DNA Technical Leader approval.

If Mx priors are used, the case notes will indicate the mean and variance used for each contributor. This may be most easily achieved via the use of printed screen shots from STRmix, but other means may be used at the discretion of the analyst.

Comparisons and LR Calculations

The likelihood ratio (LR) compares the probability of observing the evidence profile given two alternate hypotheses: one in which the person of interest (POI) is a contributor to the evidentiary profile, called H_I, and one in which a random person, unrelated to the POI, is a contributor to the evidentiary profile, called the Alternate Hypothesis (H_E).

Evidentiary profiles will be compared to all informative reference samples. If there is no expectation that DNA from an individual would be present on an exhibit of evidence, then no comparison to that individual is necessary for that sample. This will be documented in the case notes and/or the report.

Comparisons between evidentiary profiles and references determined to be probative in the context of a case will be achieved via the STRmix™ *LR from Previous* function. Manual comparisons may be made to complete (100% weighting at every locus) single source DNA profiles or mixtures where a complete single source major component can be discerned. If the individual is not excluded, the LR will be calculated via STRmix™. An individual may be excluded as a contributor to a partial profile (single source or major component of a mixture) by comparison to only those loci that produced a 100% weighted genotype.

Comparisons between questioned samples may be achieved in STRmix™ as appropriate, provided one of the questioned samples is single-source and complete at all GlobalFiler STR loci. When this is performed, only the conclusion (support for inclusion, limited support for inclusion, uninformative, limited support for exclusion, or excluded) will be reported. The associated LR will not be reported unless it is probative.

Manual comparisons between two questioned samples is permitted as described above for manual comparisons of evidentiary profiles to references.

Setting up Propositions

Propositions are typically set to minimize the LR. This can be done by minimizing the number of unknowns within the different propositions.

Non-conditioned LRs will first be run against a single POI at a time. If the case scenario dictates that the multiple persons of interest are probative in combination, then LRs should be calculated for each person individually and for the individuals all together. However, only the individual LRs will be reported.

Likelihood ratios will be calculated for the Caucasian, African American, and Hispanic populations using the allele frequencies published by Hill, et al. Other population groups may be considered on a case-by-case basis, but will require setting up the appropriate STRmix™ parameters as described in the STRmix™ Operation Manual.

> Hill, C.R., Duewer, D.L., Kline, M.C., Coble, M.D., Butler, J.M. (2013) U.S. population data for 29 autosomal STR loci. Forensic Sci. Int. Genet. 7: e82-e83.
>
> Steffen, C.R., Coble, M.D., Gettings, K.B., Vallone, P.M. (2017) Corrigendum to 'US Population Data from 29 Autosomal STR Loci' [Forensic Sci. Int. Genet. 7 (2013) e82-e83)]. Forensic Sci. Int. Genet. 31: e36-e40.
>
> Population parameters are listed in the Appendix. The population size is based on the 2016 US Census report. The number of children per family is set at 3 to be conservative.

Likelihood ratio calculations will include the Factor of N!, HPD sampling variations at 1000 iterations, and 1-sided MCMC uncertainty at the 99th quantile. The lowest 99% 1-sided Lower HPD LR across the three population groups will be reported.
When multiple single source profiles (from the same or separate exhibits), all containing profiles with only one weighted genotype at each locus and matching a single individual are obtained, only one *LR from Previous* may be run. This LR will be used for reporting purposes.

Individual LRs will be run for single source profiles having at least one locus with two or more weighted genotypes, regardless of whether or not they have all been determined to match a single individual.

If positive likelihood ratios are obtained for all loci except one where the LR is 0, the interpretation, input file, and weights should be scrutinized to ensure no STRmix™ input errors were made. If an error is detected, re-run either the deconvolution or the *LR from Previous* as needed. Documentation for both deconvolutions shall be retained with a note describing the reason for the second deconvolution. If no input errors were made, consult with the DNA Technical Leader before proceeding.

After the release of the analytical report, additional propositions may be evaluated upon customer request. Only those requests for which the propositions are determined to be reasonable by the Laboratory in the context of the case will be granted, or by court order. Granting this request is at the discretion of the casework analyst. These additional analyses shall be documented in the case record and undergo a technical review.

If the case scenario does not dictate that multiple contributors together are probative, but inclusions (> limited support) to a number of contributors equal to the assumed number of contributors have been made, a final LR evaluating the likelihood of the individuals contributing to the mixture together will be calculated. This assessment is for verification

of the contributor combination only, and is not performed to provide weight to the combined contribution.

Example scenarios are as follows:

A single source partial DNA profile is obtained from possible ridge detail on a threat letter.

- The DNA profile is analyzed with STRmix™.
- A suspect (POI) standard is received for comparison.
- H_I: DNA contributor is POI.
- H_E: DNA contributor is one unknown

A two-person mixture is obtained from the grips of a firearm.

- Deconvolution is performed without conditioning.
- One suspect (POI) is received for comparison.
- H_I: DNA contributors are POI and an unknown.
- H_E: DNA contributors are two unknowns.

A two-person mixture is obtained from a blood stain on a shirt recovered from the victim (V) of a stabbing.

- Deconvolution is first performed without conditioning.
- An *LR from Previous* is then performed to determine if the victim can be conditioned.
- If the LR for the victim supports inclusion, then a second deconvolution can be performed conditioned on the victim.
- A suspect (POI) is received for comparison.
- H_I: DNA contributors are V and POI.
- H_E: DNA contributors are V and unknown.

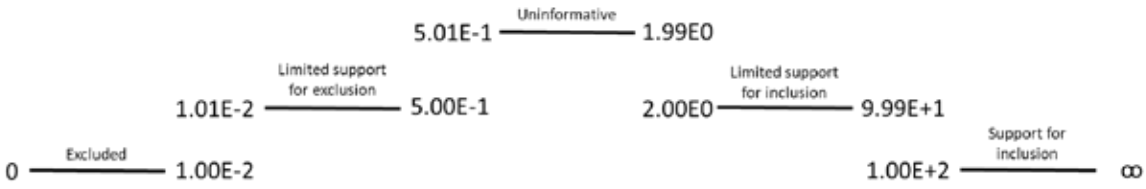
A single source DNA profile (P1) was obtained from the front sight of a firearm. A two-person mixture (P2) was obtained from the grips of the same firearm.

- The P2 profile is initially deconvoluted without conditioning.
- An LR can then be calculated to determine if the P1 profile is included as a possible contributor to the P2 mixed profile.
- If the LR for the P1 profile supports inclusion in the P2 profile, the P2 profile can be deconvoluted conditioned on the P1 profile to obtain a profile for CODIS entry purposes.
- A suspect (POI) is received for comparison. The hypotheses for reporting purposes for the P2 profile are below:
- H_I: The DNA contributors are the POI and an unknown individual.
- H_E: The DNA contributors are two unknown individuals.

Persons of Interest

Conclusions regarding a person of interest's contribution to a sample will be made based upon the 99% 1-sided Lower HPD likelihood ratio. One of five conclusions can be made for a comparison based on the LR:

Support for inclusion (LR supporting inclusion)
Excluded (LR supporting exclusion)
Limited support for exclusion (LR supporting exclusion, but within a range at higher risk for false exclusions)
Limited support for inclusion (LR supporting inclusion, but within a range at higher risk for false inclusions)
The comparison is uninformative (LR does not reliably support the Inclusionary Hypothesis (H_I) or Exclusionary Hypothesis (H_E), see below)



The LRs shall be truncated to three significant figures. For LRs between 1 and 100, the LR shall be truncated to a whole number.

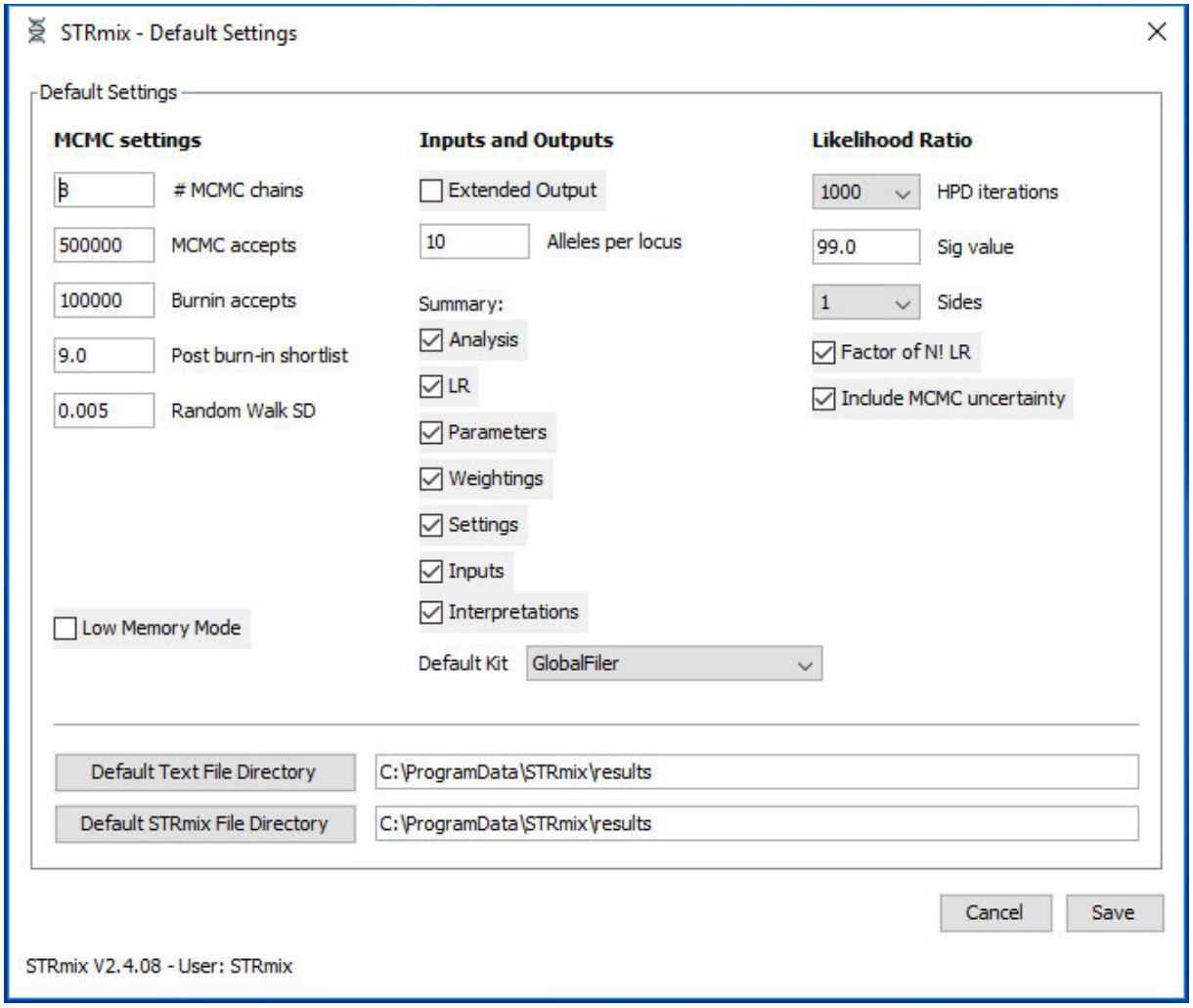
Additional Guidelines on Interpretation and Comparison:

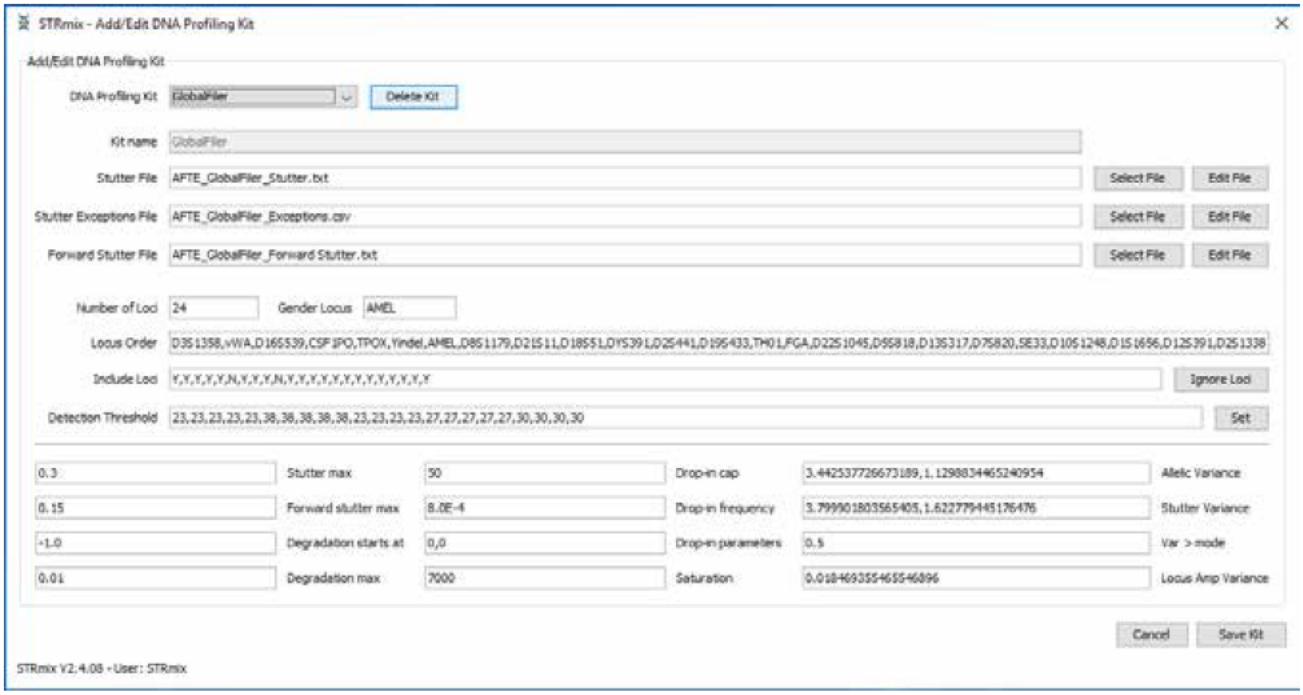
Non-numeric values such as OL, < or > and R are not permitted within the STRmix™ input files.

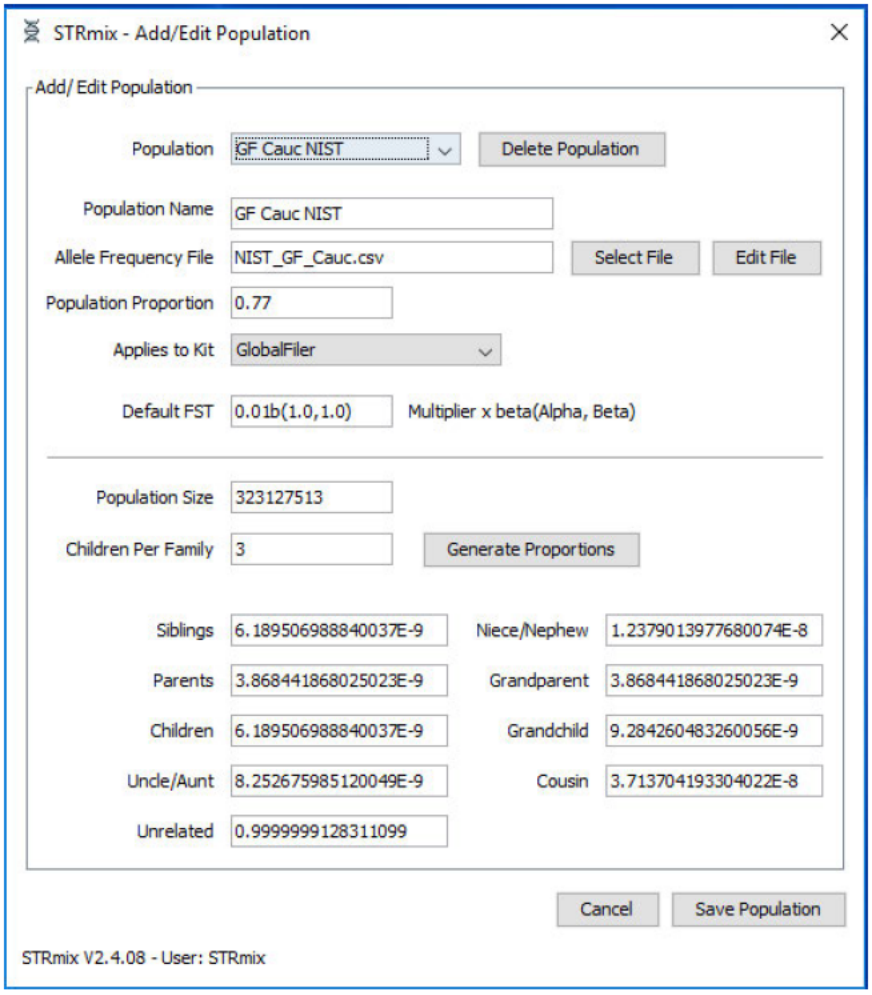
Unambiguous alleles including those that are rare should appear in the corresponding input file as their actual allelic size designation, for example D21 30.1. If alleles fall more than two repeat units off the end of the ladder, they are unable to be sized accurately or labelled correctly. These loci should be removed from the deconvolution using the *Ignore Locus* option. If an LR is calculated, the POI should be manually compared at this locus to ensure this is not an exclusion.

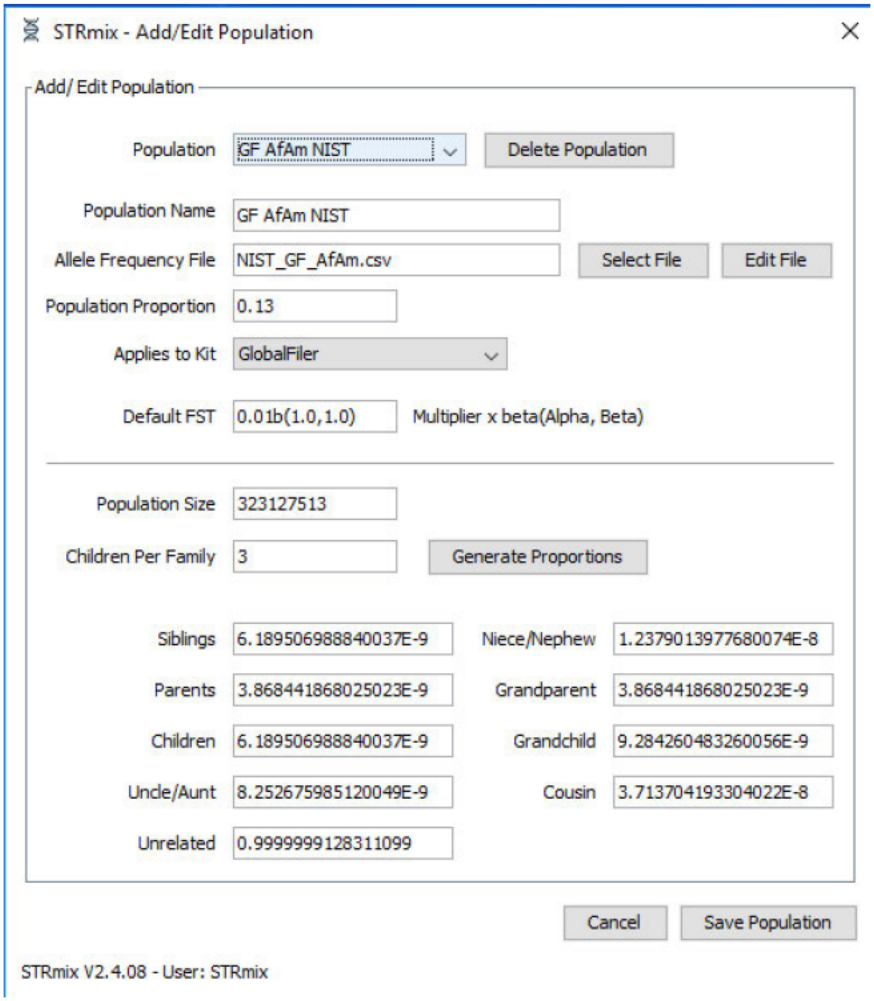
If multiple amplifications of the same extract are performed, the concordance of the genotyping results should be evaluated. In general, both samples will require STRmix™ deconvolution, unless one has significantly more genetic information than the other. Only the most informative profile shall be analyzed further.

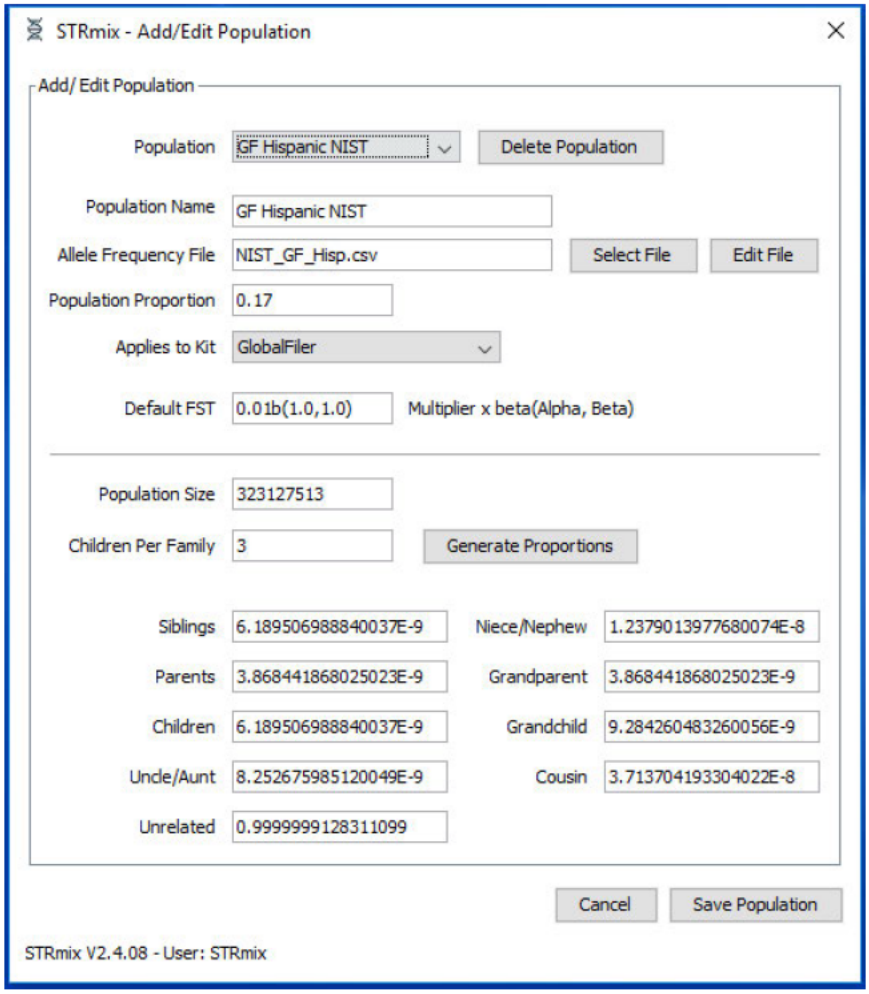
Appendix


STRmix - Default Settings
Default Settings
MCMC settings
3
MCMC chains
500000
MCMC accepts
100000
Burnin accepts
9.0
Post burn-in shortlist
0.005
Random Walk SD
Low Memory Mode
Inputs and Outputs
Extended Output
10
Alleles per locus
Summary:
Analysis
LR
Parameters
Weightings
Settings
Inputs
Interpretations
Default Kit
GlobalFiler
Likelihood Ratio
1000
HPD iterations
99.0
Sig value
1
Sides
Factor of N! LR
Include MCMC uncertainty
Default Text File Directory
C:\ProgramData\STRmix\results
Default STRmix File Directory
C:\ProgramData\STRmix\results
Cancel
Save
STRmix V2.4.08 - User: STRmix


STRmix - Add/Edit DNA Profiling Kit
Add/Edit DNA Profiling Kit
DNA Profiling Kit
GlobalFiler
Delete Kit
Kit name
GlobalFiler
Stutter File
AFTE_GlobalFiler_Stutter.txt
Select File
Edit File
Stutter Exceptions File
AFTE_GlobalFiler_Exceptions.csv
Select File
Edit File
Forward Stutter File
AFTE_GlobalFiler_Forward Stutter.txt
Select File
Edit File
Number of Loci
24
Gender Locus
AMEL
Locus Order
D3S1358,vWA,D16S539,CSF1PO,TPOX,Yindel,AMEL,D8S1179,D21S11,D18S51,DYS391,D2S441,D19S433,TH01,FGA,D22S1045,D5S818,D13S317,D7S820,SE33,D10S1248,D1S1656,D12S391,D2S1338
Include Loci
Y,Y,Y,Y,Y,N,Y,Y,Y,N,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y
Ignore Loci
Detection Threshold
23,23,23,23,23,38,38,38,38,38,23,23,23,23,27,27,27,27,27,30,30,30,30
Set
0.3
Stutter max
50
Drop-in cap
3.4425377266731 89, 1.1298834465240954
Allelic Variance
0.15
Forward stutter max
8.0E-4
Drop-in frequency
3.7999018035654 05, 1.6227794451764 76
Stutter Variance
-1.0
Degradation starts at
0,0
Drop-in parameters
0.5
Var > mode
0.01
Degradation max
7000
Saturation
0.0184693554655 46896
Locus Amp Variance
Cancel
Save Kit
STRmix V2.4.08 - User: STRmix


STRmix - Add/Edit Population
Add/ Edit Population
Population
GF Cauc NIST
Delete Population
Population Name
GF Cauc NIST
Allele Frequency File
NIST_GF_Cauc.csv
Select File
Edit File
Population Proportion
0.77
Applies to Kit
GlobalFiler
Default FST
0.01b(1.0,1.0)
Multiplier x beta(Alpha, Beta)
Population Size
323127513
Children Per Family
3
Generate Proportions
Siblings
6.189506988840037E-9
Niece/Nephew
1.2379013977680074E-8
Parents
3.868441868025023E-9
Grandparent
3.868441868025023E-9
Children
6.189506988840037E-9
Grandchild
9.284260483260056E-9
Uncle/Aunt
8.252675985120049E-9
Cousin
3.713704193304022E-8
Unrelated
0.9999999128311099
Cancel
Save Population
STRmix V2.4.08 - User: STRmix


STRmix - Add/Edit Population
Add/ Edit Population
Population
GF AfAm NIST
Delete Population
Population Name
GF AfAm NIST
Allele Frequency File
NIST_GF_AfAm.csv
Select File
Edit File
Population Proportion
0.13
Applies to Kit
GlobalFiler
Default FST
0.01b(1.0,1.0)
Multiplier x beta(Alpha, Beta)
Population Size
323127513
Children Per Family
3
Generate Proportions
Siblings
6.189506988840037E-9
Niece/Nephew
1.2379013977680074E-8
Parents
3.868441868025023E-9
Grandparent
3.868441868025023E-9
Children
6.189506988840037E-9
Grandchild
9.284260483260056E-9
Uncle/Aunt
8.252675985120049E-9
Cousin
3.713704193304022E-8
Unrelated
0.9999999128311099
Cancel
Save Population
STRmix V2.4.08 - User: STRmix


STRmix - Add/Edit Population
Add/ Edit Population
Population
GF Hispanic NIST
Delete Population
Population Name
GF Hispanic NIST
Allele Frequency File
NIST_GF_Hisp.csv
Select File
Edit File
Population Proportion
0.17
Applies to Kit
GlobalFiler
Default FST
0.01b(1.0,1.0)
Multiplier x beta(Alpha, Beta)
Population Size
323127513
Children Per Family
3
Generate Proportions
Siblings
6.189506988840037E-9
Niece/Nephew
1.2379013977680074E-8
Parents
3.868441868025023E-9
Grandparent
3.868441868025023E-9
Children
6.189506988840037E-9
Grandchild
9.284260483260056E-9
Uncle/Aunt
8.252675985120049E-9
Cousin
3.713704193304022E-8
Unrelated
0.9999999128311099
Cancel
Save Population
STRmix V2.4.08 - User: STRmix

1. Scope

1.1 The following guidelines outline the wording to be used when reporting the results of Forensic Biology analyses. These guidelines cannot encompass all possible circumstances encountered during casework analysis. Therefore, it is assumed that it may be necessary to deviate from the guidelines in certain circumstances.

2. References

2.1 Department of Justice, "Uniform Language for Testimony and Reports for Autosomal DNA Examinations Using Probabilistic Genotyping Systems", effective March 18, 2019.
2.2 Current version of SWGDAM Quality Assurance Standards for Forensic DNA Testing Laboratories
2.3 Department of Justice, "Uniform Language for Testimony and Reports for Forensic Serological Examinations", effective March 18, 2019.

3. Safety / Quality Assurance

3.1 Reports will conform with ATF Laboratory Services Quality Manual Reporting of Results
3.2 Reports will undergo technical and administrative review in accordance with ATF Laboratory Services Quality Manual Review of Case Records, Reports, and Notifications

4. Equipment

4.1 Not applicable

5. Procedure

5.1 The report format will comply with ATF Laboratory Services Quality Manual Reporting of Results.
5.2 The analysis methods used for each exhibit shall be listed in the report.
5.3 The following sub-sections are included under the "Examination/Analysis and Interpretation of Results" section:
- 5.3.1 Serological Analysis (if applicable)
- 5.3.2 DNA Analysis
- 5.3.3 Statistical Analysis (if applicable)
- 5.3.4 Combined DNA Index System (CODIS)

5.4 Results will be reported in a tabular form under the DNA Analysis sub-section, with references listed first.

5.4.1 In general, the table will contain the following: exhibit number (StarLims), sub-exhibit number, description, result, suitability for comparison, conclusions, and statistical analysis number.

5.4.1.1 The five categories of conclusions for a comparison of a reference sample to an evidence sample are: support for inclusion; limited support for inclusion; uninformative; limited support for exclusion; and excluded.

5.4.1.2 If no comparison results in an "uninformative" conclusion, this column may be left out of the table.

5.4.1.3 The statistical calculation shall be reported as described below separate from the table(s).

5.5 Results of serological examinations

5.5.1 If serological testing was performed on at least one exhibit, a "Serological Analysis" sub-section will be included under the "Examination/Analysis and Interpretation of Results" section.

5.5.2 Reporting of results will be on the exhibit level; therefore, each exhibit will be either positive, negative, or inconclusive.

5.5.3 If an item is tested multiple times with both positive and negative results, the result and conclusion will be positive for the exhibit and reported as detailed below.

5.5.4 Results of testing for the presence of blood

5.5.4.1 Positive Result:

5.5.4.1.1 Presumptive testing for blood was positive; therefore, blood is indicated on the following exhibit(s):

- Exhibit X
  - Stain on left sleeve
  - Stain on back, lower seam
- No confirmatory testing was performed.

OR

5.5.4.1.2 Presumptive testing for blood was positive for stained areas A through E on Exhibit X; therefore, blood is indicated. No confirmatory testing was performed.

5.5.4.2 Negative Result:

5.5.4.2.1 Presumptive testing for blood was negative on the following exhibit(s):

- Exhibit X
- Exhibit Y

5.5.4.3 Inconclusive Result:

5.5.4.3.1 Presumptive testing for blood was inconclusive on the following exhibit(s):

- Exhibit X

5.5.4.4 If serological analysis was requested for an exhibit, but no stains were observed:

5.5.4.4.1 No biological stains were observed on Exhibit X.

5.6 Evidence sampling

5.6.1 If an item is not suitable for Forensic Biology examination:

5.6.1.1 If no areas suitable for DNA collection were observed (e.g. no ninhydrin staining on a document):

5.6.1.1.1 No areas suitable for DNA collection were observed on the *item* (Exhibit *X*).

5.6.1.2 If evidence condition is not suitable for Forensic Biology examination (e.g. the evidence is a completely melted and charred plastic gas can):

5.6.1.2.1 The *item* (Exhibit *X*) is not suitable for Forensic Biology analysis due to the [charred, melted, etc.] condition of the evidence.

5.6.2 If the evidence is sampled in one or more areas for DNA analysis, the following statement shall precede the table:

5.6.2.1 The following evidence was sampled for DNA analysis: Exhibits X and Y. See Table(s) 1 through Z for descriptions and results.

5.6.3 If two or more extracts are combined into a single DNA extract, sub-exhibit cells and the description cells for each sample will be combined in the table. A new sub-exhibit number will reflect the combined designation, and the following statement shall be made in the combined description cell:

5.6.3.1 Combined sample of *swabs/cuttings/etc.* of *area 1* (Exhibit *X.Z*) and *swabs/cuttings/etc.* of *area 2* (Exhibit *X.Y*).

5.7 If no DNA analysis was performed on the swabs/sampling of the exhibit, the following statement will be used:

5.7.1 No DNA analysis was performed on the swabs/sampling of Exhibit X at this time.

5.7.2 The reason for not performing DNA analysis must be stated (e.g. the request for examination was withdrawn).

5.8 Results of DNA examination

5.8.1 Each DNA profile will have a description of the result and determination of suitability for comparison purposes.

5.8.1.1 A DNA profile is considered complete if there is at least one allele detected at all loci. A profile is still considered complete if there is possible allelic drop-out at one or more loci or no results are obtained for the Y indel and DYS391 loci for female profiles. When a major component is discerned from a mixed DNA profile, the major component will be considered complete if there is at least one allele at all loci for the overall profile.

5.8.1.2 A DNA profile is considered partial if there is no allelic data present at one or more loci (complete locus drop-out), including the Y indel or DYS391 loci for male profiles. When a major component is discerned from a mixed DNA profile, the major component will be considered partial if there is no allelic data present at one or more loci for the overall profile. However, if all autosomal loci contain weighted genotype sets and it can be reasonably assumed that the major component is female (clear X to Y differentiation and at least one 99% allele at all autosomal loci), the major component will not be considered partial.

5.8.1.2.1 The presence of the "Y" allele at the Amelogenin locus and/or an allele detected at the Y indel or DYS391 loci is indicative of the presence of male DNA.

5.8.1.2.2 The lack of a "Y" allele at the Amelogenin locus and the lack of results at both the Y indel and DYS391 loci are indicative of female DNA when it is reasonable to assume drop-out has not occurred (approximately 250 RFU).

5.8.1.2.3 If a "Y" allele at the Amelogenin locus is not detected and no alleles are detected at both the Y indel and the DYS391 loci, but it is reasonable to assume drop-out may have occurred at these loci, no indication of male or female will be listed in the results.

5.8.2 DNA profiles where all components have been determined to be suitable for comparison purposes shall state "…consistent with X contributors…" when describing the assumed number of contributors.

5.8.3 DNA profiles where one or more components have been determined to be not suitable for comparison purposes shall state "…consistent with at least X contributors…" when describing the assumed number of contributors.

Sample	Result Listed in Table
Known Reference Samples	Single source *male/female* DNA profile
No DNA Detected (analysis stopped at quantification)	No DNA detected
No Profile Obtained	No results
Single Source Evidence Profiles	
A partial, single source profile	Partial (male/female) DNA profile consistent with a single contributor
A complete single source profile	(Male/Female) DNA profile consistent with a single contributor
Mixed Evidence Profiles	
A partial mixed profile was obtained for an item and it is reasonable to assume the number of contributors	Partial DNA profile consistent with (assumed # of contributors) contributors, at least (*# of males*) *male/males* present.
The number of contributors cannot be reasonably assumed in a partial mixture	Partial DNA profile consistent with at least (*minimum # of contributors*) contributors, at least (*# of males*) *male/males* present.
It is reasonable to assume the number of contributors in a mixed profile with no locus dropout	DNA profile consistent with (assumed # of contributors) contributors,, at least (*# of males*) *male/males* present.
The number of contributors cannot be reasonably assumed in a mixture with no locus dropout	DNA profile consistent with at least (*minimum # of contributors*) contributors, at least (*# of males*) *male/males* present

Table 1: Results as listed in the report table for reference samples and evidence samples.

5.8.4 Mixed DNA profiles

5.8.4.1 The presence of the "Y" allele at the Amelogenin locus and/or an allele(s) detected at the Y indel or DYS391 loci is indicative of the presence of male DNA.

5.8.4.1.1 In general, if the number of contributors can be reasonably assumed and the Y chromosome loci indicate the same number of contributors, that number of male contributors will be reported. For example, if two alleles are detected at DYS391 and two individuals are assumed to have contributed to the mixture, then the results shall indicate two males contributed to the profile.

5.8.4.1.2 In general, if the number of contributors can be reasonably assumed and the Y chromosome loci indicate a number of male individuals less than the assumed number of contributors, then the results shall state at least *X* number of males are present.

5.8.4.1.3 If there is a clear female major component based on Amelogenin and the Y chromosome loci, the presence of the female will be indicated. In general, a clear indication of a female major component is indicated by a mix ratio of at least 4:1 based on the mixture proportions and a 10:1 ratio of X:Y peak heights. In some instances, it may be possible to state the number of female contributors equal to the total number of contributors to the profile or major component of a profile (e.g. high level two person mixture with no indication of a male contribution). If a female is reported in the major component of a mixture, the presence of at least one female will be reported for the overall mixture.

5.8.5 If the DNA profile or major component contains sufficient data and the number of contributors can reasonably be assumed (therefore, suitable for comparison purposes), this will be stated in the suitability column of the table with "Yes". In some instances, the profile may be analyzed/deconvoluted using STRmix™ or DBLR™ to aid in the determination of whether it is suitable for comparison purposes. All deconvolutions and simulations will be maintained in the case record.

5.8.6 DNA profiles can be determined to not be suitable for comparison purposes with or without further evaluation using STRmix™ and/or DBLR™. For example, in situations where limited loci are detected (e.g. only three loci with alleles detected in an apparent two person mixture) or there are clearly > 5 contributors, no further evaluation is necessary.

5.8.6.1 If a DNA profile is determined to be not suitable for comparison purposes during the initial evaluation without further evaluation (STRmix™ and/or DBLR™), only allelic peaks above the analytical threshold shall be used to estimate the number of contributors reported. If further evaluation was performed, then the assumed number of contributors used for the evaluation will be reported. One of the following statements shall be made in the suitability column of the table:

5.8.6.1.1 If the DNA profile has been determined to be not suitable for comparison and contains three or fewer contributors, then the following shall be stated: "No, due to limited results".

5.8.6.1.2 If the DNA profile has been determined to be not suitable for comparison purposes and contains four or more contributors, then the following shall be stated: “No, due to the complexity of the profile".

5.8.7 If a major component can be discerned from a mixed DNA profile, the table shall individually address the suitability of the major and minor components in separate cells in the suitability column:

5.8.7.1 Major component:

5.8.7.1.1 Statements listed in Table 1 for single source and mixed profiles will be used, preceded by "Major – Yes,", and indicating male or female contributors as appropriate.

5.8.7.2 Minor component:

5.8.7.2.1 If the overall assumed number of contributors to the DNA profile is three or less and the minor component has been determined to not be suitable for comparison purposes, then the suitability of the minor component shall be stated in the table as "*Minor – No, due to limited results*".

5.8.7.2.2 If the overall assumed number of contributors to the DNA profile is four or five, then the suitability of the minor component shall be stated in the table as "*Minor – No, due to the complexity of the profile"*.

5.8.8 In rare instances, STRmix™ will be unable to complete the deconvolution of a DNA profile. When this occurs, the DNA profile will be determined to be not suitable for comparison purposes due to the complexity of the profile.

5.9 Immediately before the Statistical Analysis subsection, if at least one DNA profile suitable for comparison purposes was obtained, the following statement will be included:

5.9.1 If [additional] comparisons to the above-mentioned DNA profile[s] are desired, please submit two buccal (cheek) swabs from the relevant individual(s) to the ATF National Laboratory Center.

5.10 Statistical analysis

5.10.1 If it has been determined that a DNA profile is suitable for comparison purposes and comparisons to one or more reference samples have been performed, the results of the statistical analysis shall be stated in the "Statistical Analysis" sub-section of the report, and the conclusion and calculation number shall be stated in the table. If the result of the comparison is an exclusion, it is not necessary to add the calculation number to the table since the statistical analysis will not be included in the report. The assumed number of contributors, any individual(s) assumed to be a contributor for conditioning purposes, proposed hypotheses (H_I and H_E), and the conclusion shall be stated for each statistical analysis

performed using STRmix™. Conclusions shall be based on the lowest 99% 1-sided lower HPD interval across the three populations (see relevant interpretation protocol), truncated to three significant figures. One of five conclusions can be made (See Figure 1): excluded, limited support for exclusion, uninformative, limited support for inclusion, and support for inclusion. It is not necessary to report the statistical analysis for DNA profiles associated to an individual where that individual can be reasonably assumed to be present (e.g. owner of the stolen car found on the steering wheel of a car).

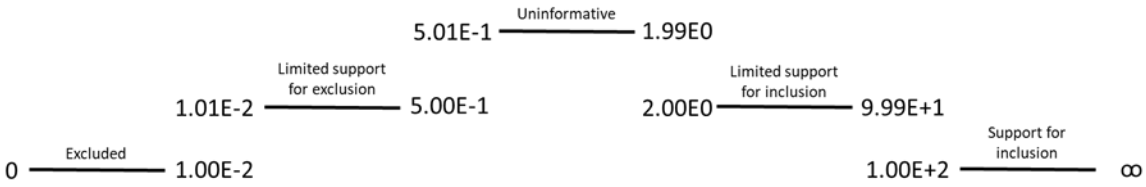


Figure 1: Scale for conclusions based on LR

5.10.2 **Excluded:** If an individual can be clearly excluded through a manual comparison or the LR falls in the exclusionary range, the conclusion can be listed in the table and the statistical analysis does not need to be reported. However, if the exclusion is made based on a statistical calculation where the assumptions are different than the general assumptions listed in the Appendix (e.g. an individual is an assumed contributor and the analysis is conditioned), then the statistical analysis will be reported using the following statements after the assumptions and hypotheses:

5.10.2.1 The DNA profile is 1/LR times more likely if it originated from *Exclusionary Hypothesis* than if it originated from *Inclusionary Hypothesis*.

5.10.2.2 Based on this calculation, *POI* (Exhibit X) is excluded as a possible contributor of (single source)/to (mixture) the DNA profile obtained from Exhibit *Y.Y*.

5.10.3 **Limited Support for Exclusion:** If the LR provides limited support for exclusion, the following shall be stated after the assumptions and hypotheses:

5.10.3.1 The DNA profile is 1/LR times more likely if it originated from *Exclusionary Hypothesis* than if it originated from *Inclusionary Hypothesis*.

5.10.3.2 Based on this calculation, there is limited evidentiary support for the exclusion of *the POI* (Exhibit X) as a possible contributor of (single source)/to (mixture) the DNA profile obtained from Exhibit *X.X*.

5.10.4 **Support for Inclusion:** if the lowest LR is greater than the upper bound of the limited support for inclusion range, the POI is included.

5.10.4.1 The maximum LR reported is 1 trillion (10^{12}). An LR greater than the maximum will be reported as "at least 1 trillion…".

5.10.4.2 The DNA profile is *LR* times more likely if it originated from *Inclusionary Hypothesis* than if it originated from *Exclusionary Hypothesis*.

5.10.4.3 The probability of an unrelated individual in the population, who has not contributed DNA to this sample, yielding this level of support, is less than 1 in LR.

OR, when the LR exceeds 1 trillion (1.00E12)

5.10.4.4 The probability of an unrelated individual in the population, who has not contributed DNA to this sample, yielding this level of support, is less than 1 in one trillion.

5.10.4.5 Based on this calculation, there is evidentiary support for the inclusion of *the POI* (Exhibit X) as a possible contributor of (single source)/to (mixture) the DNA profile obtained from Exhibit *X.X*.

5.10.5 **Limited Support for Inclusion:** If the LR provides limited support for inclusion, the following shall be stated after the assumptions and hypotheses:

5.10.5.1 The DNA profile is LR times more likely if it originated from *Inclusionary Hypothesis* than if it originated from *Exclusionary Hypothesis*.

5.10.5.2 The probability of an unrelated individual in the population, who has not contributed DNA to this sample, yielding this level of support, is less than 1 in LR.

5.10.5.3 Based on this calculation, there is limited evidentiary support for the inclusion of *the POI* (Exhibit X) as a possible contributor of (single source)/to (mixture) the DNA profile obtained from Exhibit *X.X*.

5.10.6 **Uninformative:** If the LR provides approximately equal support for both hypotheses and has been determined to be uninformative, the following shall be stated after the assumptions and hypotheses:

5.10.6.1 The statistical analysis resulted in a likelihood ratio equivalent to 1, which means that the comparison is uninformative. Therefore, the evidence provides no greater support for either the inclusion or exclusion of *the POI* (Exhibit X) as a possible contributor of (single source)/to (mixture) the DNA profile obtained from Exhibit *X.X*.

5.10.7 **Combined LR:** in instances where more than one individual is included as a possible contributor to a mixture and a combined LR was performed, the resulting LR will not be reported if the inclusion of both individuals is still supported. If, however, the resulting LR does not support the inclusion of both individuals, the LR will be reported (See Supplemental Information - Report Wording Examples).

5.10.7.1 A DNA profile consistent with a mixture of N individuals was obtained from Exhibit A.

5.10.7.2 N individuals are assumed to have contributed to the DNA profile.

5.10.7.3 Inclusionary Hypothesis (H_I): the DNA originated from Individual 1 (Exhibit Y) and Individual 2 (Exhibit Z) (and N-2 unknown, unrelated individuals if necessary).

5.10.7.4 Exclusionary Hypothesis (H_E): the DNA originated from N unrelated, unknown individuals.

5.10.7.5 Although independently there is evidentiary support for the inclusion of Individual 1 (Exhibit Y), and limited evidentiary support for the inclusion of Individual 2 (Exhibit Z), the statistical analysis does not support the proposition that Individual 1 (Exhibit Y) and Individual 2 (Exhibit Z) together, are both contributors to the DNA profile obtained from Exhibit A.

5.10.8 Loci not used in the LR calculation (*Ignore Locus* function used due to pull-up, tri-allele, primer site imbalance, etc.) will be listed after the conclusion in the statistical analysis.

5.10.8.1 The following locus[i] were not used for the statistical analysis because the locus[i] did not meet the required interpretation criteria: Locus 1.

5.10.9 See “Supplemental Information - Report Wording Examples” for examples of the common scenarios.

5.11 Reporting multiple consistent profiles from the same exhibit or different exhibits

5.11.1 If the same single source DNA profile and/or the same >99% contributor #X DNA profile was obtained from multiple samples from the same exhibit or from multiple exhibits from the same case, but each has a different statistical weight, the group of profiles may be reported together

using the lowest statistical weight to describe the entire group as long as the LR for each has the same order of magnitude. All must use the same hypotheses. In addition, a group of comparisons to the same individual with the same hypotheses where there is no >99% contributor can be reported together using the lowest statistical weight to describe the entire group as long as the LR has the same order of magnitude.

5.11.1.1 Example: Comparisons of Person A to Exhibit 1.1 results in an LR of $1.65x10^9$, Exhibit 1.2 results in an LR of $2.43x10^9$, and Exhibit 1.3 results in an LR of $3.23x10^5$, all using the same hypotheses. The comparisons to Exhibits 1.1 and 1.2 can be reported together and the comparison to Exhibit 1.3 must be reported separately.

5.11.2 Each of the sub-exhibits being reported together will be listed in the statistical analysis paragraph.

5.12 CODIS Eligibility

5.12.1 A "Combined DNA Index System (CODIS)" sub-section will be included in the report after the "Statistical Analysis".

5.12.1.1 The CODIS eligibility for all DNA profiles suitable for comparison purposes will be addressed in a separate CODIS table.

5.12.1.2 The entered profile exhibit number, description of the sub-exhibit, other sub-exhibits with consistent DNA profiles where applicable, and the CODIS level will be listed in the table.

5.12.1.3 The following footnote will follow the table:

5.12.1.3.1 CODIS Eligibility: A DNA profile may be uploaded to one of three levels of the Combined DNA Index System (CODIS), depending on the quality and quantity of genetic data present. DNA profiles entered at the LDIS level are searched against ATF Laboratory-generated profiles. DNA profiles uploaded to the SDIS level are routinely searched against federal laboratory-generated profiles. DNA profiles uploaded to the NDIS level are routinely searched against the national database.

5.12.1.4 The following is an example of a CODIS table with the appropriate columns.

Sub-Exhibit	Evidence Description	CODIS Level
1.1 - Contributor 1	Swabs of grips of pistol	NDIS
1.1 - Contributor 2	Swabs of grips of pistol	LDIS
3.1	Swabs of mouth of bottle	NDIS

A DNA profile may be uploaded to one of three levels of the Combined DNA Index System (CODIS), depending on the quality and quantity of genetic data present. DNA profiles entered at the LDIS level are searched against ATF Laboratory-generated profiles. DNA profiles uploaded to the SDIS level are routinely searched against federal laboratory-generated profiles. DNA profiles uploaded to the NDIS level are routinely searched against the national database.

5.12.1.5 When a component of a mixture is listed in the CODIS table, the STRmix™-generated contributor order number will be used to describe the sub-exhibit.

5.12.1.6 Immediately following the CODIS table, the following statement will be listed:

5.12.1.6.1 Exhibits not listed in the table either did not meet the eligibility requirements for CODIS entry or are consistent with another profile in CODIS.

5.12.1.7 If no CODIS-eligible profiles were obtained, then the following statement will replace the table.

5.12.1.7.1 No DNA profiles meeting the eligibility requirements for CODIS entry were obtained.

5.13 Immediately before the Disposition of Evidence section, the following statement shall be included:

5.13.1.1 These conclusions conform with the relevant Department of Justice policy on Uniform Language for Testimony and Reports available at *www.justice.gov*.

5.14 In the Disposition of Evidence Section, one of the following statements shall be made, as appropriate:

5.14.1 The evidence will be returned to the investigating agent.

5.14.2 The evidence was returned to the investigating agent on *month day, year.*

5.15 The disposition of all DNA extracts shall be noted in the Disposition of Evidence section of the report.

5.15.1 DNA Extracts Retained: The following DNA extracts will be retained by the ATF Laboratory: Exhibits *1.1, 2.1, etc.*

5.15.2 DNA Extracts Consumed in Analysis: The following DNA extracts were consumed in analysis: Exhibits *1.1, 2.1, etc.*

5.16 At the end of the report, if any potential biological evidence is remaining on an exhibit, the following statement shall be made:

5.16.1 Note: Exhibit *X* may potentially contain biological evidence subject to specific storage and preservation requirements. Please reference ATF O 3400.1D and the *ATF Property Taken into Bureau Custody Manual* to review the storage and preservation requirements of this evidence for the purposes of possible future DNA analysis.

5.17 An "Appendix" will be attached to all reports where a comparison has been made. The "Appendix" will contain the following, at a minimum:

5.17.1 Description of a Likelihood Ratio (LR)

5.17.2 The typical hypotheses used in the calculation of an LR

5.17.3 The LR ranges for the possible conclusions

5.17.4 Definition of limited support for inclusion/exclusion

5.17.5 Definition of uninformative results

5.18 If an additional submission is reported separately, the new report will include prior reported information and statistical analyses as follows:

5.18.1 Reference samples from previous submissions that have already been reported will be added to the new submission report if comparisons are being made to the evidence in the new submission.

5.18.2 Evidence samples (only those suitable for comparison purposes) from previous submissions that have already been reported will be added to the new submission report if comparisons are being made to reference samples in the new submission.

5.18.3 If the new submission contains both reference samples and evidence, then reference samples and evidence samples that were suitable for comparison from previous submissions will be added to the report for the new submission. Any statistical analyses from the reference samples and evidence reported from the previous submission will be included in the report for the new submission.

5.18.4 When a reference sample from a previous submission is included in the new submission report table, the reference exhibit and result will be listed at the top of the table.

5.18.5 A note will be added to the new report referencing the source of the previously reported data, and the date the original report was issued.

5.18.5.1 "Reference [and/or evidence] samples listed in Table X (Exhibits A, B, and C) were compared to [reference and/or] evidence samples in this submission. These reference [and/or evidence] samples were originally analyzed and reported under ATF Lab # [CASE] on [DATE]."

5.19 Evidentiary profiles associated with contamination or failed controls where re-analysis is not possible.

5.19.1 For evidentiary samples associated with contamination or failed controls where it is reasonable to assume that the integrity of the DNA analysis was not affected and the interpretation of the associated DNA profiles was not affected, the results shall be reported as described above.

5.19.2 For evidentiary samples associated with contamination or failed controls where it is reasonable to assume that the integrity of the DNA analysis may have been affected, the results shall be reported as described above. In addition, a note shall be added to the report briefly describing the issue and stating the results should be interpreted with caution after the results table.

5.19.3 For mixed DNA evidentiary profiles demonstrating the presence of a staff member or known contaminant, the results of the comparison shall be listed in the table. When it is determined that contamination occurred based on the LR and case information, the LR shall be calculated conditioning on the presence of the staff member or known contaminant based on the analyst's discretion and with approval of the DNA Technical Leader.

5.19.4 For evidentiary profiles in which staff contamination has occurred and the staff member has generated an LR that demonstrates support for inclusion, the following shall be stated in the Support for Inclusion column for that sub-exhibit: "ATF Staff Index (FSLX-XXX)".

5.19.5 If a staff member has been detected as a possible contributor to one or more DNA profiles, the staff member will be compared to all other relevant evidence samples (e.g. other exhibits handled or examined by the same staff member at the same time). The results of the comparisons to the staff member will be listed in the table of results and the statistical analyses will be reported, if applicable.

5.20 Comparisons to reference DNA profiles from another laboratory

5.20.1 If a reference DNA profile generated by another laboratory was used for comparison to an ATF-generated evidentiary profile, a note shall be made in the report prior to the results table describing the source of the reference DNA profile.

5.20.1.1 Example: The DNA profiles obtained from the evidence in this case were compared to DNA profiles identified as coming from Jonathan Doe (SERI Exhibit 2A) and Christopher Doe (SERI Exhibit 4) analyzed and reported by the Serological Research Institute, SERI (see SERI report dated May 5, 2014, signed by Thomas Brown). SERI.

1. Scope

This supplemental protocol lists the template paragraphs for reporting the statistical calculations for most common scenarios. This list is not exhaustive and minor changes may be needed for scenarios not covered.

2. References

Not applicable

3. Equipment

Not applicable

4. Safety/Quality Assurance

Not applicable

5. Procedure

#1	
Scenario	**Single source/partial single source DNA profile, inclusionary LR**
Statistical statement	A [partial] DNA profile consistent with a single contributor was obtained from Exhibit 1.1. A. A single individual is assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1). C. Exclusionary Hypothesis (H_E): The DNA originated from an unrelated, unknown individual. D. The DNA profile is [at least] X times more likely if it originated from John Doe (Exhibit 2.1) than if it originated from an unrelated, unknown individual. E. The probability of an unrelated individual in the population, who has not contributed DNA to this sample, yielding this level of support, is less than 1 in X. F. Based on this calculation, there is [limited] evidentiary support for the inclusion of John Doe (Exhibit 2.1) as a possible contributor of the DNA profile obtained from Exhibit 1.1.

#2	
Scenario	**Single source/partial single source DNA profile, limited support for exclusion**
Statistical statement	A [partial] DNA profile consistent with a single contributor was obtained from Exhibit 1.1. A. A single individual is assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1). C. Exclusionary Hypothesis (H_E): The DNA originated from an unrelated, unknown individual. D. The DNA profile is [1/X] times more likely if it originated from an unrelated, unknown individual than if it originated from John Doe (Exhibit 2.1). E. Based on this calculation, there is limited evidentiary support for the exclusion of John Doe (Exhibit 2.1) as a possible contributor of the DNA profile obtained from Exhibit 1.1.

#3	
Scenario	**Single source/partial single source DNA profile, uninformative**
Statistical statement	A [partial] DNA profile consistent with a single contributor was obtained from Exhibit 1.1. A. A single individual is assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1). C. Exclusionary Hypothesis (H_E): The DNA originated from an unrelated, unknown individual. D. The statistical analysis resulted in a likelihood ratio equivalent to 1 which means that the comparison is uninformative. Therefore, the evidence provides no greater support for either the inclusion or exclusion of John Doe (Exhibit 2.1) as a possible contributor of the DNA profile obtained from Exhibit 1.1.

#4	
Scenario	**Mixed/partial mixed DNA profile, inclusionary LR**
Statistical statement	A [partial] DNA profile consistent with a mixture of N individuals was obtained from Exhibit 1.1. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) and N-1 unrelated, unknown individual[s]. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The DNA profile is [at least] X times more likely if it originated from John Doe (Exhibit 2.1) and N-1 unrelated, unknown individual[s] than if it originated from N unrelated, unknown individuals. E. The probability of an unrelated individual in the population, who has not contributed DNA to this sample, yielding this level of support, is less than 1 in X. F. Based on this calculation, there is [limited] evidentiary support for the inclusion of John Doe (Exhibit 2.1) as a possible contributor to the DNA profile obtained from Exhibit 1.1.

#5	
Scenario	**Mixed/partial mixed DNA profile, limited support for exclusion**
Statistical statement	A [partial] DNA profile consistent with a mixture of N individuals was obtained from Exhibit 1.1. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) and N-1 unrelated, unknown individual[s]. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The DNA profile is [1/X] times more likely if it originated from N unrelated, unknown individuals than if it originated from John Doe (Exhibit 2.1) and N-1 unrelated, unknown individual[s]. E. Based on this calculation, there is limited evidentiary support for the exclusion of John Doe (Exhibit 2.1) as a possible contributor to the DNA profile obtained from Exhibit 1.1.

#6	
Scenario	**Mixed/partial mixed DNA profile, uninformative**
Statistical statement	A [partial] DNA profile consistent with a mixture of N individuals was obtained from Exhibit 1.1. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) and N-1 unrelated, unknown individual[s]. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The statistical analysis resulted in a likelihood ratio equivalent to 1 which means that the comparison is uninformative. Therefore, the evidence provides no greater support for either the inclusion or exclusion of John Doe (Exhibit 2.1) as a possible contributor to the DNA profile obtained from Exhibit 1.1.

#7	
Scenario	**Single source major component, inclusionary LR**
Statistical statement	A [partial] DNA profile consistent with a mixture of at least N individuals was obtained from Exhibit 1.1. The discernible major component is consistent with a single contributor. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) as the major component and N-1 unrelated, unknown individual[s]. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The DNA profile is [at least] X times more likely if it originated from John Doe (Exhibit 2.1) as the major component and N-1 unrelated, unknown individual[s] than if it originated from N unrelated, unknown individuals. E. The probability of an unrelated individual in the population, who has not contributed DNA to this sample, yielding this level of support, is less than 1 in X. F. Based on this calculation, there is [limited] evidentiary support for the inclusion of John Doe (Exhibit 2.1) as a possible contributor of the major component of the DNA profile obtained from Exhibit 1.1.

#8	
Scenario	**Single source major component, limited support for exclusion**
Statistical statement	A [partial] DNA profile consistent with a mixture of at least N individuals was obtained from Exhibit 1.1. The discernible major component is consistent with a single contributor. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) as the major component and N-1 unrelated, unknown individual[s]. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The DNA profile is [1/X] times more likely if it originated from N unrelated, unknown individuals than if it originated from John Doe (Exhibit 2.1) as the major component and N-1 unrelated, unknown individual[s]. E. Based on this calculation, there is limited evidentiary support for the exclusion of John Doe (Exhibit 2.1) as a possible contributor of the major component of the DNA profile obtained from Exhibit 1.1.

#9	
Scenario	**Single source major component, uninformative**
Statistical statement	A [partial] DNA profile consistent with a mixture of at least N individuals was obtained from Exhibit 1.1. The discernible major component is consistent with a single contributor. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) as the major component and N-1 unrelated, unknown individual[s]. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The statistical analysis resulted in a likelihood ratio equivalent to 1 which means that the comparison is uninformative. Therefore, the evidence provides no greater support for either the inclusion or exclusion of John Doe (Exhibit 2.1) as a possible contributor of the major component of the DNA profile obtained from Exhibit 1.1.

#10	
Scenario	**Mixed major component, inclusionary LR**
Statistical statement	A [partial] DNA profile consistent with a mixture of at least N individuals was obtained from Exhibit 1.1. The discernible major component is consistent with a mixture of M individuals. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) as a contributor to the major component and N-1 unrelated, unknown individuals. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The DNA profile is [at least] X times more likely if it originated from John Doe (Exhibit 2.1) as a contributor to the major component and N-1 unrelated, unknown individuals than if it originated from N unrelated, unknown individuals. E. The probability of an unrelated individual in the population, who has not contributed DNA to this sample, yielding this level of support, is less than 1 in X. F. Based on this calculation, there is [limited] evidentiary support for the inclusion of John Doe (Exhibit 2.1) as a possible contributor to the major component of the DNA profile obtained from Exhibit 1.1.

#11	
Scenario	**Mixed major component, limited support for exclusion**
Statistical statement	A [partial] DNA profile consistent with a mixture of at least N individuals was obtained from Exhibit 1.1. The discernible major component is consistent with a mixture of M individuals. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) as a contributor to the major component and N-1 unrelated, unknown individuals. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The DNA profile is [1/X] times more likely if it originated from N unrelated, unknown individuals than if it originated from John Doe (Exhibit 2.1) as a contributor to the major component and N-1 unrelated, unknown individuals. E. Based on this calculation, there is limited evidentiary support for the exclusion of John Doe (Exhibit 2.1) as a possible contributor to the major component of the DNA profile obtained from Exhibit 1.1.

#12	
Scenario	**Mixed major component, uninformative**
Statistical statement	A [partial] DNA profile consistent with a mixture of at least N individuals was obtained from Exhibit 1.1. The discernible major component is consistent with a mixture of M individuals. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) as a contributor to the major component and N-1 unrelated, unknown individuals. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The statistical analysis resulted in a likelihood ratio equivalent to 1 which means that the comparison is uninformative. Therefore, the evidence provides no greater support for either the inclusion or exclusion of John Doe (Exhibit 2.1) as a possible contributor to the major component of the DNA profile obtained from Exhibit 1.1.

#13	
Scenario	**The same single source DNA profile was obtained from multiple samples from the same exhibit and a sample from another exhibit**
Statistical statement	[Partial] DNA profiles consistent with a single contributor were obtained from Exhibits 1.1, 1.2, 1.3 and 4.1. The statistical analysis was performed for each DNA profile individually and the lowest value is reported. A. A single individual is assumed to have contributed to each DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1). C. Exclusionary Hypothesis (H_E): The DNA originated from an unrelated, unknown individual. D. The DNA profiles are at least X times more likely if they originated from John Doe (Exhibit 2.1) than if they originated from an unrelated, unknown individual. E. The probability of an unrelated individual in the population, who has not contributed DNA to these samples, yielding this level of support, is less than 1 in X. F. Based on this calculation, there is [limited] evidentiary support for the inclusion of John Doe (Exhibit 2.1) as a possible contributor of the DNA profiles obtained from Exhibits 1.1, 1.2, 1.3 and 4.1.

#14	
Scenario	**A group of comparisons for similar mixed DNA profiles from the same exhibit to the same individual with the same hypotheses where there is no >99% contributor**
Statistical statement	[Partial] DNA profiles consistent with a mixture of N individuals were obtained from Exhibits 1.1, 1.2, and 1.3. The statistical analysis was performed for each DNA profile individually and the lowest value is reported. A. N individuals are assumed to have contributed to each DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) and N-1 unrelated, unknown individual[s]. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The DNA profiles are at least X times more likely if they originated from John Doe (Exhibit 2.1) and N-1 unrelated, unknown individual[s] than if they originated from N unrelated, unknown individuals. E. The probability of an unrelated individual in the population, who has not contributed DNA to these samples, yielding this level of support, is less than 1 in X. F. Based on this calculation, there is evidentiary support for the inclusion of John Doe (Exhibit 2.1) as a possible contributor to the DNA profiles obtained from Exhibits 1.1, 1.2, and 1.3.

#15	
Scenario	**Two or more individuals are included as possible contributors to a mixture when compared individually. However, there is no support when the comparison is performed to determine if both could be possible contributors together.**
Statistical statement	A [partial] DNA profile consistent with a mixture of N individuals was obtained from Exhibit 1.1. A. N individuals are assumed to have contributed to each DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1), John Smith (Exhibit 3.1), and N-2 unrelated, unknown individual[s]. C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. Although independently there is evidentiary support for the inclusion of John Doe (Exhibit 2.1) and John Smith (Exhibit 3.1), the statistical analysis does not support the proposition that together, John Doe (Exhibit 2.1) and John Smith (Exhibit 3.1) are both contributors to the DNA profile obtained from Exhibit 1.1.

#16	
Scenario	**Mixed / partial mixed DNA profile with a conditioned component, inclusionary LR**
Statistical statement	A [partial] DNA profile consistent with a mixture of N individuals was obtained from Exhibit 1.1. A. N individuals are assumed to have contributed to the DNA profile. B. Bob Smith (Exhibit 3.1) is assumed to be a contributor to the mixture. C. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1), Bob Smith (Exhibit 3.1), and N-2 unrelated, unknown individual[s]. D. Exclusionary Hypothesis (H_E): The DNA originated from Bob Smith (Exhibit 3.1) and N-1 unrelated, unknown individuals. E. The DNA profile is at least X times more likely if it originated from John Doe (Exhibit 2.1), Bob Smith (Exhibit 3.1) and N-2 unrelated, unknown individual[s] than if it originated from Bob Smith (Exhibit 3.1) and N-1 unrelated, unknown individuals. F. The probability of an unrelated individual in the population, who has not contributed DNA to this sample, yielding this level of support, is less than 1 in X. G. Based on this calculation, there is [limited] evidentiary support for the inclusion of John Doe (Exhibit 2.1) as a possible contributor to the DNA profile obtained from Exhibit 1.1.

#17	
Scenario	**Two comparisons for similar mixed DNA profiles with major components from the same exhibit to the same individual with the same hypotheses where there is no >99% contributor**
Statistical statement	[Partial] DNA profiles consistent with a mixture of at least N individuals were obtained from Exhibits 1.1 and 1.2. The discernible major components are consistent with a mixture of M individuals. The statistical analysis was performed for each DNA profile individually and the lowest value is reported. A. N individuals are assumed to have contributed to the DNA profile. B. Inclusionary Hypothesis (H_I): The DNA originated from John Doe (Exhibit 2.1) as a contributor to the major component and N-1 unrelated, unknown individual(s). C. Exclusionary Hypothesis (H_E): The DNA originated from N unrelated, unknown individuals. D. The DNA profiles are at least X times more likely if they originated from John Doe (Exhibit 2.1) as a contributor to the major components and N-1 unrelated, unknown individuals than if they originated from N unrelated, unknown individuals. E. The probability of an unrelated individual in the population, who has not contributed DNA to these samples, yielding this level of support, is less than 1 in X. F. Based on this calculation, there is [limited] evidentiary support for the inclusion of John Doe (Exhibit 2.1) as a possible contributor to the major components of the DNA profiles obtained from Exhibit 1.1 and 1.2.

Appendix:

"A Likelihood Ratio (LR) is a statistic for the comparison of the probability of the evidence (E), given two competing propositions. The inclusionary proposition (H_I) includes the person of interest (POI) and, for mixed samples, known and/or unknown, unrelated individuals. The total count of individuals included in the proposition is equal to the number of contributors interpreted to be in the sample. The exclusionary proposition (H_E) generally consists of unknown, unrelated individuals, equaling the total number of contributors interpreted to be in the sample."[1] Additional hypotheses can be considered upon request.

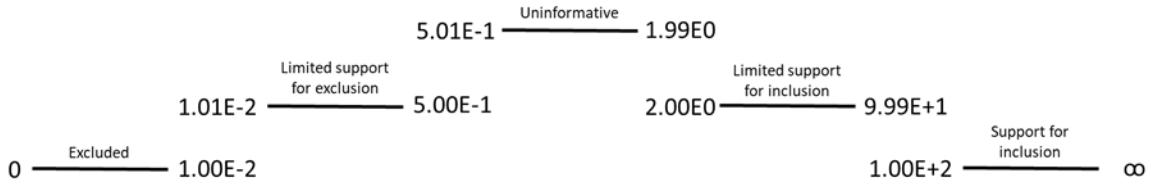
Likelihood Ratios occur on a continuum. Equal (or nearly equal) support for both propositions results in a likelihood ratio of 1, which is qualified as Uninformative. The magnitude of the LR reflects the support for one proposition over the other. LRs greater than one reflect a greater support for the inclusionary proposition. LRs less than one reflect a greater support for the exclusionary proposition.[2] The maximum LR reported is one trillion. LRs greater than one trillion are reported as "at least one trillion."

The typical assumption and propositions or hypotheses are listed below:

A. X number of individuals are assumed to have contributed to the DNA profile.
B. Inclusionary Hypothesis (H_I): POI and X-1 unknown individuals are assumed to have contributed to the DNA profile.
C. Exclusionary Hypothesis (H_E): X unknown individuals are assumed to have contributed to the DNA profile.

The assumptions and hypotheses are not listed in the report when the POI is excluded from the evidentiary profile.

The conclusions drawn from the LR are based on the scale below:



Limited Support for Inclusion/Exclusion: an examiner's conclusion that the evidence provides greater support for one hypothesis over the other; however, there is a greater risk of adventitious support (false inclusion/exclusion) in this range.[2]

Uninformative results: an examiner's conclusion that the evidence provides no greater support for either the inclusion or the exclusion of the POI as a possible contributor to the DNA typing results obtained from an evidentiary sample.[1]

[1] Department of Justice Uniform Language for Testimony and Reports for Forensic Autosomal DNA Examinations Using Probabilistic Genotyping Systems – adopted September 18, 2018, effective March 18, 2019
[2] Recommendations of the SWGDAM Ad Hoc Working Group on Genotyping Results Reported as Likelihood Ratios, approved July 12, 2018

1. Scope

This protocol is to be used to estimate the concentration of DNA in an extract. The amount of amplifiable human DNA in a sample can be determined both quickly and accurately using Real-Time PCR with the Applied Biosystems™ Quantifiler™ HP DNA Quantification Kit. The Quantifiler™ HP Kit combines two human DNA-specific assays and an internal PCR control (IPC) assay. The human DNA-specific assays includes primers specific for two human (or higher primate) DNA sequences, one short PCR amplicon and one long PCR amplicon, and TaqMan® probes that bind to one of those sequences. The IPC assay includes a unique synthetic DNA sequence as well as primers and a TaqMan® probe specific for that synthetic sequence. This method can be used to detect DNA concentrations between 0.005 ng/µL and 50 ng/µL. The IPC can detect the presence of inhibitory substances. However, a normal IPC result only indicates that no inhibition was detected. If a larger volume of extract is added to the STR amplification reaction, some inhibition may still be observed that was not detected by the IPC.

The TaqMan® probes contain a reporter dye at one end (VIC™ for the small autosomal human DNA target, ABY™ for the large autosomal human DNA target, and JUN™ for the IPC) and a non-fluorescent quencher at the other. Due to the physical proximity of the quencher, the reporter dye is unable to be detected. During amplification, the DNA polymerase enzyme cleaves TaqMan® probes that are bound to the target sequence, separating the fluorescent reporter from the quencher. When this separation occurs, the resulting fluorescence can be detected by the Applied Biosystems™ 7500 Real-Time PCR System. A reading is taken during each amplification cycle and fluorescence from the samples is compared to the fluorescence from known quantities of DNA Standards. The amount of fluorescence detected is in direct proportion to the amount of DNA present in the sample. The PCR cycle number at which the level of fluorescence in a sample exceeds a user-defined cycle threshold (C_T) is used in this comparison.

While the amount of human DNA varies between samples, the amount of IPC template does not. Therefore, the C_T determined for the IPC should be similar from one sample to the next. If the IPC C_T values are not similar, there is more than one possible explanation:

If a sample contains a very high quantity of human DNA, that human DNA is preferentially amplified showing reduced IPC amplification.

If a sample contains inhibitors, amplification of both the human DNA and the IPC DNA will be reduced. Examination of the IPC amplification results is critical to distinguishing between samples with DNA concentrations below detectable levels and those that are inhibited.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

2.1. Applied Biosystems™ Quantifiler HP and Trio Quantification Kits User Guide, 2017.
2.2. Applied Biosystems™ 7500 Real-Time PCR Systems Chemistry Guide and the Quantifiler™ HP and Trio DNA Quantification Kits User Guide.
2.3. Prince George's County (MD) Police, Forensic Services Division, Serology/DNA Laboratory Short Tandem Repeat (STR) Analysis Protocol, 2006.
2.4. R. Higuchi, C. Fockler, G. Dollinger, R. Watson, Kinetic PCR analysis: real-time monitoring of DNA amplification reactions, Biotechnology 11 (1993) 1026-1030.
2.5. J.M. Butler, Sample collection, DNA extraction, and DNA quantitation, in: Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers, second ed., Elsevier Academic Press, Burlington, 2005, pp. 50-56.
2.6. J.S. Waye, L.A. Presley, B. Budowle, G.G. Shutler, R.M. Fourney, A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts, BioTechniques 7 (1989) 852-855.
2.7. H. Andréasson, M. Nilsson, B. Budowle, H. Lundberg, M. Allen, Nuclear and mitochondrial DNA quantification of various forensic materials, Forensic Science International 164 (2006) 56-64.
2.8. R.L. Green, I.C. Roinestad, C. Boland, L.K. Hennessy, Developmental validation of the Quantifiler™ Real-Time PCR kits for the quantification of human nuclear DNA samples, J. Forensic Sci. 50 (2005) 1-17.
2.9. A. Holt, S. Wootton, J. Mulero, P. Brzoska, E. Langit, R. Green, Developmental validation of the Quantifiler™ HP and Trio Kits for human DNA quantification in forensic samples, Forensic Science International: Genetics 21 (2016) 145-157.

3. Equipment

3.1. Disposable gloves
3.2. Eye protection
3.3. Lab coat
3.4. TE^{-4} (10 mM Tris-HCl , 0.1 mM EDTA, pH 8.0)
3.5. MicroAmp™ Optical Reaction Plate
3.6. MicroAmp™ Splash Free Support Base
3.7. MicroAmp™ Optical Adhesive Film
3.8. MicroAmp™ Adhesive Film Applicator
3.9. Applied Biosystems™ 7500 Real-Time PCR System
3.10. Microcentrifuge tubes
3.11. 1.5 mL Low DNA Bind tubes (Sarstedt, part # # 72.706.700)
3.12. Pipettors

3.13. Benchtop hood
3.14. Disposable aerosol-resistant pipettor tips
3.15. Plate centrifuge
3.16. Vortexer
3.17. Centrifuge
3.18. Quantifiler™ HP DNA Kit
3.19. 10% bleach solution
3.20. 70% ethanol

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling reagents and sample tubes.
4.2. Lab coat and eye protection must be worn at all times while performing this procedure.
4.3. Sample setup must be performed in a benchtop hood. Clean surfaces with 10% bleach solution followed by 70% ethanol before and after use. After exiting the hood, turn on the UV light for 15 minutes.
4.4. Only one sample tube shall be open at a time.
4.5. Use aerosol-resistant pipettor tips when transferring liquids containing DNA.
4.6. Change pipettor tips after transferring any liquids potentially containing DNA.
4.7. Exercise caution when opening tubes.
4.8. Record the lot number of each reagent or kit used in notes. Do not use the reagents after the expiration date.
4.9. Ensure that the MicroAmp™ Optical Reaction Plate sits in the MicroAmp™ Splash Free Support Base and does not touch the surface of the hood. Also, make sure not to touch the bottom of the Optical Reaction Plate or the Optical Adhesive Cover as this can affect the instrument reading.
4.10. Ensure that Standards have not been stored in the refrigerator for more than two weeks.
4.11. The Standards will be run in duplicate.
4.12. Samples thought to contain lower levels of DNA will be handled before those thought to contain higher amounts of DNA.
4.13. A plate blank containing the master mix and 2 µL of TE^{-4} shall be added to the Optical Reaction Plate. The results must be below the detection limit of the system (5 pg/µL) to be acceptable.
4.14. No bubbles should be present in the wells of the Optical Reaction plate when it is added to the instrument. Any bubbles present will pop during the initial cycling stages on the instrument and affect the baseline fluorescence for those samples.
4.15. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Procedure

5.1. Remove the Quantifiler™ HP Primer Mix and THP PCR Reaction Mix from the freezer and thaw.

5.1.1. If the Quantifiler™ HP Primer Mix and THP PCR Reaction Mix were previously thawed, remove them from the refrigerator and continue with step 5.2.

5.1.2. Add 1 µL of the Quantifiler™ Automation Enhancement Kit buffer to the PCR Reaction Mix tube and vortex gently.

NOTE: The PCR Reaction Mix expires six months after the addition of the Quantifiler™ Automation Enhancement Kit buffer. Therefore, the PCR Reaction Mix will expire six months from the date of the addition or the manufacturer's expiration date, whichever is sooner.

5.2. If using Quantifiler™ HP DNA Standards that have been previously prepared and stored in the refrigerator, remove them from the refrigerator and proceed to Step 5.4.

5.3. Prepare the Quantifiler™ HP DNA Standards.

NOTE: During the QC procedure for Quantifiler™ HP Kits, the actual concentration of the Quantifiler™ THP DNA Standard is measured. Based on this value, the volume of Quantifiler™ THP DNA Dilution Buffer used for Standard 1 is adjusted to give Standard 1 a value of 50 ng/µL.

The following listed volumes can be increased (doubled, tripled, etc.) as long as the ratio remains the same.

5.3.1. Label five (5) 1.5 mL Low DNA Bind microcentrifuge tubes to correspond to the five Standards.

5.3.2. Vortex the Quantifiler™ THP DNA Standard and spin the tube briefly in the microcentrifuge to remove the liquid from the cap of the tube.

5.3.3. Check the Reagent Logbook to see how much Quantifiler™ THP DNA Dilution Buffer is needed to create ***Standard 1*** based on the lot number of the Quantifiler™ THP DNA Standard.

5.3.4. Add the required volume of DNA Dilution Buffer to each labeled tube (see table below).

5.3.5. Using a new pipette tip, add 10 µL of the Quantifiler™ THP DNA Standard to the labeled tube for ***Standard 1***.

5.3.6. Vortex ***Standard 1*** thoroughly.

5.3.7. Prepare ***Standards 2 through 5*** as indicated in the following table, making sure to vortex the tubes between each Standard.

Standard	Concentration	Amounts
1	50.0	10 µL (Quant. THP DNA Std) + variable uL Quant. THP DNA dilution buffer
2	5.0	10 µL (**Standard 1**) + 90 µL dilution buffer
3	0.5	10 µL (**Standard 2**) + 90 µL dilution buffer
4	0.05	10 µL (**Standard 3**) + 90 µL dilution buffer
5	0.005	10 µL (**Standard 4**) + 90 µL dilution buffer

5.4. Prepare the samples as follows:

5.4.1. Calculate the volumes of the Primer Mix (8 uL per sample) and PCR Reaction Mix (10 uL per sample). Add an appropriate number of additional reactions to compensate for pipettor error.

NOTE: Do not forget to add the Standards when calculating the number of samples.

5.4.2. Vortex the Primer Mix and briefly centrifuge to remove liquid from the lid of the tube.

5.4.3. Gently vortex the PCR Reaction Mix.

5.4.4. Create a master mix by adding the appropriate volumes of Primer Mix and PCR Reaction Mix to a microcentrifuge tube.

5.4.5. Vortex the PCR Reaction Mix and centrifuge briefly.

5.5. Dispense 18 µL of master mix to the appropriate wells in the 96 well Optical Reaction Plate.

NOTE: The 96 well Optical Reaction Plate should remain in its base at all times. When handling the plate, do not touch the bottom of the plate or allow it to come into contact with the counter.

5.6. Add 2 µL of sample, control, or standard to the appropriate wells in the Optical Reaction Plate.

5.7. Hold the Optical Adhesive Cover by the edges and remove the backing from the center being careful not to touch the part of Optical Adhesive Cover that will cover the samples.

5.8. Place the Optical Adhesive Cover on the Optical Reaction Plate and seal it using the Adhesive Film Applicator.

5.9. While the plate is inside the base, tap the base on the benchtop to bring the bubbles to the liquid surface. Lift the plate, then inspect each well for bubbles; tap each well with a marker, pen, or gloved fingertip.

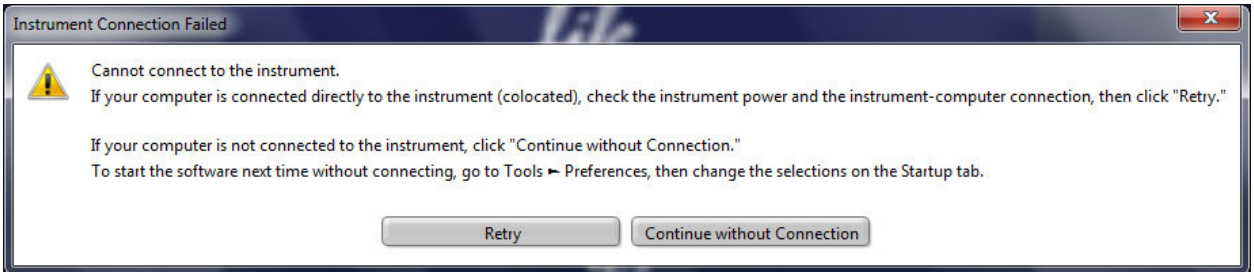
5.10. Carry the Optical Reaction Plate into the post-amplification room, and centrifuge the plate for approximately 20 seconds at 3000 rpm.

5.10.1. Check each well for the presence of bubbles. If bubbles are present, tap and re-centrifuge the plate until they have all been removed.

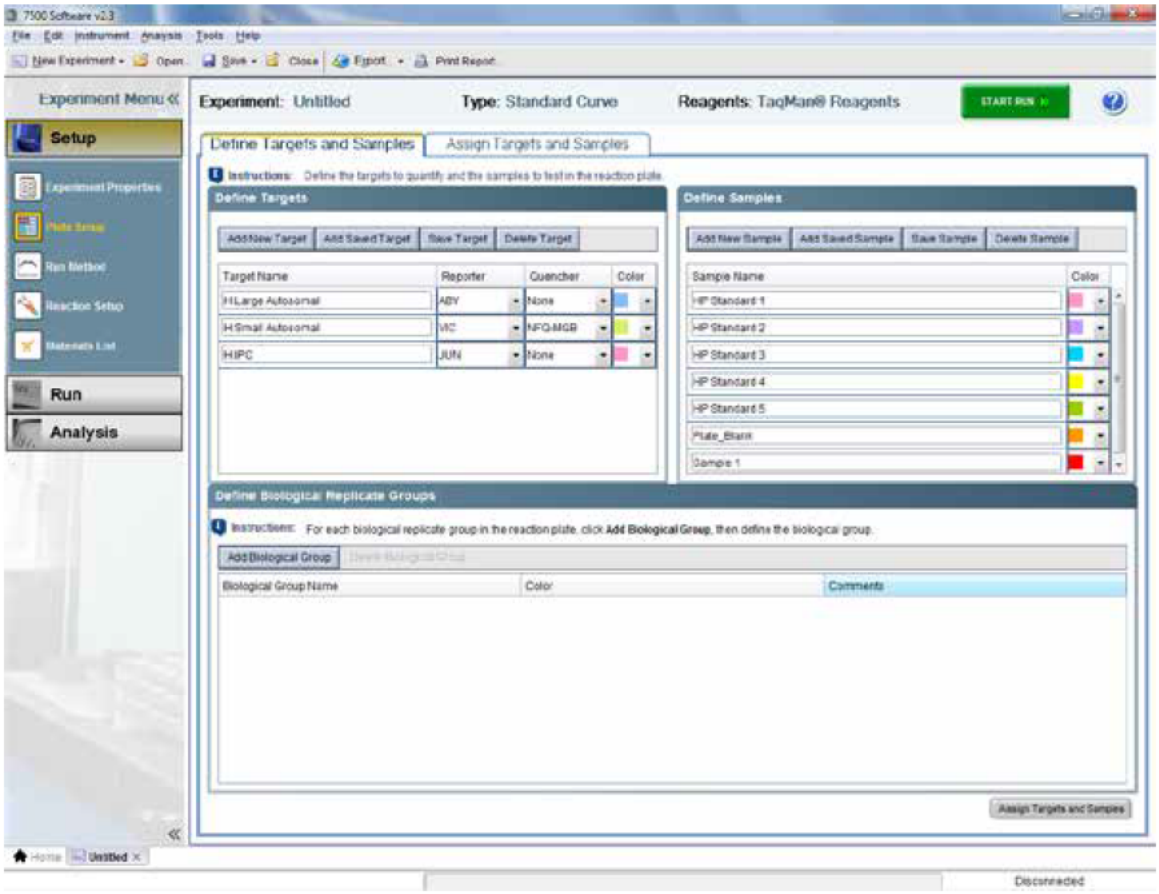
5.11. Turn on the AB 7500 Real-Time PCR System.

5.12. Open the sample tray of the AB 7500 and place the Optical Reaction Plate inside, noting that the notch is in the upper right corner of the plate. Close the sample tray.
5.13. On the computer, double click on the 7500 Software icon to open the software.
5.14. At the log-in screen, log-in as Guest.

NOTE: If you are using the software on a computer that is NOT connected to an instrument, click "Continue without Connection" to pass the following screen:



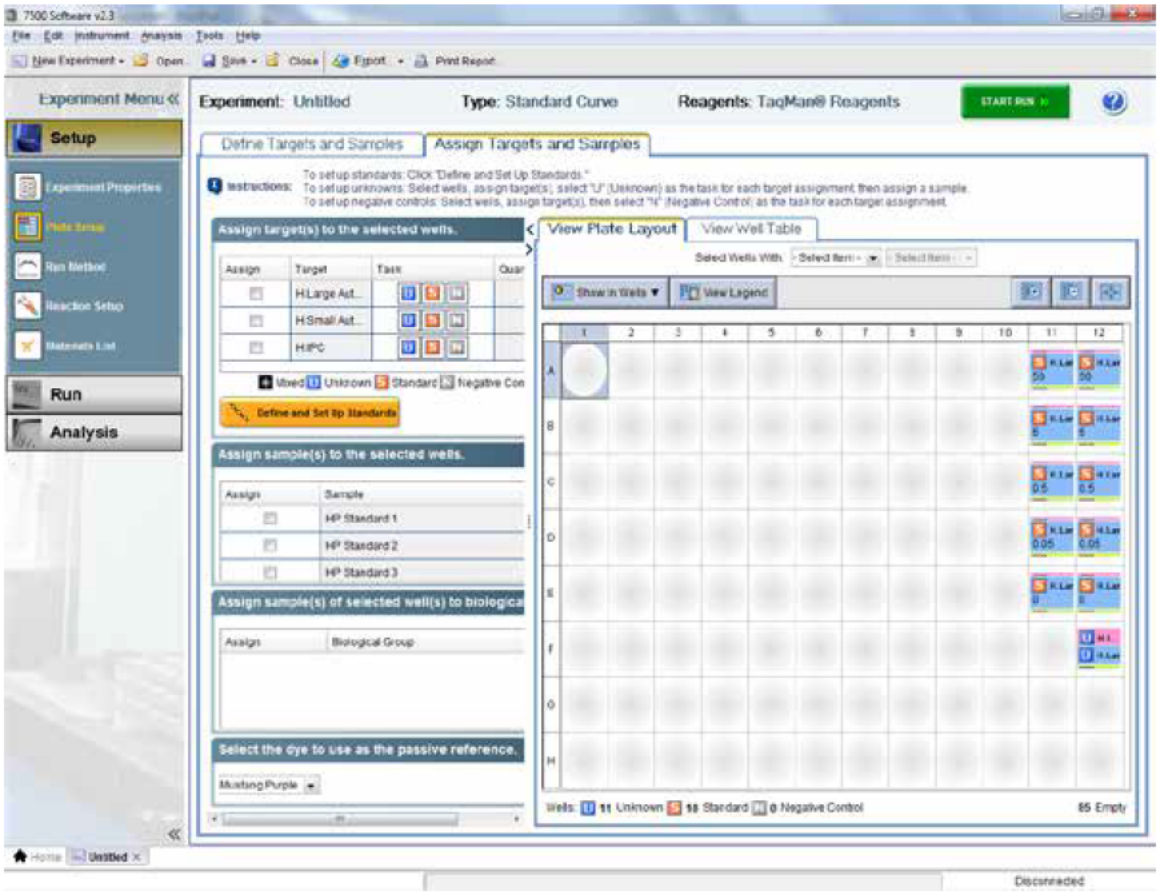
5.15. To create a new project from a template, select "New Experiment" > "From Template" from the menu bar.
  5.15.1. Select "QuantifilerHP_RightStandards_Import" from the templates folder and click Open.
5.16. To name the project, navigate to the "Experiment Properties" tab from the tool bar on the left side of the screen and type in the following:
  5.16.1. Experiment Name: include the date, operator's initials, and instrument name (e.g. 011618_TWB_Orioles)
  5.16.2. User Name: operator's initials
  5.16.3. Comments: any comments desired
5.17. To import sample information from an existing file, skip to **step 5.22**.
5.18. To enter sample data, navigate to the "Plate Setup" tab from the tool bar on the left side of the screen:
  5.18.1. Click on the "Define Targets and Samples" tab.
  5.18.2. Under the "Define Samples" heading click "Add New Sample".
  5.18.3. Enter the sample name in the box that opens.
  5.18.4. Click on the "Save Sample" button to save the sample name.
  5.18.5. Repeat the previous steps for each new sample name.
5.19. Verify the following:
  5.19.1. IPC Detector is associated with JUN™ Reporter.
  5.19.2. Large Autosomal Target Detector is associated with ABY™ Reporter.
  5.19.3. Small Autosomal Target Detector is associated with VIC™ Reporter.



5.20. To assign samples to plate wells, click on the "Assign Targets and Samples" tab.
  5.20.1. Highlight a single well to add sample information for that well (i.e. A1).
  5.20.2. While the plate well is highlighted, click the check box next to the sample of interest from the "Assign sample(s) to the selected wells" section.
  5.20.3. Repeat the previous two steps for each sample you wish to add to the plate.

5.21. To assign targets to the sample wells, highlight all of the sample wells that contain samples and click the check boxes next to the IPC and targets in the "Assign target(s) to the selected wells" section.

5.22. Verify the following:
  5.22.1. For the Standard wells, verify that the Detector Task is set to Standard (Orange 'S' Box) and the appropriate value is listed in the Quantity field (in ng/µL).
  5.22.2. For the unknown samples and plate blank wells, verify that the Detector Task is set to Unknown (Blue 'U' box) and leave the Quantity field blank.
  5.22.3. For all samples, verify that the IPC Detector Task is set to Unknown (Blue 'U' box).



5.23. To import sample information from an existing file:

5.23.1. Select “File” > “Import”.

5.23.2. Navigate to your existing sample import file and select “Import”.

5.23.3. When prompted to overwrite existing data select “YES”.

5.24. Under the ‘Run Method’ tab on the left side of the screen, make sure that the settings are as follows:

5.24.1. Sample Volume (µL): 20

5.24.2. Holding Stage:

5.24.2.1. 95 degrees, 2 minutes

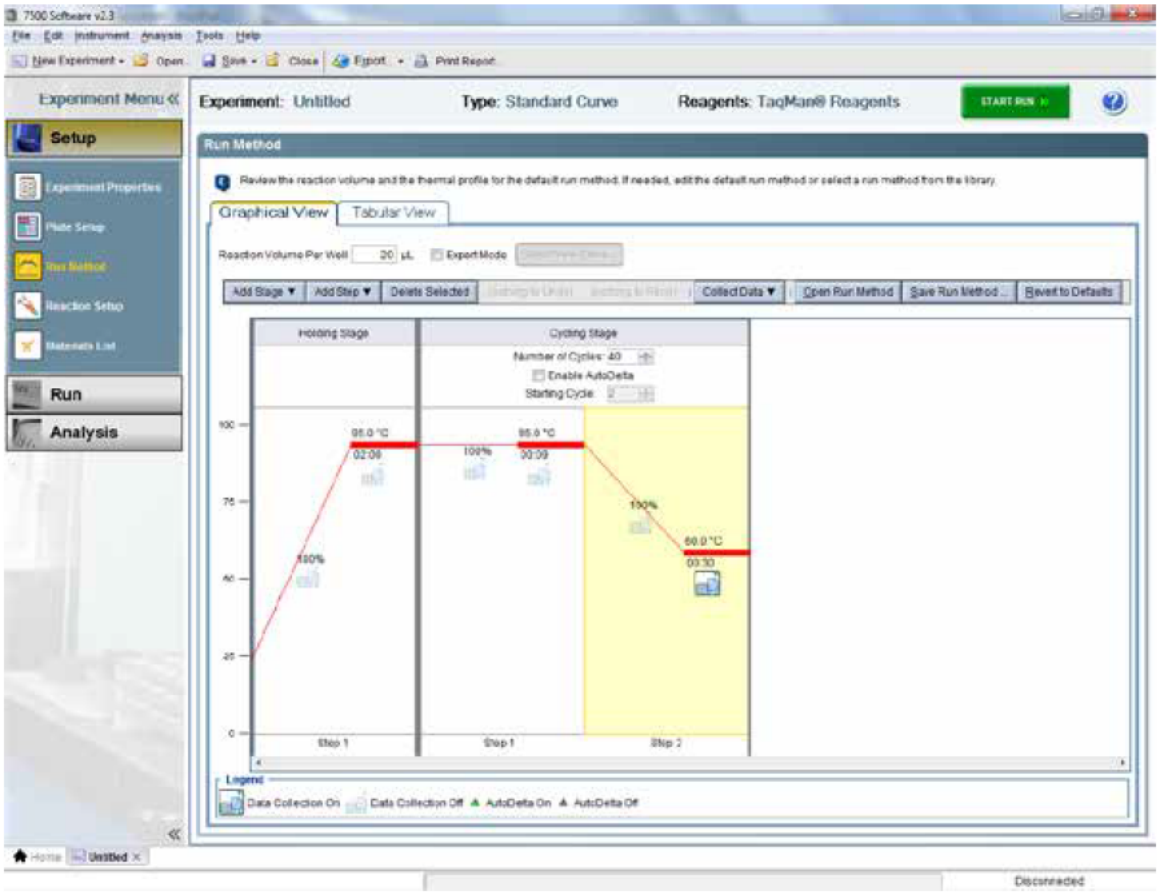
5.24.3. Cycling Stage:

5.24.3.1. Number of Cycles: 40

5.24.3.2. Step 1: 95 degrees, 9 seconds

5.24.3.3. Step 2: 60 degrees, 30 seconds

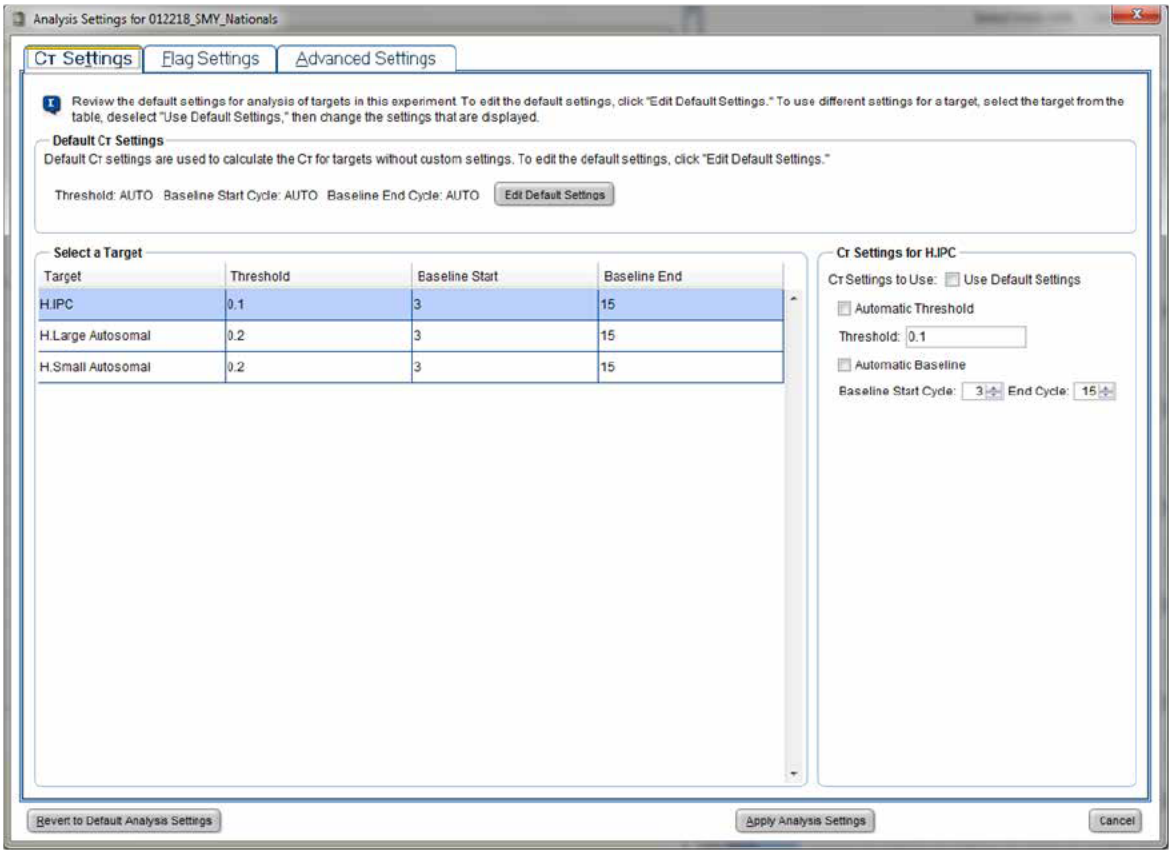
5.24.3.4. Starting Cycle: 2



5.25. To start the run: Select the green "Start Run" button at the top of any tab.

5.25.1. The software will prompt you to save the plate before thermal cycling begins. Use the run date, analyst's initials, and instrument name in the file name.

5.26. At the conclusion of the run, the program will switch to the analysis screen. Prior to analyzing the data, confirm the following analysis settings:



5.27. Click the large green "Analyze" button on the top right.

5.28. Using the buttons on the left side of the screen, select "Standard Curve".

5.28.1. Using the dropdown for "target" in the plot settings choose "All".

5.28.2. Ensure that the wells containing the quantification standards are selected.

5.28.3. Evaluate the Standard Curve to determine that the quantification results are accurate and reliable by examining the slope and the R^2 values.

5.28.3.1. Small Autosomal Slope: The value shall be between -3.0 and -3.6. A slope of -3.3 indicates 100% amplification efficiency.

5.28.3.2. Large Autosomal Slope: The value shall be between -3.1 and -3.7. A slope of -3.4 indicates 100% amplification efficiency.

5.28.3.3. R^2: The value shall be greater than 0.98.

5.29. The analyst may use his/her knowledge and experience to omit outlying data from both replicates for one data point and/or one replicate at each data point in the standard curve. To omit a data point:

5.29.1. Right click on the sample and select "Omit".

5.29.2. After you omit a data point, you must click the green "Analyze" button.

5.29.3. If the Standard Curve does not fall within the above listed specifications after omitting data points, it may be necessary to rerun Quantifiler™ HP.

5.30. To print the results:

5.30.1. Select the samples you want to be included in the printed report.

5.30.2. On the menu bar, select “File” > “Print Report” or click the “Print Report” icon.

5.30.3. Select the options you want printed.

5.30.3.1. Click “Print Preview”.

5.30.3.2. Using the icons on the top left of the pop-up window either print or save if the computer is not connected to a printer.

5.30.3.3. Only the Results Table and the Standard Curve must be printed.

5.31. To export the results:

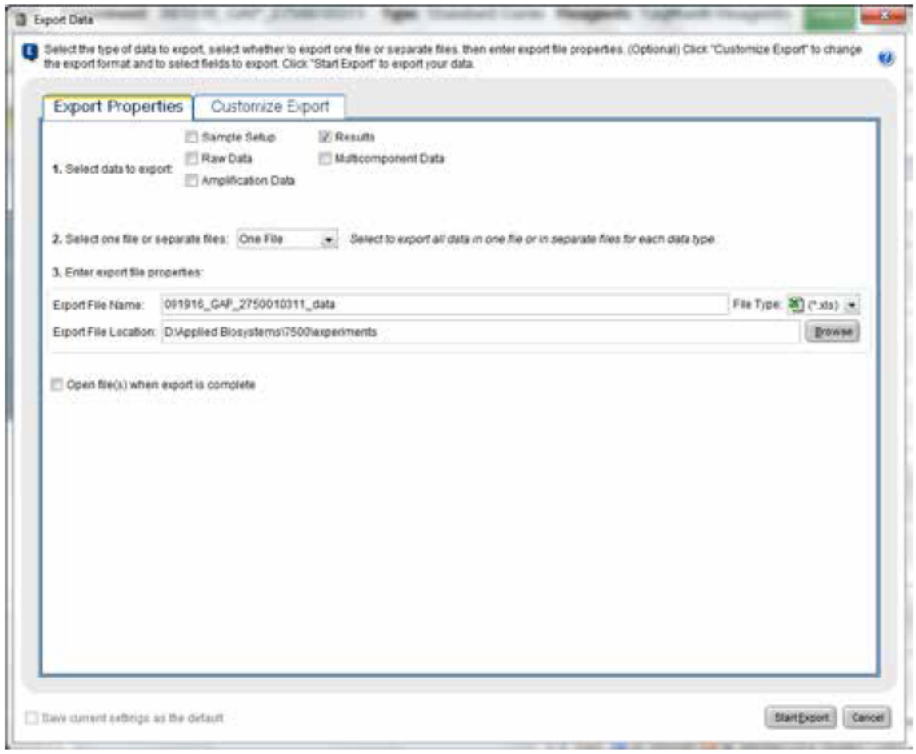
5.31.1. Highlight the samples you wish to export or the full sample plate.

5.31.2. On the menu bar, select “File” > “Export” or click the “Export” icon.

5.31.3. Ensure that the “Results” box is checked.

5.31.4. Select where you would like to save the file using the “Browse” button.

5.31.5. Export the results by clicking on the “Start Export” button.



5.32. Check the C_T values of the IPC to evaluate possible inhibition:

5.32.1. Import the exported results into the Quantifiler™ HP Data Worksheet using Microsoft Excel.

5.32.2. The Average IPC for the Standards is automatically calculated.

5.32.3. If the IPC value for a sample differs significantly from the Average IPC, there may be inhibition present and appropriate steps should be taken during the subsequent analysis. These steps include, but are not limited to, performing re-quantification or purifying samples using Microcon® Concentrators.
NOTE - If a sample contains a very high quantity of human DNA, that human DNA is preferentially amplified, showing reduced IPC amplification. If a sample contains inhibitors, amplification of both the human DNA and the IPC DNA will be reduced. Examination of the IPC amplification results is critical to distinguishing between samples with DNA concentrations below detectable levels and those that are inhibited.

5.33. Samples that produce "Undetermined" (0 ng/µl) results for both the Small and Large Autosomal Target and where there is no indication of inhibition may not be analyzed further at the analyst's discretion. Generally, if the difference between the sample IPC C_T value and the average IPC C_T value (for standards 3-5) is less than 1, it can be assumed there is no indication of significant inhibition. Other factors such as discoloration of the extract or the presence of inhibitors detected in samples from the

same exhibit may be considered. DNA extracts not processed further will be retained by the laboratory for possible future analysis.

1. Scope

This protocol describes an approach to collecting DNA from fired cartridge cases. The protocol is not meant to encompass all possible circumstances that may be encountered in forensic casework. An analyst may perform minor deviations from this protocol as circumstances necessitate based on his/her training and experience. Significant deviations must be approved by the DNA Technical Leader.

2. References

2.1. ATF Validation Documents – Validation of Modified Rinse/Swab Method with Copper Binding Additives for Collection of DNA from Fired Brass Cartridge Cases and subsequent modifications

2.2. ATF Validation Documents – Modification of Rinse and Swab Collection from Fired Cartridge Cases (ATF-LS-FB37)

2.3. D. Sweet, M. Lorente, J.A. Lorente, A. Valenzuela, E. Villanueva. An Improved Method to Recover Saliva from Human Skin: The Double Swab Technique. Journal of Forensic Sciences. 42 (1997): 320-322.

2.4. B.C.M. Pang, B.K.K. Cheung. Double swab technique for collecting touched evidence. Legal Medicine. 9 (2007): 181-184.

2.5. K. Yokawa, T. Kagenishi, T. Kawano. Prevention of Oxidative DNA Degradation by Copper-Binding Peptides. Bioscience, Biotechnology, and Biochemistry. 75 (2011): 1377-1379.

2.6. M. Holland, R. Bonds, C. Holland, J. McElhoe. Recovery of mtDNA from unfired metallic ammunition components with an assessment of sequence profile quality and DNA damage through MPS analysis. Forensic Science International: Genetics. 39 (2019): 86-96.

2.7. P. Dieltjes, R. Mieremet, S. Zuniga, T. Kraaijenbrink, J. Pijpe, P. de Knijff. A sensitive method to extract DNA from biological traces present on ammunition for the purpose of genetic profiling. International Journal of Legal Medicine. 125 (2011): 597-602.

2.8. S. Montpetit, P. O'Donnell. An optimized procedure for obtaining DNA from fired and unfired ammunition. Forensic Science International: Genetics. 17 (2015): 70-74.

2.9. T.C.R. Wan, L. MacDonald, Y. Perez, T.W. Bille, D.S. Podini. Recovering Touch DNA from Cartridge Casings Using a Method of Tape Lifting. Proceedings of the American Academy of Forensic Sciences. 67th Annual Scientific Meeting, Orlando, FL, 2015. B135.

2.10. T.W. Bille, G. Fahrig, S.M. Weitz, G.A. Peiffer. An improved process for the collection and DNA analysis of fired cartridge cases. Forensic Science International: Genetics. 46 (2020).

3. Equipment

3.1. Disposable gloves
3.2. Lab coat
3.3. Sterile swabs (foam)
3.4. Sterile water
3.5. Disposable 15 mL beakers (or suitable alternative)
3.6. Rubber-tipped forceps or suitable replacement
3.7. 70% ethanol or alcohol wipes
3.8. 10% bleach solution
3.9. Cartridge case rinse solution, containing (per sample):
  3.9.1. 500 µL Qiagen® Buffer ATL
  3.9.2. 30 µL BTmix solution (2 mg/mL BSA and 62.5 mg/mL GGH tripeptide)
3.10. P100/P200, and P1000 pipettes
3.11. Disposable aerosol-resistant P200 and P1000 pipette tips
3.12. Qiagen® Investigator Lyse & Spin baskets and microcentrifuge tubes
3.13. Bench paper

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling supplies, reagents, and evidence.
4.2. Use aerosol-resistant pipette tips when transferring liquids containing DNA.
4.3. Record the lot number and expiration date of each reagent used in notes. Do not use reagents after the expiration date.
4.4. Lab coat must be worn at all times while performing this procedure.
4.5. Exercise caution when opening evidence packaging and handling sharp objects.
4.6. The laboratory bench surface shall be cleaned before use with 10% bleach solution or other sanitizing agent and may be followed by 70% ethanol. Fresh bench paper shall then be placed on the surface prior to evidence examination.
4.7. Any utensils used to cut or manipulate the evidence must be cleaned between uses with 10% bleach solution and followed by 70% ethanol or alcohol wipe.
4.8. Disposable 15 mL beakers must be irradiated in the ultraviolet crosslinker prior to use.
4.9. Lyse & Spin tubes containing swabs and rinse solution will be labeled with sufficient detail to distinguish individual items of evidence.

5. Procedure

5.1. Evaluate each cartridge case for caliber, headstamp, metal type, condition, and firing pin estimation, if applicable, and record this information in the notes.

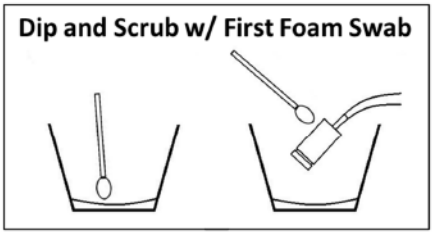
5.1.1. Collection may be performed on each cartridge case individually or on two cartridge cases together depending on the characteristics noted above and case information.

5.2. Prepare the rinse/lysis solution by combining 500 µL of Qiagen® Buffer ATL and 30 µL of BTmix per sample. Gently mix the solution (add at least one extra sample to ensure sufficient volume).

5.3. Draw 500 µL of rinse/lysis solution into a P1000 pipette tip and dispense into the 15 mL beaker.

5.4. Hold the cartridge case from the inside using rubber-tipped forceps. Hold the case with the head stamp facing down over the disposable 15 mL beaker.

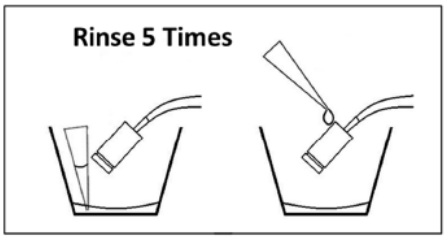
5.5. Repeatedly dip a sterile foam swab into the rinse/lysis solution and scrub the outer surfaces of the FCC while holding it over the 15 mL beaker.
NOTE: If performing collection on two FCCs together, complete steps 5.4, 5.5, 5.7, and 5.8 sequentially for both FCCs prior to steps 5.6, 5.9, and 5.10, breaking the foam swabs into the Lyse and Spin baskets and adding the rinse solution after the final use of the swabs.



5.6. Place swab head into the Lyse & Spin basket by breaking the shaft near the foam head.

5.7. Perform five rinses with the rinse/lysis solution by dispensing four times over the cartridge case body, rotating ¼ turn after each rinse, and then dispensing once over the head stamp area. The rinse/lysis solution is collected in the 15 mL beaker and re-used for each rinse.

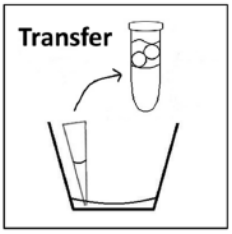
5.7.1. For each rinse, use approximately 80% of the rinse volume (e.g. 400 µL). The reduced volume prevents the formation of foam during rinsing.



5.8. Swab all outer surfaces of the cartridge case with one sterile foam swab.

5.9. Place the second foam swab head into the same Lyse & Spin basket by breaking the shaft near the foam head.

5.10. Pipette the entire volume of rinse/lysis solution into the Lyse & Spin basket.



5.11. Add 25 µL Proteinase K, in accordance with an ATF Laboratory protocol for DNA extraction (ATF-LS-FB09 or ATF-LS-FB23).

5.12. Immediately following this procedure, thoroughly rinse lysis buffer from cartridge cases in ethanol (at least 70%) to prevent corrosion.

5.12.1. This can be accomplished by submerging the cartridge case in ethanol and agitating for at least 5 seconds. Cartridge cases can be air-dried or dried with a lab wipe prior to packaging.

1. Scope

This protocol is used to analyze the amplified DNA product on the Applied Biosystems™ (AB) 3500xL Genetic Analyzer. The DNA fragments amplified with the AB GlobalFiler™ PCR Amplification Kit are separated according to size by capillary electrophoresis using the AB 3500xL Genetic Analyzer, a multi-capillary instrument. An internal lane standard (ILS) is injected along with the amplified DNA fragments. The ILS fragments are then used to determine the base pair size for each amplified DNA fragment. The use of an ILS provides consistency in sizing between samples.

The amplified fragments are detected by laser excitation and the subsequent emission spectra are captured by a charge-coupled device (CCD) camera that displays the signals as peaks. The resulting data is graphically displayed as colored peaks noted by height in relative fluorescence units and time (scan number). This display is called an electropherogram.

The reference allelic ladders for the short tandem repeat (STR) loci, indel marker, and Amelogenin are also subjected to capillary electrophoresis. These allelic ladders contain the more common alleles in the general population for each locus. Using the ladders, the alleles present in known and questioned DNA specimens may be determined.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from the references cited below.

2.1. Applied Biosystems™ GlobalFiler™ PCR Amplification Kit – PCR Amplification and CE, Revision C, October 23, 2018.
2.2. Applied Biosystems™ 3500 and 3500xL Genetic Analyzers Quick Reference Card, Revision B, May, 2009.
2.3. Applied Biosystems™ 3500/3500xL Genetic Analyzer User Guide with 3500 Series Data Collection Software 3.1, Revision C, October, 2018.
2.4. Applied Biosystems™ 3500/3500xL Genetic Analyzer User Bulletin, Revision A, June, 2011.
2.5. J.M. Butler, DNA separation methods, DNA detection methods, instrumentation for STR typing, and STR genotyping issues, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 313-388.
2.6. J.M. Butler, B.R. McCord, J.M. Jung, R.O. Allen, Rapid analysis of the short tandem repeat HUMTH01 by capillary electrophoresis, Biotechniques 17 (1994) 1062-1070.
2.7. M.M. Holland, L.A. Turni, S.A. Del Rio, M.A. Marino, R.S. Lofts, D.L. Fisher, J. Ross, J.W. Schumm, P.E. Williams, Typing human DNA using capillary

electrophoresis: comparison of slab gel and capillary formats, in: W. Bär, A. Fiori, U. Rossi (Eds.), Advances in Forensic Haemogenetics, volume 5, Springer, Berlin, 1994, pp. 156-159.

2.8. T.R. Moretti, A.L. Baumstark, D.A. Defenbaugh, K.M. Keys, A.L. Brown, B. Budowle, Validation of STR typing by capillary electrophoresis, J. Forensic Sci. 46 (2001) 661-676.

2.9. J.M. Butler, E. Buel, F. Crivellente, B.R. McCord, Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis, Electrophoresis 25 (2004) 1397-1412.

2.10. A.R. Isenberg, R.O. Allen, K.M. Keys, J.B. Smerick, B. Budowle, B.R. McCord, Analysis of two multiplexed short tandem repeat systems using capillary electrophoresis with multiwavelength fluorescence detection, Electrophoresis 19 (1998) 94-100.

2.11. McCord, B.R., Budowle, B., Isenberg, A.R., and Allen, R.O. (1997) Capillary electrophoresis for the automated analysis of multiplexed STRs using multiwavelength fluorescence detection. Seventh International Symposium on Human Identification (1996). pp.116-122.

2.12. ATF "Internal Validation of the 3500xl Genetic Analyzer Using the Globalfiler™ PCR Amplification Kit and GeneMapper™ ID-X Software."

3. Equipment

3.1. Disposable gloves
3.2. Lab coat
3.3. Eye protection
3.4. Deionized water
3.5. Computer with data collection software
3.6. AB 3500xL genetic analyzer with 24-capillary array, 36 cm
3.7. AB Anode buffer container (ABC) 3500 Series
3.8. AB Cathode buffer container (CBC) 3500 Series
3.9. AB Cathode buffer septa
3.10. AB POP-4 polymer for 3500/3500xL genetic analyzers
3.11. AB Conditioning reagent, 3500 series
3.12. AB Hi-Di™ Formamide
3.13. AB GlobalFiler™ allelic ladder
3.14. AB GS600 LIZ™ sizing standard, v2.0
3.15. MicroAmp™ Optical Reaction Plate
3.16. MicroAmp™ Splash Free Support Base
3.17. Retainer and base set (standard) for 3500/3500xL genetic analyzers, 96 well
3.18. 96 well plate septa

3.19. 10% bleach solution
3.20. 70% ethanol
3.21. 1.5 mL microcentrifuge tubes
3.22. Pipette
3.23. Disposable aerosol-resistant pipette tips
3.24. Centrifuge
3.25. Heat block
3.26. Cold block
3.27. Vortexer

4. Safety/Quality Assurance

4.1. Disposable gloves shall be worn when handling reagents and amplified DNA.
4.2. Lab coat must be worn at all times while performing this procedure.
4.3. Sample setup must be performed on bench paper, tissue wipes, or laboratory bench surfaces cleaned with 10% bleach solution followed by 70% ethanol.
4.4. Exercise caution when opening tubes.
4.5. Only one tube of amplified DNA shall be open at a time.
4.6. Use aerosol-resistant pipette tips when transferring liquids containing DNA and change pipette tips after transferring any liquids potentially containing DNA.
4.7. Record the lot number of each reagent used if they are not recorded in exported data. Do not use the reagents after the expiration date.
4.8. Check the AB 3500xL dashboard to ensure that anode buffer, cathode buffer, and POP-4 polymer have not been on the instrument for more than two weeks.
4.9. Clean plate base and retainer with tap water followed by deionized water between runs. Do not clean with bleach.
4.10. At least one positive amplification control (Control DNA 007), one negative control, and one allelic ladder must be included with each sample run (set of injections).
4.11. It is good practice to include multiple wells with allelic ladder and positive control to account for variations in electrophoresis conditions.
4.12. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and should be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.
4.13. The spatial calibration determines the position on the CCD camera where the signal emitted by each capillary is expected to fall. A new spatial calibration is required when the capillary array is replaced or temporarily removed from the instrument detection block, or when the instrument is moved.
4.14. The spectral calibration accounts for the overlapping emission spectra of the six dyes detected by the instrument. If optical components of the instrument (laser, CCD

camera, capillary array) have been realigned/replaced, a new spectral calibration is required. Additionally, if there is a noticeable degradation in spectral separation (increased pull-up and/or pull-down peaks) in the electropherograms, a new spectral calibration may be needed.

4.14.1. For instructions on performing spatial and spectral calibrations, refer to *Applied Biosystems™ 3500/3500xL Genetic Analyzer User Guide with 3500 Series Data Collection Software 3.1, Revision C, 2018.*

5. Procedure

PREPARING THE INSTRUMENT

5.1. Power on the computer workstation; wait until the log-in screen is reached.

5.2. Power on the instrument and wait for the green status light to come on.

5.3. Log in to the computer workstation and wait 1-2 minutes for the 3500 server monitor to load.

5.4. Launch and log in to the 3500 Series Data Collection Software.

5.5. Check the dashboard for instrument and reagent statuses as well as reagent levels. Perform any necessary maintenance tasks (refer to ATF-LS-FB29).

5.5.1. Maintenance tasks include, but are not limited to, installing or replacing the capillary array, polymer, or buffer. When possible, follow wizards contained in the data collection software (see *Applied Biosystems™ 3500/3500xL Genetic Analyzer User Guide with 3500 Series Data Collection Software v3.1* for specific instructions).

5.5.1.1. When replacing the cathode buffer, allow it to reach room temperature prior to use. Gently tilt the CBC to distribute buffer evenly between both chambers. Fully remove the plastic film seal and wipe buffer or moisture off of the container. Stretch the new septa to open septa holes prior to placing on the container, pushing firmly to ensure that the septa are properly seated on each chamber. Once the CBC is in use, care should be taken to ensure liquid does not shift between waste and wash chambers.

5.5.1.2. When replacing the anode buffer, allow it to reach room temperature prior to use. Invert the container to move all liquid to the larger side of the container, then ensure that the plastic film seal is fully removed from the container prior to loading on the instrument.

5.5.1.3. If polymer must be replaced, ensure that new polymer is warmed to room temperature before using. Use the **Replenish Polymer** wizard to replenish polymer.

5.5.1.4. After any reagents are replaced, wait for the tray to reach the home position then click **Refresh** on the dashboard to pull in new reagent information.

5.6. Check for bubbles in the pump block and channels. Use the **Remove Bubbles** wizard to remove bubbles. Ensure sufficient polymer is present for completing the scheduled runs.

5.7. Check for leaks around the buffer pin valve, check valve, and array locking lever. Clean dried polymer if present.

SETTING UP FOR A RUN

5.8. From the dashboard, click the **Start Pre-heat** button to begin a 60°C preheat in advance of your run. The pre-heat will automatically shut off after 2 hours. In order to mitigate first-run effects, a ***30 minute pre-heat is recommended*** if running on a cold instrument.

PREPARING SAMPLES

5.9. Determine the plate configuration including placement of samples, allelic ladders, and controls. Three columns (1 through 3) consisting of 24 wells (rows A through H) are injected simultaneously. For each injection, ensure that all 24 wells contain either sample, allelic ladder, control, or formamide blank.

5.10. Prepare a formamide/GS600 LIZ v2.0 sizing standard master mix with 9.6 µL formamide and 0.4 µL GS600 LIZ v2.0 sizing standard per sample (add several extra samples to ensure adequate volume).

5.11. Vortex and briefly spin the master mix tube.

5.12. Dispense 10 µL master mix into each well of the optical reaction plate that will contain a sample, allelic ladder, or control. Fill remaining wells with excess master mix or formamide to complete a 24-sample injection.

5.13. Add 1 µL of amplified product or allelic ladder to appropriate wells.

5.14. Cover the plate with a new plate septa.

5.15. Briefly spin the plate on a plate centrifuge to remove bubbles and collect the volume at the bottom of each well.

5.16. Place the plate on a heat block at ~95˚C for 3-5 minutes.

5.17. Place the plate in a frozen cold block for 3-5 minutes.

5.18. Place the optical reaction plate in a clean 3500 plate base, ensuring that it is correctly aligned. The plate will only align with the notch in the plate mirroring the notch in the plate base.

5.19. Snap the plate retainer onto the optical reaction plate/plate base assembly, ensuring that the holes in the septa align with those in the plate retainer.

5.20. Place the plate assembly on the plate deck of the instrument; the plate will only rest on the deck in one orientation, with the notch at the front right corner.

CREATING OR IMPORTING A PLATE RECORD

5.21. From the dashboard, click the **Create New Plate** button.

5.21.1. **Create Plate from Template** can be used with the **ATF_Globalfiler_12sec** template.

5.22. Type in the name of your plate (*Figure 1*). The plate name shall include at a minimum the date, analyst's initials, and instrument name.

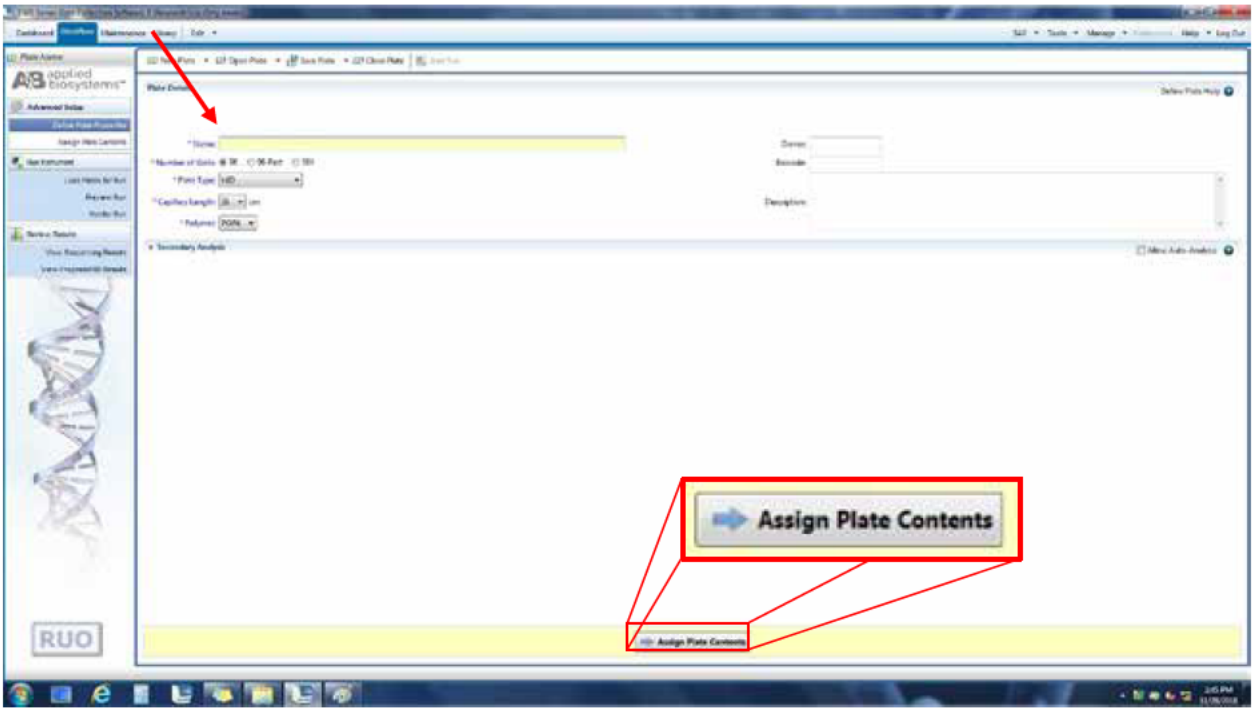
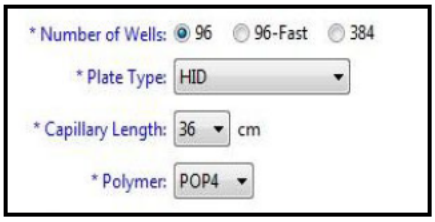


Figure 1

5.22.1. Ensure plate settings are as follows:



5.23. Click **Assign Plate Contents** (*Figure 1*).

5.24. To import a plate, click the orange **Import** button (*Figure 2*), locate your plate import file, and click **Open**.

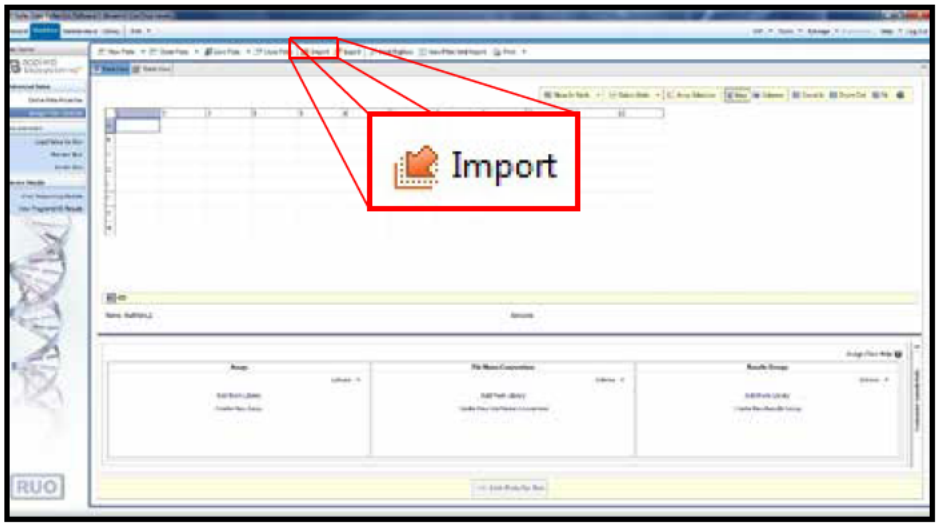


Figure 2

5.25. Verify that sample names and positions imported correctly. Also verify that the correct assay, file name convention, and results group are assigned to each sample (see Table 1 for a list of the analysis parameters).

Assay: Half_Inj_GF_POP4_xl
File Name Convention: ATF_FNC
Results Group: ATF_Globalfiler

Genetic Analyzer	AB 3500xL
Operating System	Windows 7 version 6.1.7601
Data Collection Software	3500 Series Collection Software 3
Instrument Protocol	Half_INJ_HID36_POP4xl_J6_NT3200
Dye Set	J6
Run Modules	HID36_POP4
Injection Parameters	1.2 kV, 12 seconds
Run Conditions	13 kV, 1550 seconds
Oven Temperature	60° C

Table 1: 3500xL analysis parameters

5.25.1. Alternative validated assays can be accessed by clicking **Actions** in the **Assays** box, selecting **Add from Library**, choosing an assay, and then

assigning it to desired wells by selecting the well and ticking the box next to the Assay.

5.26. If you are *not* importing plate contents, double-click a well to type in a sample name. Select the desired Assay, File Name Conventions, and Results Groups, or choose new options from the respective Actions menus.

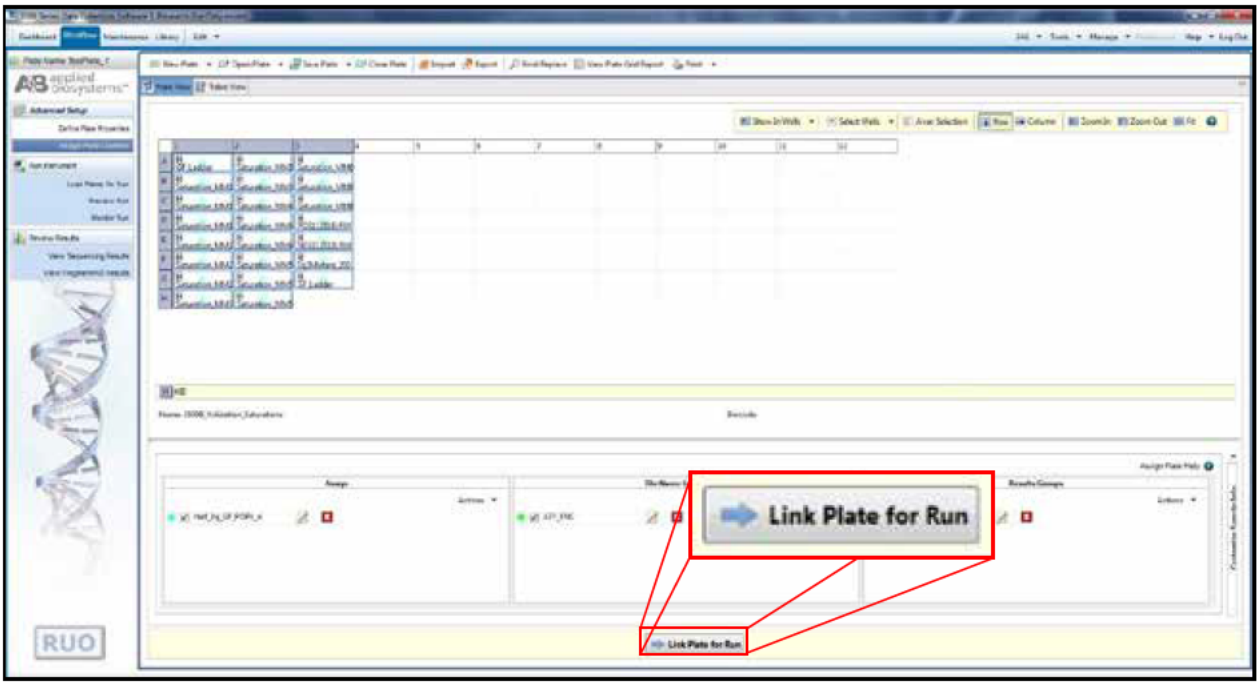


Figure 3

5.27. Click **Save Plate** to store the plate settings, then click **Link Plate for Run** (*Figure 3*).

5.28. Verify that the information in the desired plate position (A or B) is correct (*Figure 4*): The **Run Name** is a time stamp of the run and should not be adjusted. "**Name**" should match the imported plate name and "**Type**" should read "HID".

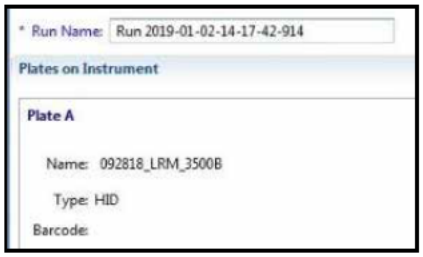


Figure 4

5.28.1. If the plate is not assigned to the correct location, Plate A and Plate B can be swapped by clicking the plate swap icon: 

5.29. Click **Create Injection List**.

5.30. Verify desired injections are listed. Injections can be prioritized by selecting an injection from the list then clicking **Move Up in List** or **Move Down in List**.

5.30.1. Toggle between Plates A and B using the tabs above the plate view window.

5.30.2. Injections can be added to a run by selecting the desired injection from the list and clicking the **Duplicate** button. Select whether to add the injection after the original injection, or following all injections.

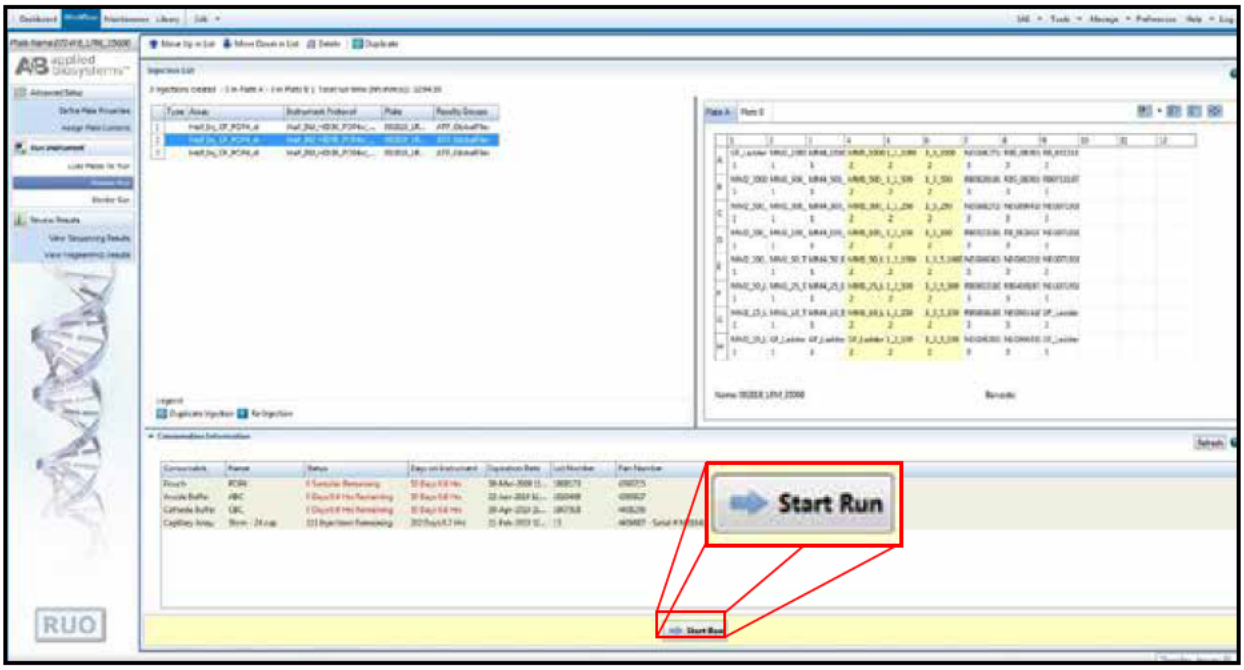


Figure 5

5.31. Click **Start Run** (Figure 5).

MONITORING A RUN

5.32. During a run, navigate to the *Monitor Run* section in the Workflow tab (*Figure 6*).

5.33. View individual sample results by selecting a well in the plate view, or view injection results by selecting from the injection list. Failed samples can be easily identified in the **Flags** summary table.

5.34. If desired, re-inject a sample, change the injection order, or delete or terminate an injection.

5.34.1. To re-inject a sample, select the sample from the **Plate View** or **Flags** summary table. Select multiple samples by holding the CTRL key while selecting samples. Click the **Re-Inject** button in the *Instrument Run Views and Flags window* (*Figure 6*). Select any ladders from the provided list if needed, and click OK. The selected sample wells will update in the **Plate View** window with the injection numbers below the sample name:

	1	2
A	GF_Ladder 1, 2, 4	4Mixture_5_1_1_ 1, 2, 5, 6
B	2Mixture_5_1_10 1, 2	4Mixture_5_1_1_ 1, 2

5.34.2. To duplicate an entire injection, select an injection from the **Injection** list. Click the **Duplicate** button (Figure 6). Selecting the **Re-Inject** button instead will allow for changes to the run assay.

5.34.3. To delete an injection, select an injection from the **Injection** list. Click the **Delete** button.

5.34.4. If the run has ended, click **Resume Run** to start newly added injections. Ensure the added injections include at least one positive control, negative control, and allelic ladder (this is not necessary if the added injections are initiated prior to the run ending).

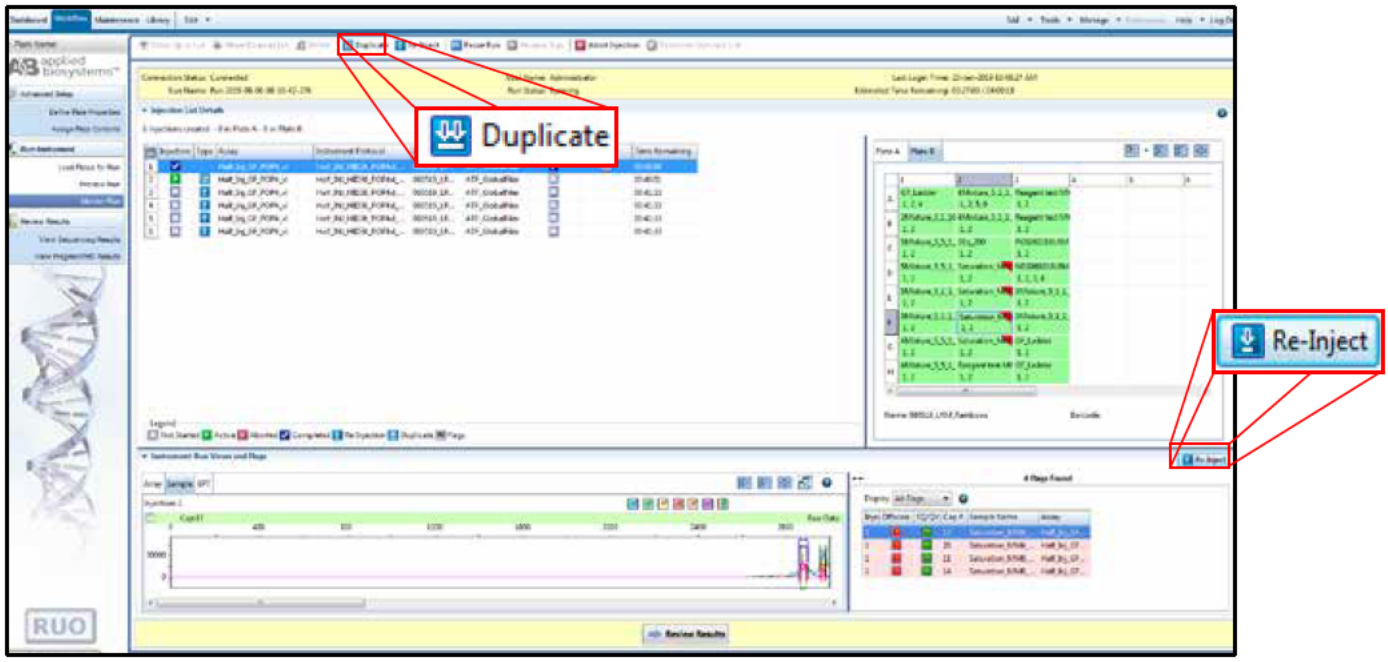


Figure 6

5.35. *(Optional)* After a run, click **Review Results** at the bottom of the window to advance to the *View Fragment Results* section and examine data from any completed injection.

WORKING FROM A PREVIOUSLY RUN PLATE

5.36. To re-inject a plate or samples from a plate that has completed a run and been unlinked from the instrument, navigate to the **Dashboard**.

5.37. Place the desired plate on the instrument in position A or B.
5.38. Click **Edit Existing Plate**. Select the desired plate and click **Open**.
5.39. Make edits to the plate contents as needed. Samples can be deleted or modified.
5.40. Click the drop-down arrow next to **Save Plate**, and select **Save As** to save the plate with a new date or plate name (*Figure 7*).
    5.40.1. Proceeding to **Link Plate for Run** without saving will maintain the original plate name, and any edits to the plate will be permanent.
5.41. Click **Link Plate for Run**.
5.42. Create the injection list as specified from step 5.29.

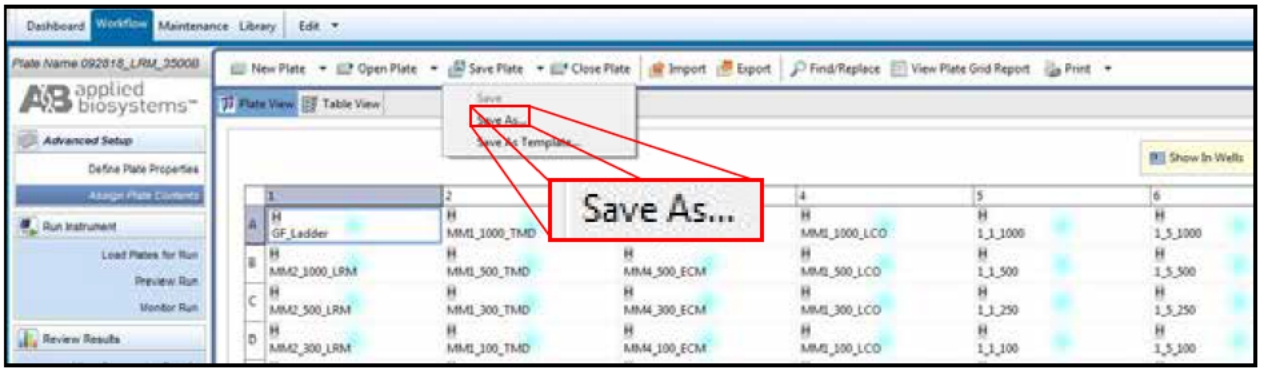


Figure 7

Shutting Down the Instrument

5.43. Close the 3500 Series Data Collection Software.
5.44. Power down the instrument using the power button on the front.
5.45. Power down the computer workstation.

Contents

1. Scope 2
2. References 2
3. Equipment 4
4. Safety/Quality Assurance 4
5. Procedure 5
5.1 General DNA Profile Interpretation Steps 5
5.2 Evaluation of Controls 6
Verification of Internal Size Standard (GS-600 v2.0) 6
Verification of the GlobalFiler™ Allelic Ladder 6
Evaluation of Negative Controls 7
Evaluation of Amplification Positive Controls (Control DNA 007) 8
Single Injection/Capillary Events 10
5.3 Evaluation of Sample Profiles 11
Analytical Threshold (AT) 11
Stochastic Threshold 11
Off-Scale Peaks 12
Artifacts 14
Heterozygous Balance (Hb) 19
Preliminary Evaluation of Sample DNA Profiles 22
5.4 STRmix™ Analysis 25
Positive Control 25
Conditioning 25
Running STRmix™ 27
Reviewing STRmix™ Outputs 28
Comparisons and LR Calculations 31
Additional Guidelines on Interpretation and Comparison 38
APPENDIX 35

1. Scope

The interpretation guidelines are intended to be a general guide for the evaluation and statistical analysis of the typing results for DNA amplified with the GlobalFiler™ PCR Amplification Kit and analyzed with the ABI 3500xL Genetic Analyzer and GeneMapper® ID-X analysis software. The statistical analysis is performed, when necessary, using STRmix™ probabilistic genotyping software. The guidelines will increase consistency in the evaluation of typing results and calculation of the statistical weight of the evidence between DNA analysts. These guidelines cannot encompass the entire range of samples or circumstances that will be encountered in forensic casework and exceptions may be made based on the analyst's training and experience. Significant deviations from the guidelines must be approved by the DNA Technical Leader. The guidelines are based on manufacturer's recommendations, manufacturer's user's manuals, internal validation studies, interpretation guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDAM), Department of Justice Uniform Language for Testimony and Reports, and the scientific literature.

2. References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

2.1. Applied Biosystems GeneMapper® *ID-X* Software Version 1.3 User Bulletin, revision A, October 2011.
2.2. Applied Biosystems GeneMapper® *ID-X* Software Version 1.0.1/1.1 Reference Guide, revision B, April 2009.
2.3. GlobalFiler™ PCR Amplification Kit User's Manual, revision E, July 2016.
2.4. Internal validation studies
2.5. STRmix™ Operation Manual
2.6. STRmix™ V2.6 User's Manual, August 2018.
2.7. ACME lab STRmix™ SOP, Institute of Environmental Science and Research Limited, 14 December 2015, obtained via the STRmix™ support website.
2.8. Oregon State Police GlobalFiler™: Interpretation and Reference Profile Comparison protocol.
2.9. J.M. Butler, DNA separation methods, DNA detection methods, and Instrumentation for STR typing, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 313-372.
2.10. J.M. Butler, Commonly used short tandem repeat markers and commercial kits, Biology of STRs, and Forensic issues, in: Forensic DNA Typing, second ed., Elsevier Academic Press, Burlington, 2005, pp 85–179.

2.11. A. Edwards, A. Civitello, H.A. Hammond, C.T. Caskey, DNA typing and genetic mapping with trimeric and tetrameric tandem repeats, Am. J. Hum. Genet. 49 (1991) 746–756.
2.12. H.A. Hammond, L. Jin, Y. Zhong, C.T. Caskey, R. Chakraborty, Evaluation of 13 short tandem repeat loci for use in personal identification applications, Am. J. Hum. Genet. 55 (1994) 175–189.
2.13. E. S. Mansfield, J. M. Robertson, M. Vainer, A. R. Isenberg, R. R. Frazier, K. Ferguson, S. Chow, D.W. Harris, D.L. Barker, P.D. Gill, B. Budowle, B.R. McCord, Analysis of multiplexed short tandem repeat (STR) systems using capillary array electrophoresis, Electrophoresis 19 (1998) 101-107.
2.14. P.S. Walsh, N.J. Fildes, R. Reynolds, Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA, Nucleic Acids Res. 24 (1996) 2807-2812.
2.15. B. Leclair, C.J. Frégeau, K.L. Bowen, R.M. Fourney, Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples, J. Forensic Sci. 49 (2004) 968-980.
2.16. T.M. Clayton, J.P. Whitaker, R. Sparkes, P. Gill, Analysis and interpretation of mixed forensic stains using DNA STR profiling, Forensic Sci. Int. 91 (1998) 55-70.
2.17. P.M. Schneider, R. Fimmers, W. Keil, G. Molsberger, D. Patzelt, W. Pflug, T. Rothämel, H. Schmitter, H. Schneider, B. Brinkmann, The German Stain Commission: recommendations for the interpretation of mixed stains, Int. J. Legal Med. 123 (2009) 1-5.
2.18. J. Bright, J. Turkington, J. Buckleton, Examination of the variability in mixed DNA profile parameters for the Identifiler™ multiplex, Forensic Sci. Int. Genet. 4 (2010) 111-114.
2.19. P. Gill, C.H. Brenner, J.S. Buckleton, A. Carracedo, M. Krawczak, W.R. Mayr, N. Morling, M. Prinz, P.M. Schneider, B.S. Weir, DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures, Forensic Sci. Int. 160 (2006) 90-101.
2.20. Scientific Working Group on DNA Analysis Methods, Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories, 2017.
2.21. B. Budowle, A.J. Onorato, T.F. Callaghan, A.D. Manna, A.M. Gross, R.A. Guerrieri, J.C. Luttman, D.L. McClure, Mixture interpretation: Defining the relevant features for guidelines for the assessment of mixed DNA profiles in forensic casework, J. Forensic Sci. 54 (2009) 810-821.
2.22. J. Bright, D. Taylor, C. McGovern, S. Cooper, L. Russell, D. Abarno, J. Buckleton, Developmental validation of STRmix™, expert software for the

interpretation of forensic DNA profiles, Forensic Sci. Int. Genet. 23 (2016) 226-239.

2.23. D. Hares, Expanding the CODIS core loci in the United States, Letter to the Editor; Forensic Sci. Int. Genet. 6 (2012) e52-e54.

2.24. D. Hares, Selection and implementation of expanded CODIS core loci in the United States, Letter to the Editor; Forensic Sci. Int. Genet. 17 (2015) 33-34.

2.25. V. Bogas, M. Carvalho, F. Corte-Real, M.J. Porto, Testing the behavior of GlobalFiler PCR amplification kit with degraded and/or inhibited biological samples, Forensic Sci. Int. Genet. Suppl. Ser. 5 (2015) e21-e23.

2.26. D. Taylor, J. Bright, C. McGoven, C. Hefford, T. Kalafut, J. Buckleton, Validating multiplexes for use in conjunction with modern interpretation strategies, Forensic Sci. Int. Genet. 20 (2016) 6-19.

2.27. J. Bright, D. Taylor, J. Curran, J. Buckleton, Developing allelic and stutter peak height models for a continuous method of DNA interpretation, Forensic Sci. Int. Genet. 7 (2013) 296–304.

2.28. C. Brookes, J. Bright, S. Harbison, J. Buckleton, Characterising stutter in forensic STR multiplexes, Forensic Sci. Int. Genet. 6 (2012) 58-63.

2.29. H. Kelly, J. Bright, J. Curran, J. Buckleton, Modelling heterozygote balance in forensic DNA profiles, Forensic Sci. Int. Genet. 6 (2012) 729-734.

2.30. H. Haned, L. Péne, J. Lobry, A. Dufour, D. Pontier, Estimating the number of contributors to forensic DNA mixtures: Does maximum likelihood perform better than maximum allele count? J. Forensic Sci. 56 (2011) 23-28.

2.31. J. Buckleton, J. Bright, D. Taylor, I. Evett, T. Hicks, G. Jackson, J. Curran, Helping formulate propositions in forensic DNA analysis, Science and Justice 54 (2014) 258-261.

2.32. D. Taylor, J. Buckleton, I. Evett, Testing likelihood ratios produced from complex DNA profiles, Forensic Sci. Int. Genet. 16 (2015) 165-171.

2.33. Department of Justice Uniform Language for Testimony and Reports for Forensic Autosomal DNA Examinations Using Probabilistic Genotyping Systems, adopted 9/18/18, effective 3/18/19.

2.34. T. Moretti, R. Just, S. Kehl, L. Willis, J. Buckleton, J. Bright, D. Taylor, A. Onorato, Internal validation of STRmix™ for the interpretation of single source and mixed DNA profiles, Forensic Sci. Int. Genet. 29 (2017) 126-144.

2.35. Bille T, Weitz S, Buckleton JS, Bright J-Anne, Interpreting a major component from a mixed DNA profile with an unknown number of minor contributors, Forensic Sci Int: Genet (2019) May; 40:150-159.

2.36. Work instructions for the evaluation of DNA profiles using STRmix™ and DBLR™.

3. Equipment

3.1. Computer with GeneMapper® ID-X analysis software
3.2. Computer with STRmix™ v2.6 software
3.3. Computer with DBLR™ software

4. Safety/Quality Assurance

4.1. Safety: Not applicable
4.2. Quality Assurance: See Procedure section

5. Procedure

5.1 General DNA Profile Interpretation Steps

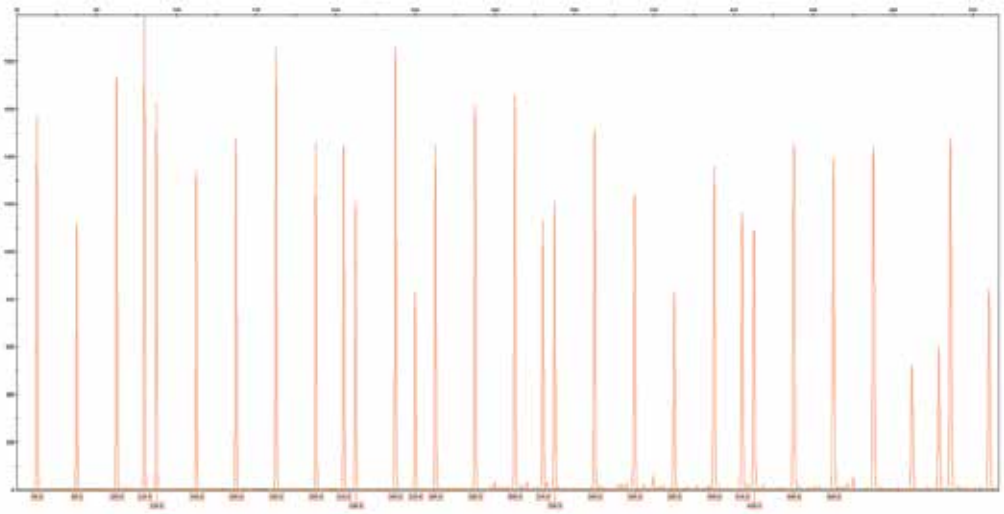
- Evaluation of standards, allelic ladders, and controls.
- Evaluation of evidence sample DNA profiles.
- Analysis and/or deconvolution of DNA profiles using STRmix™ software.
- Analysis of known DNA sample profiles.
- Comparison of known DNA profiles to evidentiary DNA profiles.
- Calculation of the statistical weight (likelihood ratio) when an individual cannot be excluded as a possible contributor to an evidentiary DNA profile, except when noted below.
- Prior to being reported, all profiles suitable for comparison purposes and meeting the minimum qualifications to be entered into CODIS will be compared to the Staff Index, Profiles Generated Index, and LDIS database. This serves as a way of detecting possible contamination. See *CODIS Manual* for instructions on entering and searching profiles in CODIS.
- The results of the DNA analysis are then detailed in a laboratory report.

5.2 Evaluation of Controls

Verification of Internal Size Standard (GS-600 v2.0)
The Internal Size Standard (GS-600 v2.0, LIZ dye labeled) consists of 36 peaks of known base pair (bp) size ranging from 20bp to 600bp.

All Internal Size Standard peaks ranging from 60 to 460 base pairs must be present, have the expected peak morphology and peak balance across the range of sizing, and be labeled correctly, with peak heights greater than 73 relative fluorescence units (RFU) for the associated profile to be acceptable.

Extraneous peaks observed in the Internal Size Standard can be disregarded as long as they are not incorrectly labeled as peaks of the Internal Size Standard.



Typical Internal Size Standard (GS-600 v2.0)

Internal Size Standard (GS-600 v2.0) peaks necessary for the analysis of ABI GlobalFiler™ amplified products	
Base Pair (bp) Sizes	60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, and 460

Verification of the GlobalFiler™ Allelic Ladder
The presence of all alleles of the allelic ladder must be verified. Allelic ladders missing one or more alleles or containing allele peaks that are marked off-ladder are not valid.

Sample profiles must be associated with one or more valid allelic ladders to be considered for interpretation.

The Sample Type of allelic ladders that are found not to be valid may be changed from "Allelic Ladder" to "Sample." If this is done, the project must be re-analyzed.

Evaluation of Negative Controls

Reagent Blank

The reagent blank is a negative control initiated at the extraction step and is processed in parallel with the associated evidentiary sample(s). It consists of all the reagents used during the extraction. The reagent blank is used to detect DNA contamination from the reagents, disposables, or environment that may affect the evidentiary samples. Contamination detected in the reagent blank may be systemic in nature or a randomly occurring event. If contamination is detected, an effort should be made to identify the source to prevent future contamination events.

Amplification Negative Control

The amplification negative control is a negative control initiated at the amplification step and consists of all reagents used in the amplification, except TE^{-4} is used in place of template DNA.

Re-inject: addition of injection to a run in progress or a run completed within the past 24 hours

Re-plate: creation of a new sample on a new plate containing new CE reagents and re-sampling of the original amplification product

Re-analyze: catch all term that includes any of the following – re-extraction, re-amp, re-injection, or re-plate

An acceptable negative control will contain no peaks above the analytical threshold.

A negative control containing a peak above the analytical threshold between 60 and 460bp which is believed to be an artifact such as a spike or spectral pull-up from the internal size standard shall be re-analyzed. If, upon re-analysis, no peaks above the analytical threshold are present, the negative control is acceptable. Artifacts outside the 60 to 460bp window are not significant and do not necessitate re-analysis of the control.

If the issue is determined to be sample-specific (e.g. a spike) and not systemic, the associated samples do not need to be re-analyzed as long as there was at least one valid negative control in the run. If the issue is determined to be potentially systemic, the associated samples must be re-analyzed (re-plated), unless the associated samples were determined to be unsuitable for comparison purposes for other reasons (e.g. no results, limited results, or too complex for interpretation).

In some instances, it may not be necessary to re-inject a negative control containing peaks above the analytical threshold to confirm the peaks. For example, the reagent blank produced a quantifiable amount of DNA and full profile consistent with the analyst is detected in the electropherogram, it is not necessary to re-analyze the sample to confirm the presence of the peak(s).

If at least one non-artifact peak above the analytical threshold or a recognizable pattern of peaks below the threshold is present (with or without re-plating/analysis), this will be considered a contamination event (see below for further guidance on the detection of contamination).

A printout of the negative control raw data demonstrating the presence of the primer peaks will be included with the case material along with a printout of the electropherogram with a maximum Y-axis of 100 RFU.

Re-analysis of a Reagent Blank

- Based on the results of the other controls (e.g. amplification negative control, positive control, etc.) associated with the evidentiary samples, the re-analysis of a reagent blank may consist of re-injection, re-amplification, or re-extraction of all associated samples.
- All re-injections must contain a valid positive control, negative control, and ladder at a minimum, unless the re-injection(s) is added to a run in progress.
- If it is not possible to re-extract a sample associated with a contaminated reagent blank and if the sample results differ from adventitious DNA observed in the control sample, the original typing results will be reported following the standard protocol.
    - A note must be added to the report describing the contamination event if the interpretation of the evidentiary profile is affected (see *Report Wording Protocol*).
    - If the DNA profile obtained from the evidence sample is consistent with the contaminating profile in the reagent blank, then the evidentiary sample is not suitable for comparison purposes. An exception can be made with the approval of the DNA Technical Leader if the average peak heights of the evidence sample profile are significantly higher than the contaminating DNA profile and the profile does not appear to be systemic. This would indicate that the negative control was contaminated by the evidence sample.
    - In the event of low-level contamination/drop-in, if a peak in an evidentiary profile cannot be distinguished from a contaminating peak, the locus should be evaluated to determine if the evidentiary locus/profile is potentially affected by the contamination. If not, the evidentiary locus/profile can be used for comparison purposes. If the evidentiary

locus/profile is potentially affected by the contamination, the locus shall be ignored for statistical purposes, but may be used for exclusionary purposes with approval by the DNA Technical Leader.
    - The entire contaminating profile should be used when determining if an evidence profile is affected. For example, if the negative control contains data at three loci and the contaminating profile and evidence profile both have a TPOX 8,11, but the genotypes at the other two loci differ, it can be reasonably assumed that the contaminating profile is not affecting the evidence profile (i.e. TPOX would not have to be ignored for comparison purposes).
- If a reagent blank associated with a set of samples to be re-amplified cannot be re-amplified due to limited volume, and it was determined to be valid from a previous analysis (at the same level of sensitivity or greater), the samples associated with the reagent blank can still be analyzed.

Re-analysis of a Negative Amplification Control

- The re-analysis of a negative amplification control may consist of re-injection, re-plating, or re-amplification of all associated samples with a new amplification negative control.
- All re-injections must contain a valid positive control, negative control, and ladder at a minimum, unless the re-injection(s) is added to a run in progress.
- If it is not possible to re-amplify an evidentiary sample and if the sample results differ from adventitious DNA observed in the control sample, the original typing results will be reported following the standard protocol.
    - A note must be added to the report describing the contamination event if the interpretation of the evidentiary profile is affected (see *Report Wording Protocol*).
    - If the DNA profile obtained from the evidence sample is consistent with the contaminating profile in the negative amplification control, then the evidentiary sample is not suitable for comparison purposes. An exception can be made with the approval of the DNA Technical Leader if the average peak heights of the evidence sample profile are significantly higher than the contaminating DNA profile and the profile does not appear to be systemic. This would indicate that the negative control was contaminated by the evidence sample. For example, the evidence DNA profile's average peak height is approximately 500 RFU and the contaminating DNA profile's peaks are all near or below the analytical thresholds.
    - In the event of low-level contamination/drop-in, if a peak in an evidentiary profile cannot be distinguished from a contaminating peak, the locus should be evaluated to determine if the evidentiary locus/profile is

potentially affected by the contamination. If not, the evidentiary locus/profile can be used for comparison purposes. If the evidentiary locus/profile is potentially affected by the contamination, the locus shall be ignored for statistical purposes, but may be used for exclusionary purposes with approval by the DNA Technical Leader.

- The entire contaminating profile should be used when determining if an evidence profile is affected. For example, if the negative control contains data at three loci and the contaminating profile and evidence profile both have a TPOX 8,11, but the genotypes at the other two loci differ, it can be reasonably assumed that the contaminating profile is not affecting the evidence profile (i.e. TPOX would not have to be ignored for comparison purposes).

Evaluation of Amplification Positive Controls (Control DNA 007)

The amplification positive control is initiated at the amplification step and is processed in parallel with the evidentiary samples. It consists of all the reagents used in the amplification reaction and template DNA of a known profile (Control DNA 007). The amplification positive control monitors the amplification reaction and ensures the reliability of the results of the associated evidentiary samples.

The typing results for the positive control should match the expected profile below.

Locus	Genotype	Locus	Genotype
D3S1358	15,16	D19S433	14,15
vWA	14,16	TH01	7,9.3
D16S539	9,10	FGA	24,26
CSF1PO	11,12	D22S1045	11,16
TPOX	8, 8	D5S818	11,11
Y Indel	2	D13S317	11,11
Amelogenin	X,Y	D7S820	7,12
D8S1179	12,13	SE33	17,25.2
D21S11	28,31	D10S1248	12,15
D18S51	12,15	D1S1656	13,16
DYS391	11	D12S391	18,19
D2S441	14,15	D2S1338	20,23

Expected typing results for Control DNA 007

If the allelic peak heights are greater than the relevant thresholds, match the expected profile, and generally fall within the expected heterozygous balance, the positive control is valid. On rare occasions, the heterozygote balance may fall outside expectations. The positive control is still valid in this situation. If multiple loci demonstrate greater than

expected imbalance, this may be an indication of an issue with amplification or capillary electrophoresis.

If the typing results do not match the expected profile (incorrect profile, additional alleles due to possible contamination or partial/no results), the positive control is not valid and all evidentiary samples associated with the positive control shall be re-analyzed. Contamination in a positive control should be evaluated to determine if it is a sample-specific issue or possibly a systemic issue. If it is potentially a systemic issue, the associated samples shall be re-analyzed, where possible.

- The re-analysis may consist of re-injection or re-amplification.
- If it is not possible to re-amplify an evidentiary sample associated with a positive control sample displaying contamination that cannot be resolved by re-injection or re-plating and if the results differ from the adventitious DNA observed in the control sample, the original typing results will be reported following the standard protocol.
- If it is not possible to re-amplify an evidentiary sample associated with a positive control sample displaying a partial profile or no profile and the issue cannot be resolved by re-injection or re-plating, the sample(s) will be re-plated with a valid positive control sample to ensure proper sizing and allele calling. The results will be reported following the standard protocol if the analyst can reasonably assume the amplification of the associated samples was not affected.
    - A note must be added to the report describing the issue if the interpretation of the evidentiary profile is affected (see *Report Wording Protocol*).
        - If the DNA profile obtained from the evidence sample is consistent with a contaminating profile in the control sample, then the evidentiary sample is not suitable for comparison purposes. An exception can be made with the approval of the DNA Technical Leader if the average peak heights of the evidence sample profile are significantly higher than the contaminating DNA profile and the profile does not appear to be systemic. This would indicate that the positive control sample was contaminated by the evidence sample.

If the amplification positive control demonstrates the presence of a spike or other possible well/capillary-specific artifact and the expected alleles are otherwise properly detected, the amplification positive control should be re-injected or re-plated to confirm the presence of the spike/artifact, but it is not necessary to re-inject or re-plate the associated samples if the issue is not present in the re-analysis.

Single Injection/Capillary Events

In general, if a single capillary or single injection event occurs that results in a poor injection (as evidenced by the ILS peaks) the sample or samples specifically affected may

be re-injected; the entire run does not have to be re-injected and analyzed. Each run must have a valid positive control, allelic ladder, and negative control if the samples are set-up as a new analysis. If additional injections are added at the end of the current analysis (prior to the end of all the injections associated with the plate), no additional positive control sample, allelic ladder, or negative control samples must be added. Alternatively, samples can be re-injected within 24 hours of the initiation of the first injection from the original plate without re-plating, but must include a positive control, negative control, and a ladder.

5.3 Evaluation of Sample Profiles

Analytical Threshold (AT)

The Analytical Threshold is the RFU value that, when exceeded by peaks that display the expected peak morphology, allows those peaks to be considered "real" products of amplification.

Analytical Thresholds are dye-specific:

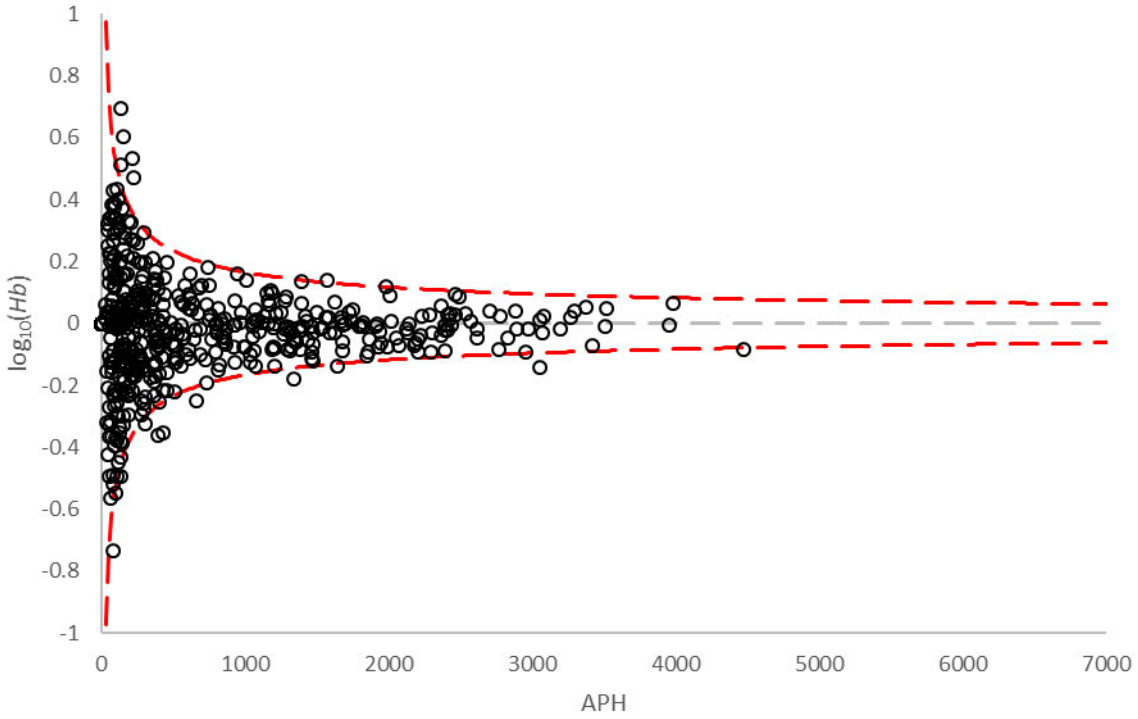
Dye	Analytical Threshold (RFU)
6-FAM	23
VIC	37
NED	21
TAZ	32
SID	37
LIZ	73

Stochastic Threshold

Due to the inherent nature of PCR amplification, the expected heterozygous balance will decrease as the levels of template DNA decrease. The two dashed red lines in the graph below represent the 95% confidence interval for the heterozygous balance data generated during validation. At higher levels of template DNA (average peak height of 1000 RFU) the expected heterozygous balance ranges from approximately 68% to 146%. At lower levels of template DNA (average peak height of 150 RFU) the expected heterozygous balance ranges from approximately 37% to 268%. Dramatic heterozygous peak height imbalance at the lower levels of template DNA may result in allele drop-out.

While a Stochastic Threshold has not been set due to the use of STRmix™ software to aid in interpretation, the graph below can be used to estimate the expected heterozygous balance when evaluating a DNA profile.

Graph: Expected Heterozygous Balance



Expected Heterozygous Balance: Data generated during the validation of GlobalFiler™ PCR Amplification Kit, Applied Biosystems™ 3500xL, and STRmix™ software v2.6. The dashed red lines represent the 95% confidence interval.

Off-Scale Peaks

An off-scale peak is a peak that exceeds the linear dynamic range of the instrument. Off-scale peaks may result in raised baselines and/or excessive "pull-up" in one or more colors.

Analyzed data from off-scale peaks should not be used for quantitative comparisons since the true quantity of signal is not known. For example, the use of off-scale peaks can result in disproportionately high stutter ratios.

The saturation level has been set at 29,000 RFU. DNA profiles containing peaks greater than 29,000 RFU should not be analyzed using STRmix™. If only a few loci are affected, then these loci should not be used in the STRmix™ analysis, but the remaining loci may be used. The affected loci may be used for exclusion.

Sample profiles containing off-scale data may be re-analyzed at the discretion of the analyst. Re-analysis may consist of adding less amplified product to the ABI 3500 set-up tray, diluting the amplified product in formamide prior to adding it to the 3500 set-up

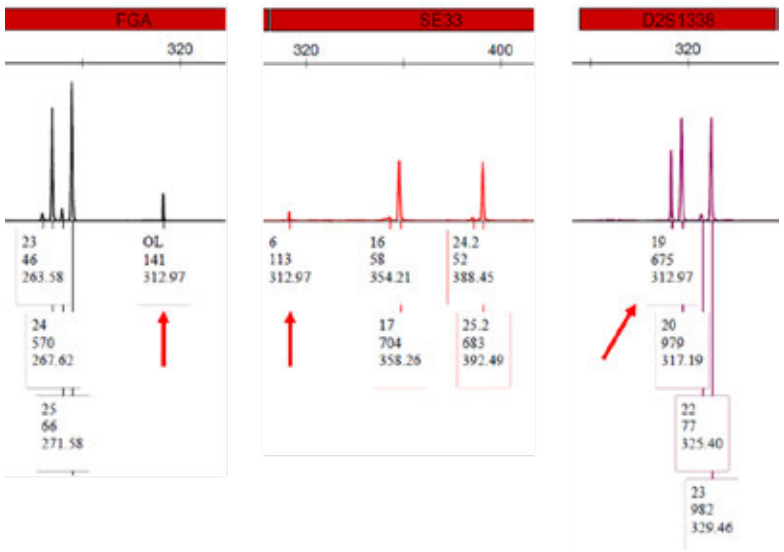
tray, or re-amplifying the sample. Re-amplification is preferred and may be done at the analyst's discretion since some artifacts (e.g. –A peaks) can still be present in dilutions of amplicons that were initially amplified with excessive DNA template.

Artifacts

Spikes

A spike is defined as a non-specific, non-reproducible, and non-allelic peak that is typically observed in one or more colors and is an artifact of the electrophoresis and signal detection. A spike does not typically display the same peak morphology as an allelic peak with respect to peak width, starting slope and ending slope.

If a spike is present in the analytical range (60 – 460 bp), the spike may be confirmed to be an artifact by re-injection or re-plating if it interferes with interpretation. Upon re-analysis, the same spike should not be present. If the spike does not interfere with interpretation (e.g. a high level single source profile containing a low level spike, a complex mixture otherwise not suitable for comparison purposes, or a sample with no results), the presence of the spike does not need to be confirmed.



Example of a spike detected in three color channels. In two instances, the spike falls within an allelic bin. In one instance, the spike is labeled as an OL.

Occasionally, a peak may be labeled as a spike. Upon closer examination, the peak will have the expected allelic peak morphology and fall within an allelic bin. However, the top of the peak will be indented (not rounded). This is due to spectral pull-down (a peak in another dye channel is negatively affecting the signal of the current channel). If the peak is determined to be an allele, the label can be edited to reflect the appropriate allele

call. If the pull-down appears to be significant, this locus may need to be ignored during the STRmix™ analysis.

Non-Template Nucleotide Addition

Some polymerases have the inherent property of non-template directed nucleotide addition. In extreme conditions (such as the addition of excessive template DNA), a portion of the amplified product will not contain the additional nucleotide, resulting in a fragment one base pair shorter than the expected allele. Since the nucleotide typically added is adenine (A), the peak representing amplified product in which the non-template directed nucleotide addition did not occur is oftentimes referred to as the –A peak. This should not be confused with true alleles that are one base shorter than full repeat alleles (e.g. D1S1656 15.3/16). If –A is observed, the affected sample should be re-amplified, if possible. Typically, re-amplification with less template DNA will resolve the issue. If re-amplification is not possible, then the *Ignore Locus* function should be used for the affected loci during the STRmix™ analysis.

Stutter

Stutter peaks are an artifact of the Polymerase Chain Reaction when amplifying repeat regions of DNA. The primary peak is representative of the actual repeat number contained in the template DNA. The stutter peak is an artifact usually one repeat shorter in length than the primary peak (reverse stutter). For tetrameric repeats, the reverse stutter peak is n-4 base pairs where n is the base pair length of the primary peak. Forward stutter (n+4), double back stutter (n-8), and 2 base pair stutter (n-2) may also be detected, but typically at a lower level than the reverse stutter (n-4). Forward stutter, double back stutter, and n-2 stutter are more prevalent at some loci, such as SE33.

Stutter peaks can be characterized both by their size and by their peak height proportion when compared to the associated primary peak, expressed as the percentage of the stutter peak height compared to the primary peak. The average stutter ratios have been experimentally defined at the ATF Laboratory and will vary for each locus. These are the values used by STRmix™. The values were calculated using one of three methods: the linear regression (allele X slope + intercept); the longest uninterrupted sequence; or the average per allele (see "INTERNAL VALIDATION OF THE 3500XL GENETIC ANALYZER USING THE GLOBALFILER™ PCR AMPLIFICATION KIT AND GENEMAPPER™ ID-X SOFTWARE" for a list of which method was used for each locus and the associated parameters). For general evaluation purposes, the table below lists the maximum stutter percentages expected for each locus.

Locus [1]	% Stutter
CSF1PO	8.77
D10S1248	11.46
D12S391	13.66
D13S317	9.19
D16S539	9.48
D18S51	12.42
D19S433	9.97
D1S1656	12.21
D1S1656 (−2 nt)	2.45
D21S11	10.45
D22S1045	16.26
D22S1045 (+3 nt)	6.69
D2S1338	11.73
D2S441	8.10
D3S1358	10.98
D5S818	9.16
D7S820	8.32
D8S1179	9.60
DYS391	7.43
FGA	11.55
SE33	14.49
SE33 (−2 nt)	3.97
TH01	4.45
TPOX	5.55
vWA	10.73

[1] These percentages are used as stutter filters in GlobalFiler_stutter.txt. GlobalFiler PCR Amplification Kit User Guide, rev E, July 2016.

Under some circumstances, stutter peaks may exceed the maximum expected stutter percentages. These peaks may be considered stutter at the analyst's discretion based on the specific circumstances.

- Stutter peak calculations associated with off-scale allele peaks will not be reflective of the true percentage.
- Stutter peaks between two alleles that differ by two repeats (e.g. 15 and 17 allelic peaks with a stutter peak at the 16 position) may exceed the maximum expected stutter percentages due to the n-4 contribution from the 17 allele and the n+4 contribution from the 15 allele.
- Stutter peak percentages may increase at lower levels of input template DNA.

Minor peaks cannot be absolutely identified as stutter peaks even though they meet both the size and stutter peak percentage criteria. If there is no other indication of a mixture in a profile, minor peaks meeting the stutter peak criteria can be attributed to stutter; however, these peaks should be left in for STRmix™ analysis. If evidence of a mixture is observed in a profile, minor peaks falling within the stutter peak criteria should be taken into consideration during the interpretation.

"It is important for analysts to be aware of iso-alleles present at the vWA locus. Specifically vWA alleles 14 and 15. This can be observed in the ATFE stutter data. Results obtained from other laboratories also demonstrate that these two alleles can exhibit multiple clusters of data due to different sequence variants that stutter in different amounts. The 'Allele Regression' explanatory variable has been selected to determine the expected vWA stutter ratio for ATFE's data. On occasion, this will lead to potential over- or under-estimation of the expected stutter ratio at vWA 14 and 15 alleles. Analysts need to be aware of this when reviewing the STRmix™ interpret [sic] of profiles containing these alleles." (From ATFE Parameters v 2.6 (© 2019 ESR))

Matrix Failure (Spectral Pull-Up)

Matrix Failure, also known as spectral pull-up, results in peaks from one color "bleeding" into another color. The wavelength ranges emitted by the fluorescent tags overlap. The software is designed to use a matrix algorithm to separate the fluorescent signal given off by each of the fluorescent tags. This separation is not always complete, resulting in minor peaks being detected in the adjacent spectral colors. These pull-up peak heights are typically less than 5% of the peak height of the source peak (usually between 1-3%). The pull-up peak is usually sized within ± 1 base pairs of the source peak.

If pull-up peaks are observed in a sample, the sample may be re-analyzed at the analyst's discretion based on the specific circumstances. It may be necessary to perform a new Spectral Calibration prior to re-analysis if spectral pull-up is excessive.

If pull-up peaks are present in a mixed DNA profile, it may not be possible to distinguish a possible pull-up peak from a minor contributor allele. If a peak that falls within the expected parameters of a pull-up peak is observed at a locus and is at a level similar to one or more minor contributor peaks, the *Ignore Locus* function in STRmix™ may be used for this locus at the analyst's discretion.

Raised Baseline

Raised baseline may be observed in samples containing excess template DNA or due to instrument failure. The raised baseline appears as non-specific elevation along the x-axis of the electropherogram.

If excess raised baseline is observed in a sample, the sample may be re-analyzed at the analyst's discretion.

Other Artifacts

Other peaks that do not represent genetic data from the human template DNA may be observed in the sample electropherogram. These peaks or series of peaks may result from dissociated fluorescent dyes or other causes due to the instrument, capillary electrophoresis, or amplification kit.

Some peaks may be the result of the presence of non-human DNA or other artifacts of the PCR. In these circumstances, the analysis of these samples will be evaluated on a case-by-case basis. Suspected non-human artifacts may affect a single locus or multiple loci in different color channels and may vary in peak height in the same DNA profile. If the interpretation of a locus is potentially affected by one or more suspected non-human artifact peaks, then the *Ignore Locus* function should be used during the STRmix™ analysis at the analyst's discretion. The analyst may use an internal artifact database, external artifact databases, or other DNA profiles from the same or similar exhibits to inform the interpretation.

Off-Ladder Peaks

Most common alleles are represented by the allelic ladder or virtual bins set by the software. Occasionally, non-artifact peaks will be observed outside of the allele bins and will be labeled as "OL." Off-ladder alleles may result from temperature fluctuations in the instrument's environment during the course of the electrophoresis. The change in temperature may cause a migration shift of allelic peaks that consequently fall outside the ±0.5 base pair bins even though the alleles are represented in the ladder. Micro-variant and rare variant alleles may also be observed that fall between alleles within a locus or the range of alleles between two loci.

Micro-variant and rare variant alleles that are off-ladder shall be re-injected to confirm the sizing unless the DNA profile is determined to be not suitable for comparison purposes.

Off-ladder (OL) allelic peaks observed between alleles within a locus should be labeled as a variant of the smaller flanking allele. If the OL peak is approximately one base pair longer than the smaller flanking allele (X), the OL peak will be designated as X.1. If the OL peak is approximately two base pairs longer than the smaller flanking allele, it will be designated as X.2. If the OL peak is approximately three base pairs longer than the smaller flanking allele, it will be designated as X.3. For example, an OL peak falling between the 8 and 9 alleles that is approximately two base pairs longer than the 8 allele would be designated as an 8.2.

An OL allelic peak that falls between the allele range of two loci first should be associated with one of the loci and then be labeled as described below.

- To associate the OL peak with one of the flanking loci, the alleles present in the flanking loci along with the base pair sizes of the alleles need to be considered.
- If one of the flanking loci contains two alleles and the other locus contains one, the OL peak will be associated with the locus that contains one.
- If both loci contain one allele, the base pair size of the OL peak should be compared to the base pair sizes of the flanking allelic ladders. If the OL peak is a "perfect repeat" of one of the allelic ladders, it will be associated with that locus. If it is not a "perfect repeat" of either flanking allelic ladder, then both loci may only be used for exclusionary purposes and the *Ignore Locus* function should be used during the STRmix™ analysis.
- If the OL peak has been associated with a locus, it will then be designated as "<" or ">" the first or last allele of the associated allelic ladder at that locus.

Heterozygous Balance (Hb)

Expected Variation in Heterozygous Balance

It is not unusual for PCR to produce imbalanced peak heights for heterozygous loci. As the amount of template DNA decreases, the imbalance may become more dramatic. The previous Expected Heterozygous Balance graph displays the log Hb derived from data generated during the internal validation. The data points represent the average peak height at the locus vs the log Hb for the heterozygous pair. The balance is calculated as a ratio of the smaller allele (repeat number) as compared to the larger allele (repeat number). The dashed red-lines represent the 95% confidence interval. At higher levels of template DNA (average peak height of 1000 RFU) the expected heterozygous balance ranges from approximately 68% to 146%. At lower levels of template DNA (average peak height of 150 RFU) the expected heterozygous balances range from approximately 37% to 268%. Dramatic heterozygous peak height imbalance at the lower levels of template DNA may result in allele drop-out.

Expected Hb based on the average peak height (APH) can be calculated by:

$$\text{Log Hb} = \pm\sqrt{2} \times 1.96 \times \sqrt{\frac{c^2}{\text{APH}}}$$

Where c^2 (3.574) is the mode of the gamma distribution for alleles from Model Maker in STRmix™.

For heterozygous loci with alleles separated by relatively few repeats, the imbalance is not significantly affected by allele size. Hence, the imbalance will affect the larger allele

as much as the smaller allele. Therefore, if a sample is amplified multiple times using the same template quantity, the imbalance may vary and may favor the larger allele one time and the smaller allele another time.

For heterozygous loci with alleles separated by a larger number of repeats (such as the locus FGA), the imbalance may be greater than expected and may favor the smaller allele.

Peak Height Imbalance due to Primer Site Mutation

A sequence mutation in the primer region may have one of three effects. If the mutation causes significant destabilization, the allele will not be amplified and therefore not detected (null allele). If the mutation does not affect the stability of the primer binding, no effect will be observed. If, however, the mutation causes some destabilization of the primer binding but not to the extent to prevent amplification, the resulting peak balance at the locus may be outside the expected range. This form of imbalance is reproducible.

If a similar imbalance is observed in an evidence sample and a reference sample, this is an indication that a primer binding site mutation is the cause (e.g. allele A – 600 RFU, allele B – 100 RFU). After the initial deconvolution, the use of the *Ignore Locus* function in STRmix™ may be justified in this instance.

If the peak height imbalance falls outside the expected range at a single locus in an assumed single source profile, several causes should be considered:

- Expected Hb is based on a 95% confidence interval, therefore it is predicted that some values will fall outside of expectations.
- Hb has greater variation at low levels of template DNA.
- The Hb may be affected by a primer binding site mutation.
- A second, low-level contributor may be present.

Expected Peak Height Balance in Mixed DNA Profiles

The Expected Heterozygous Balance graph shown previously displays the expected Hb for single source samples. If a DNA profile is the result of DNA from multiple contributors, the additive effects of allele sharing must be considered.

Composite Profiles

In some instances, it may be useful to re-inject or re-amplify a sample to obtain interpretable data for additional loci. Combining data from multiple injections/amplifications resulting from a single DNA extract is called a composite profile. For example, if a sample is severely degraded, the initial amplification using 1ng of template DNA may have produced interpretable data at a few of the smaller molecular weight loci. However, when the same DNA extract was re-amplified using 2ng of

template DNA, additional interpretable data was obtained from a few of the higher molecular weight loci. The smaller molecular weight loci in the second amplification are now not interpretable due to saturation.

Composite profiles and replicate analysis with STRmix™ are not validated for use at this time.

Preliminary Evaluation of Sample DNA Profiles

The evidence profile shall be evaluated prior to the analysis of reference samples, where feasible. This will not be possible when additional evidence has been submitted after previous DNA analyses, including the analysis of references. However, it is imperative that the evidence profile is evaluated independently of any reference profiles.

The entire DNA profile should be evaluated to determine if the profile is suitable for comparison purposes and to determine the assumed number of contributors. At a minimum, a DNA profile must have sufficient data to be able to reasonably determine the number of contributors for the profile to be considered suitable for comparison purposes. If the DNA profile is the result of four or more contributors, the mixed DNA profile is not suitable for comparison purposes except as described below in the "Discerning a Major Component" section.

Number of Contributors

The assumed number of contributors must be determined for a DNA profile or a component of a DNA profile to be suitable for comparison purposes.

In general, the following can be used to assist in the determination of the number of contributors:

The maximum number of alleles per locus (see below)
Heterozygous balance (Hb)

- The presence of multiple alleles at the Y indel and/or DYS391 loci should be taken into account when determining the number of contributors to a sample
- Peaks below the Analytical Threshold may be considered when determining the number of contributors

If the apparent number of contributors is 1, 2, or 3, then the assumed number of contributors shall be documented as the number.

If the apparent number of contributors is more than three or one or more components of a DNA profile are not suitable for comparison purposes, the assumed number of contributors shall be documented as "at least X" where X is the apparent number of contributors.

Largest # of alleles at a locus	Minimum # of contributors
2	1
3	2
4	2
5	3
6	3
7	4
8	4
9	5
10	5

Table of the minimum number of contributors based on the maximum number of alleles detected at a locus within a DNA profile.

Peaks observed below the Analytical Threshold with expected allelic peak morphology and falling within allelic bins may be considered when interpreting a low-level sample. While these peaks cannot be considered true allelic peaks, their presence may be an indication of additional contributors that could influence the interpretation of low-level samples.

Mixtures containing four or more contributors shall be considered not suitable for comparison purposes due to the complexity of the profile (except as described below in the "Discerning a Major Component" section).

Mixtures where the number of contributors cannot be reasonably determined, but have fewer than four contributors, shall be considered not suitable for comparison purposes due to limited results. If the entire DNA profile is determined to be not suitable for comparison purposes during the initial evaluation without further evaluation (STRmix™ and/or DBLR™), only allelic peaks above the analytical threshold shall be used to estimate the number of contributors reported. If further evaluation was performed, then the assumed number of contributors used for the evaluation will be reported.

Discerning a Major Component for Mixtures

To perform a Deconvolution using STRmix™, a number of contributors has to be assumed. In some mixed DNA profiles, the components of the mixture do not have equal contributions. It may not be possible to determine the total number of contributors to a mixture as the contribution from lower level components decreases. In these instances, the data for the higher-level components may still be valid and suitable for comparison and STRmix™ analysis. If there is ambiguity with respect to the total number of contributors, the apparent number of contributors should be used for the STRmix™ analysis.

Two and three person mixtures should be evaluated to determine if all of the components are suitable for comparison purposes (see work instructions for profile evaluation using STRmix™ and DBLR™). In some circumstances, only the components with greater levels of data (e.g. higher RFU or number of alleles detected) are suitable for comparison purposes. In these situations, components that do not meet suitability criteria shall be deemed not suitable for comparison purposes due to limited results. The evaluation process and the suitability for comparison of all components shall be documented. If one or more components are not suitable for comparison purposes, the suitable component(s) shall be considered the major component.

A major component of a four or five person mixture can be discerned under the following conditions:

- The major component contains no more than three contributors.
- There is no indication that the mixture contains more than a total of five contributors.
- The lowest contributor to the major component has a Mixture Proportion of at least 20%, as determined by STRmix™.
- The lowest contributor to the major component has a ratio of Mixture Proportions of at least 3:1 compared to the next highest component, as determined by STRmix™.
- Each of the contributors to the major component has a Template Amount of at least 87 RFU, as determined by STRmix™.

When a major component of a four or five person mixture is discerned, the minor component of the mixture shall be deemed not suitable for comparison purposes due to the complexity of the profile.

If a major component is discerned, results can only be reported for comparisons made to those contributors. The LR will only be reported for a comparison to a reference sample if the individual is determined by STRmix™ to fall in one of the positions of the Contributor Order that is part of the major component (components determined to be suitable for comparison purposes). For example, contributors 1 and 2 in the Contributor Order in the STRmix™ report of an assumed four person mixture can be discerned from the remaining two contributors where the number of minor contributors is somewhat ambiguous. No comparisons or LRs can be reported for individuals that are determined by STRmix™ to best fit in the third or fourth position of the Contributor Order. In these instances, the individual would be excluded as a possible contributor to the major component.

Single Source

A DNA profile may be considered to be from a single source under the following conditions:

- No more than two alleles are present at each locus (excluding stutter and other explainable artifacts).
    - Tri-allele patterns are not common but can be observed. If three alleles are observed at a single locus and all other loci display no more than two alleles, the possibility of a tri-allele pattern should be considered. However, it is also possible that the third allele is the product of a second low-level contributor only detected at a single locus or increased stutter.
- Loci containing two allelic peaks meet the expected Hb. Hb outside expectations is not always by itself indicative of a mixture.
- A sufficient number of alleles and loci are detected from which a conclusion as to the number of contributors can be reliably drawn. This determination will be made based on the analyst's training and experience.
    - Information specific to the case and the item of evidence should be taken into account when making this determination. For example, a small bloodstain collected from a sidewalk may be assumed to be from a single contributor. A swab from a doorknob in a public building will most likely contain DNA contributions from multiple individuals.

The presence of male DNA in a sample is established by a "Y" allele at Amelogenin and/or typing results at the Y indel or DYS391 locus. If it is determined that the mixture consists of two individuals and two alleles are present at DYS391 (excluding stutter) and/or the Y indel, then the analyst can conclude that the mixture contains two males. If it is determined that the mixture consists of three individuals and three alleles are present at DYS391 (excluding stutter), then the analyst can conclude that mixture contains three males. If the number of alleles present at DYS391 and the Y indel are less than the total number of individuals assumed to be in the mixture, then the analyst can only conclude that at least X (where X corresponds to the number of males indicated by male-specific loci) males are present in the mixture. A single source DNA profile can be considered consistent with originating from a female if the "X" allele at Amelogenin is greater than 250 RFU and there are no indications of alleles present at the Y indel or DYS391 loci.

Documenting Sample Evaluation

The assumed number of contributors used for the evaluation, presence of a male/female contributor (if applicable) and whether or not the DNA profile is suitable for comparison purposes will be documented on the electropherogram for evidentiary and reference samples. If the DNA profile or a component of a DNA profile is determined to be not suitable for comparison purposes, the step in the evaluation process where the component

failed to meet the required criteria, if applicable, (i.e. greater than five contributors, the STRmix™-generated template amount, DBLR overlap, or STRmix™ failed to deconvolute) and the reason shall be documented (e.g. due to limited results or due to the complexity of the profile, see above). If a mixture contains both suitable and not suitable components, the interpretation for each component shall be documented on the electropherogram (i.e. STRmix™ contributor order positions).

> Example of one way to document interpretation: 3 person mixture, C1 suitable, C2 and C3 not suitable:
>
> > NOC: 3, at least one male present
> >
> > C1: SFC
> >
> > C2: NSFC, DBLR, LRes
> >
> > C3: NSFC, Tem, LRes

Preparing for STRmix™ Evaluation

If an evidence profile has been determined to be suitable for comparison purposes, then a STRmix™ input table (text file) containing the sample name, marker, allele 1 – 20, size 1 – 20, and height 1 – 20 shall be exported for STRmix™ analysis. In some instances, it may be necessary to increase the number of alleles, etc.

Reference profiles will be exported for STRmix™ analysis using a STRmix™ input table (text file) containing the sample name, marker, allele 1-10, and size 1-10.

In most instances, the file containing reference profiles required by STRmix™ will be exported directly from GeneMapper® ID-X. However, on rare occasions (e.g., when performing a comparison to a reference DNA profile provided by an external laboratory), it may be necessary to manually create this file for STRmix™ input. In these instances, the Manual Reference Generator tool must be used, which will automatically add "Manually Generated Reference Sample" to the file name (e.g., Sample_1.X_23W000X_Manually Generated Reference Sample_Export.txt") and "MAN" to the sample name that will be displayed in STRmix™ (e.g., 1.X_23W000X MAN_REF.csv). This file will be stored in FireToss. In addition, a note shall be made in the STRmix Case Notes table in the case record indicating that the file for Exhibit X was manually created.

5.4 STRmix™ Analysis

All DNA profiles (single source, 2-person mixtures, 3-person mixtures, and up to a 3-person major component with no more than a total of five contributors) that have been determined to be suitable for comparison purposes shall initially be analyzed/deconvoluted with STRmix™ without a reference.

Positive Control
Before STRmix™ is used to analyze an evidentiary DNA profile that has been determined to be suitable for comparison, the DNA profile for the Control DNA 007 will be analyzed using STRmix™ and an LR calculated to ensure the software is functioning properly. The point LR from the current analysis will be compared to the known point LR for the Control DNA 007. If STRmix™ is functioning as expected, the values will be 3.64E28 for African American, 2.91E27 for Caucasian, and 2.77E27 for Hispanic populations. If the values are not identical, the analyst and possibly the DNA Technical Leader should investigate why and correct the issue.

Conditioning
Conditioning is a means by which an individual can be assumed to be a contributor to a mixture. This assumption should be reasonable for both hypotheses that will be considered later in the likelihood ratio.

Conditioning is appropriate in the following circumstances:

- If the sample is an intimate sample, the STRmix™ deconvolution may be conditioned upon the donor of the sample.
- If the sample is non-intimate, the STRmix™ deconvolution may be conditioned upon a non-probative contributor who may be reasonably assumed to have contributed DNA to the item (e.g. conditioning a mixture from a steering wheel on the vehicle's owner).
- In the event of staff-to-sample contamination, the deconvolution may be conditioned upon the contaminating profile.

Initially, the deconvolution of the profile shall be performed in STRmix™ without any assumed contributors. Following the deconvolution, an *LR from Previous* should be run against the conditioning candidate.

- If the likelihood ratio supports inclusion, the deconvolution may be rerun with conditioning. This new, conditioned, deconvolution will be used for subsequent comparisons.
- If the likelihood ratio does not support inclusion, the non-conditioned deconvolution will be used for subsequent comparisons.

On a case-by-case basis, a mixture may be conditioned on a potentially probative contributor purely for the purposes of determining a profile for CODIS entry.

- This may only be performed after a deconvolution of the mixture without conditioning was attempted.
- A likelihood ratio supporting inclusion of the individual upon whom the mixture will be conditioned must have been obtained prior to running the mixture with conditioning.
- For example, two profiles are detected on a firearm. A single source profile is obtained from smudging on the slide (Male #1). A mixture of two individuals is obtained from the grips consistent with Male #1 and a second unknown individual. The mixed DNA profile may be conditioned on the Male #1 profile for the purposes of obtaining a CODIS-eligible profile for the second contributor.

Running STRmix™

The initial STRmix™ deconvolution will be run independent of any reference profiles. Comparisons to references will be achieved after deconvolution via the *LR from Previous* option.

1. Select *Interpretation*.
2. Enter the case number into the *Case Number* field.
3. The *Sample ID* should be "Ex" followed by the exhibit number, an optional descriptor, and "Decon" (e.g. Ex1.1-Decon or Ex. 1.Q1-Grips-Decon).
4. The *Comments* box may be used to enter notes at the discretion of the analyst.
5. Enter the number of assumed contributors.
6. Select *Next* to proceed.
7. The profiling kit used is GlobalFiler_3500_GS.
8. Ignore Locus may be used in the following situations:
    a. STRmix™ cannot account for triallelic patterns. If a triallelic pattern (or other chromosomal abnormality such as a somatic mutation or null allele) is observed.
    b. If an allelic peak is potentially the result of spectral pull-up and is at the same level as minor component alleles at a locus.
    c. If a potential non-human peak is present that may interfere with the interpretation at a locus.
    d. If an unresolved peak is detected above the Analytical Threshold at a locus (e.g. 15.3,16).
9. The number of burn-in accepts and post burn-in accepts should be left at the default of 100,000 per chain and 50,000 per chain, respectively. These values may be raised as needed during troubleshooting of instrument diagnostics (see below).
10. In general, run settings should not be adjusted (see Appendix for screenshots of settings). In some instances, it may be necessary to adjust a run setting. In these

cases, the change and justification shall be documented. The default settings will not be changed.

11. Select the evidence profile.
    a. If appropriate, reference profiles may be added for the purposes of conditioning. If conditioning, ensure that the reference is included as a contributor to both HP and the HD.
12. Select *Start* to initiate sample deconvolution.
13. After each deconvolution, an advanced report is automatically generated.

The above steps may be achieved individually or in batch mode, which queues up the deconvolution or *LR from Previous* of multiple profiles. See the STRmix™ Operation Manual for specific instructions on the use of batch mode.

Reviewing STRmix™ Outputs

Review the run diagnostics listed below. No single run diagnostic alone is demonstrative of a problem with the STRmix™ deconvolution. When multiple diagnostics are affected (e.g. very low acceptance rate with very high Gelman-Rubin value), that may be indicative of a problem with the STRmix™ deconvolution and warrant further action.

Total Iterations (Acceptance Rate)

The total iterations value is the number of post burn-in iterations run by the MCMC in order to reach the target of 400,000 accepts.

The acceptance rate is 400,000/total iterations. An acceptance rate of 1 in thousands or millions may indicate that the analysis needs to be re-run with additional iterations.

Effective Sample Size

The effective sample size is a value derived from the MCMC. During the MCMC, each iteration is correlated to the one before. The effective sample size is the number of samples from the MCMC posterior distribution that are considered independent from each other (non-correlated).

The smaller the effective sample size is compared to the number of total iterations, the greater the potential for a large difference in genotype weights from another MCMC analysis of the same profile. The effective sample size is taken into account during the HPD interval generation in the likelihood ratio calculations when considering the MCMC variability.

Average log (likelihood)

This value is the average of the log probability values created at each of the post burn-in MCMC accepts.

The larger this value, the better STRmix™ has been able to model the data.

A low or negative value may suggest that STRmix™ has not been able to describe the data well, given the information provided. Reasons for this include:

- The profile is very low-level and there is little data making up the likelihood.
    - The average log (likelihood) is the sum of the log (likelihood) for each allele in the profile. Therefore, DNA profiles with relatively few alleles will inherently have a smaller average log (likelihood).
- The incorrect number of contributors was entered and therefore forced stochastic events in the STRmix™ run as a result (e.g. large peak height imbalances or variation in contributor ratios across the profile).
- Data has been removed that was real, particularly stutter peaks, and must now be described by drop-out.
- Artifact peaks have been left in the STRmix™ input and must be accounted for by drop-in.

Gelman-Rubin Convergence Diagnostic

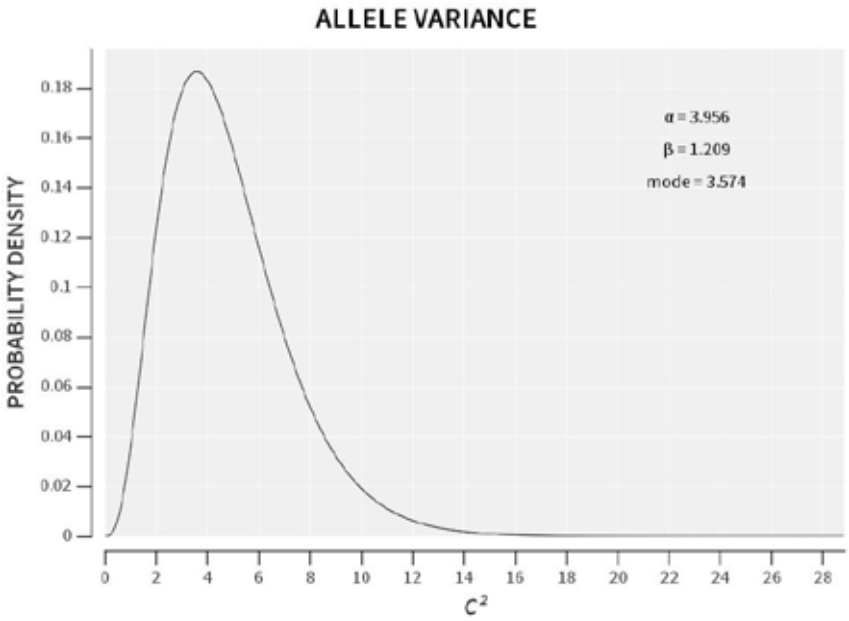
This diagnostic is a measure of how well the eight STRmix™ chains carrying out the MCMC have converged on a final profile deconvolution. For fully converged chains, this value is 1.0.

If this value is greater than 1.2, it is possible that the analysis did not run for long enough (e.g. the sample may need to be re-run with additional accepts in both burn-in and overall). A value greater than 1.2 does not automatically necessitate additional analysis, but does warrant further inspection. If the other diagnostics are acceptable and the genotype weightings, mixture proportions, and LRs meet logical expectations, then no further analysis is necessary.

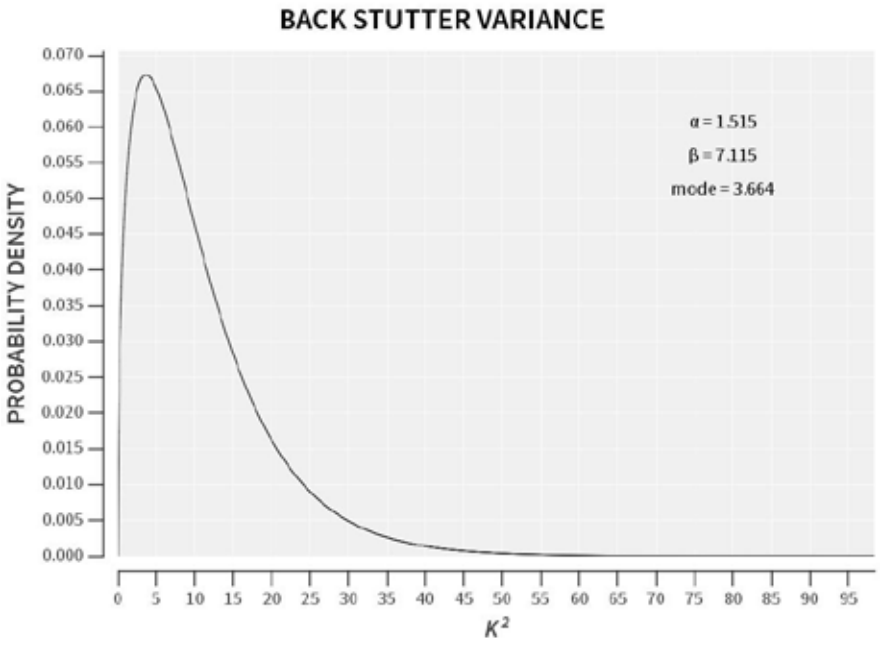
Allele Variance and Stutter Variance Constants

These values are the average values for the allele variance and stutter variance constants across the entire post burn-in MCMC accepts. These values can be indicative of the level of stochastic variation in peak heights that are present in the profile.

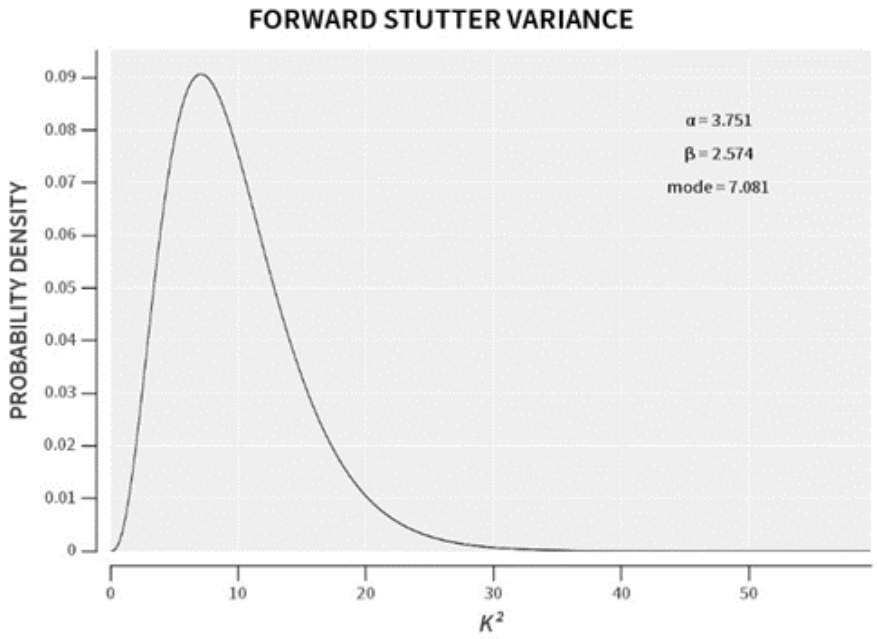
The allele variance mode is 3.574, with a gamma distribution curve shown below:



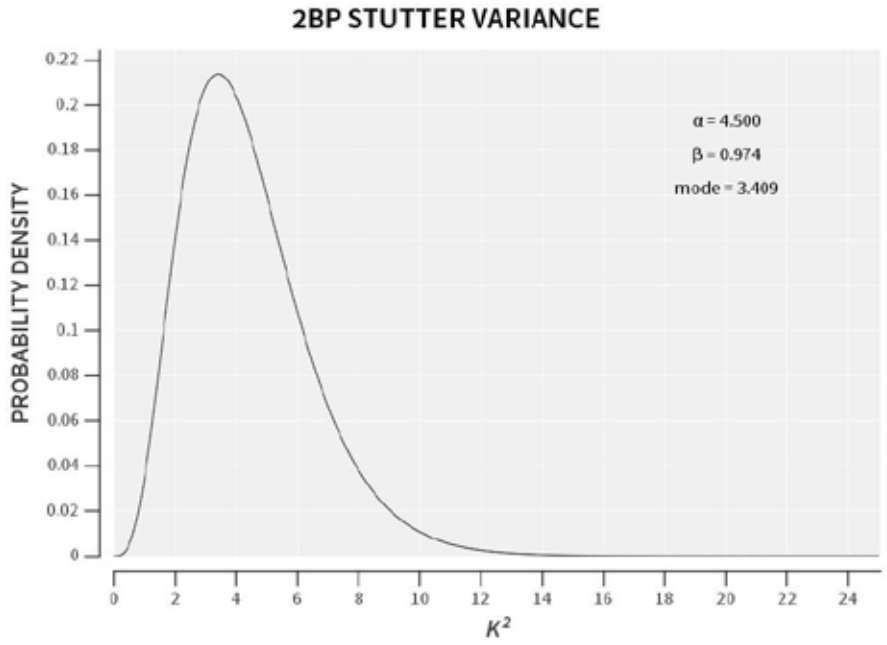
The back stutter variance mode is 3.664, with a gamma distribution curve shown below:



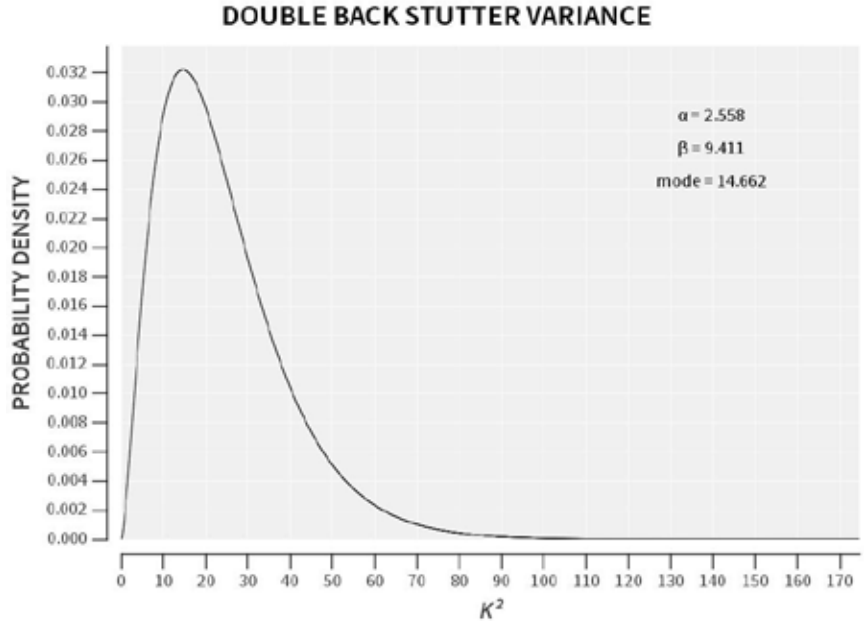
The forward stutter variance mode is 7.081, with a gamma distribution curve shown below:



The 2 base pair stutter variance mode is 3.409, with a gamma distribution curve shown below:



The double back stutter variance mode is 14.662, with a gamma distribution curve shown below:



If the allele or stutter variance values are significantly higher than the mode, this may indicate that the DNA profile itself is of poor quality or that the incorrect number of contributors was assumed.

Used in conjunction with the average (log) likelihood, a large allele or stutter variance value can indicate poor amplification.

- If the sample is simply low level, the deconvolution should yield a low average (log) likelihood and a variance constant close to the mode.
- If some data has been inappropriately omitted, artifacts left in, or the profile was otherwise misinterpreted, the deconvolution should yield a low average (log) likelihood and high allele and/or stutter variances relative to the mode.

However, stochastic effects can result in elevated stutter or increased heterozygous peak imbalance. One or two instances within a profile can significantly affect the value of the variances. Therefore, elevated variances are an indication that the profile should be further scrutinized, but are not a definitive indication that the analysis is unreliable.

Genotype Weights, DNA Amounts, and Mixture Proportions

In addition to the run diagnostics, the genotype weights, DNA amounts, and mixture proportions should be reviewed to ensure that the deconvolution meets the analyst's expectations of approximate contributor ratios and likely genotype pairs based upon their assessment of the data.

The DNA amounts are calculated by averaging the mode of the post burn-in DNA template amount per contributor per chain. The DNA amount modes are placed in bins with widths of 30. The DNA mixture proportion is calculated per chain and the mean across all chains is reported. Therefore, the DNA amounts and mixture proportions reported may not mathematically align. Due to the binning of the modes, low level profiles may not meet expectations with respect to the reported DNA amount. For example, if a single source profile consistently has allelic peak heights of 50 RFU, the reported DNA amount may be 30 since 50 falls within the 30 – 59 bin.

On rare occasions, the genotype weights will not meet the analyst's expectations at a locus due to an issue affecting only that locus. This may be due to:

- An unusually high stutter peak
- An unresolved minor component peak in instances where the minor peak has a one base pair difference to a major component peak (e.g. major 15.3 allele and a minor 16 allele)
- Other uncommon circumstances

In these instances, the deconvolution may be re-run using the *Ignore Locus* function for the affected locus.

Discrete contributor profiles will be found in the "COMPONENT INTERPRETATION SUMMARY ≥99%" section of the advanced report. This summary lists the genotype(s) that are found in the top 99% of the weights as determined by STRmix™ and may be listed as fully resolved (e.g. 17,17 or 17,18), partially-resolved (e.g. 17,F), or non-resolved (F,F).

Re-running a Deconvolution

Once the run diagnostics, contributor weights, and mixture proportions have been reviewed, the analyst may determine it is necessary to re-run the deconvolution. Reasons for a repeat deconvolution must be clearly documented on the first page of the advanced report or as a case note and retained in the case file.

Deconvolution may be re-run with an edited input file, with a greater number of MCMC accepts, with a change to the number of contributors, or by the introduction of user-informed mixture proportion priors (Mx priors). Mx priors are a means by which an analyst can provide STRmix™ with a starting-point for contributor ratios in circumstances when a mixture may contain relatives or peaks below the analytical threshold indicating a trace contributor. See the STRmix™ Operation Manual for specific instruction on the use of the Mx prior functionality.

- The use of Mx priors requires documented DNA Technical Leader approval.
- If Mx priors are used, the case notes will indicate the mean and variance used for each contributor. This may be most easily achieved via the use of printed screen shots from STRmix™, but other means may be used at the discretion of the analyst.

In addition, the analyst may decide to re-run a deconvolution based on an elevated Degradation Exponential Curve value. The standard maximum value used for this setting in STRmix™ is 0.01. Based on the observed electropherogram, STRmix™-generated DNA amounts, and/or genotype weights which may indicate that the observed profile was not appropriately modeled, the analyst can increase the Degradation Exponential Curve value to 0.1 in the settings. If this value is increased to 0.1, the number of burn-in and post burn-in accepts will be increased by 10X to 1,000,000 and 500,000, respectively.

Comparisons and LR Calculations

The likelihood ratio (LR) compares the probability of observing the evidence profile given two alternate hypotheses: one in which the person of interest (POI) is a contributor to the evidentiary profile, called the Inclusionary Hypothesis (H_I), and one in which a random person, unrelated to the POI, is a contributor to the evidentiary profile, called the Exclusionary Hypothesis (H_E).

Evidentiary profiles will be compared to all informative reference samples. If there is no expectation that DNA from an individual would be present on an exhibit of evidence based on the case circumstances, then no comparison to that individual is necessary for that sample. This will be documented in the case notes and/or the report.

Comparisons between evidentiary profiles and references determined to be probative in the context of a case will be achieved via the STRmix™ *LR from Previous* function. Manual comparisons may be made to 100% weighted genotypes for complete or partial single source DNA profiles or mixtures where a complete or partial single source major component can be discerned. If the individual is not excluded, the LR will be calculated via STRmix™. Manual exclusions will be documented in the case notes using the STRmix™ case note sheet noting that the exclusion was performed manually and at least one locus demonstrating the exclusion.

Comparisons between questioned samples may be achieved in STRmix™ as appropriate, provided one of the questioned samples is single-source and complete at all GlobalFiler™ STR loci. When this is performed, only the conclusion (support for inclusion, limited support for inclusion, uninformative, limited support for exclusion, or excluded) will be reported. The associated LR will not be reported unless it is probative.

Manual comparisons between two questioned samples is permitted as described above.

Setting up Propositions

Propositions are typically set to minimize the LR. This can be done by minimizing the number of unknowns within the different propositions.

Non-conditioned LRs will first be run against a single POI at a time. If the case scenario dictates that multiple persons of interest are probative in combination, then LRs should be calculated for each person individually and for the individuals all together. However, only the individual LRs will be reported.

Likelihood ratios will be calculated for the Caucasian, African American, and Hispanic populations using the allele frequencies published by Hill, et al. Other population groups may be considered on a case-by-case basis, but will require setting up the appropriate STRmix™ parameters as described in the STRmix™ Operation Manual.

- Hill, C.R., Duewer, D.L., Kline, M.C., Coble, M.D., Butler, J.M. (2013) U.S. population data for 29 autosomal STR loci. Forensic Sci. Int. Genet. 7: e82-e83.
- Steffen, C.R., Coble, M.D., Gettings, K.B., Vallone, P.M. (2017) Corrigendum to ‘US Population Data from 29 Autosomal STR Loci’ [Forensic Sci. Int. Genet. 7 (2013) e82-e83)]. Forensic Sci. Int. Genet. 31: e36-e40.

- Population parameters are listed in the Appendix. The population size is based on the 2016 US Census report. The number of children per family is set at 3 to be conservative.

Likelihood ratio calculations will include the sub-sub-source LR, sub-source LR, and 99% 1-Sided Lower HPD Interval. The lowest HPD LR across the three population groups will be reported.

When multiple single source profiles (from the same or separate exhibits), all containing profiles with only one weighted genotype (100% weighting) at each locus and matching a single individual are obtained, only one *LR from Previous* may be run. This LR will be used for reporting purposes.

Individual LRs will be run for single source profiles having at least one locus with two or more weighted genotypes, regardless of whether or not they have all been determined to match a single individual.

If positive likelihood ratios are obtained for all loci except one where the LR is 0, the interpretation, input file, and weights should be scrutinized to ensure no STRmix™ input errors were made. If an error is detected, re-run either the deconvolution or the *LR from Previous* as needed. Documentation for both deconvolutions shall be retained with a note describing the reason for the second deconvolution. If no input errors were made, consult with the DNA Technical Leader before proceeding.

After the release of the analytical report, additional propositions may be evaluated upon customer request. Only those requests for which the propositions are determined to be reasonable by the Laboratory in the context of the case will be granted, or by court order. Granting this request is at the discretion of the casework analyst. These additional analyses shall be documented in the case record and undergo a technical review.

In general (e.g. two and three person mixtures), if comparisons to multiple individuals produce LRs providing support for inclusion as a possible contributor to the same DNA profile, an LR evaluating the likelihood of the individuals contributing to the mixture together will be calculated. This assessment is for verification of the contributor combination only, and is not performed to provide weight to the combined contribution. More complicated (e.g. four or five person mixtures or those involving related individuals) instances will be evaluated on a case by case basis.

Example scenarios are as follows:

A single source partial DNA profile is obtained from possible ridge detail on a threat letter.

- The DNA profile is analyzed with STRmix™.
- A suspect (POI) standard is received for comparison.
- H_I: DNA contributor is POI.
- H_E: DNA contributor is one unknown

A two-person mixture is obtained from the grips of a firearm.

- Deconvolution is performed without conditioning.
- One suspect (POI) is received for comparison.
- H_I: DNA contributors are POI and an unknown.
- H_E: DNA contributors are two unknowns.

A two-person mixture is obtained from a blood stain on a shirt recovered from the victim (V) of a stabbing.

- Deconvolution is first performed without conditioning.
- An *LR from Previous* is then performed to determine if the victim can be conditioned.
- If the LR for the victim supports inclusion, then a second deconvolution can be performed conditioned on the victim.
- A suspect (POI) is received for comparison.
- H_I: DNA contributors are V and POI.
- H_E: DNA contributors are V and unknown.

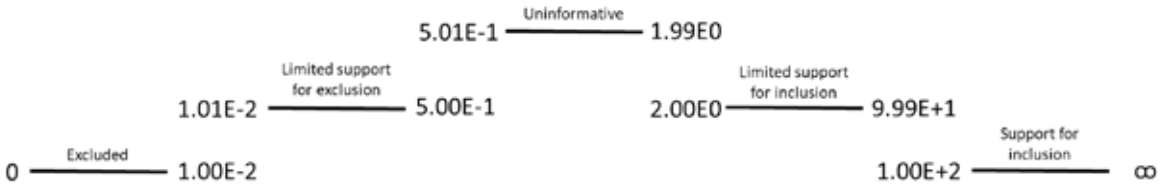
A single source DNA profile (P1) was obtained from the front sight of a firearm. A two-person mixture (P2) was obtained from the grips of the same firearm.

- The P2 profile is initially deconvoluted without conditioning.
- An LR can then be calculated to determine if the P1 profile is included as a possible contributor to the P2 mixed profile.
- If the LR for the P1 profile supports inclusion in the P2 profile, the P2 profile can be deconvoluted conditioned on the P1 profile to obtain a profile for CODIS entry purposes.
- A suspect (POI) is received for comparison. The hypotheses for reporting purposes for the P2 profile are below:
- H_I: The DNA contributors are the POI and an unknown individual.
- H_E: The DNA contributors are two unknown individuals.

Persons of Interest

Conclusions regarding a person of interest's contribution to a sample will be made based upon the 99% 1-Sided Lower HPD Interval likelihood ratio. One of five conclusions can be made for a comparison based on the LR:

- Support for inclusion (LR supporting inclusion)
- Excluded (LR supporting exclusion)
- Limited support for exclusion (LR supporting exclusion, but within a range at higher risk for false exclusions)
- Limited support for inclusion (LR supporting inclusion, but within a range at higher risk for adventitious matches)
- The comparison is uninformative (LR does not reliably support the Inclusionary Hypothesis (H_I) or Exclusionary Hypothesis (H_E), see below)



The LRs shall be truncated to three significant figures. For LRs between 1 and 100, the LR shall be truncated to a whole number.

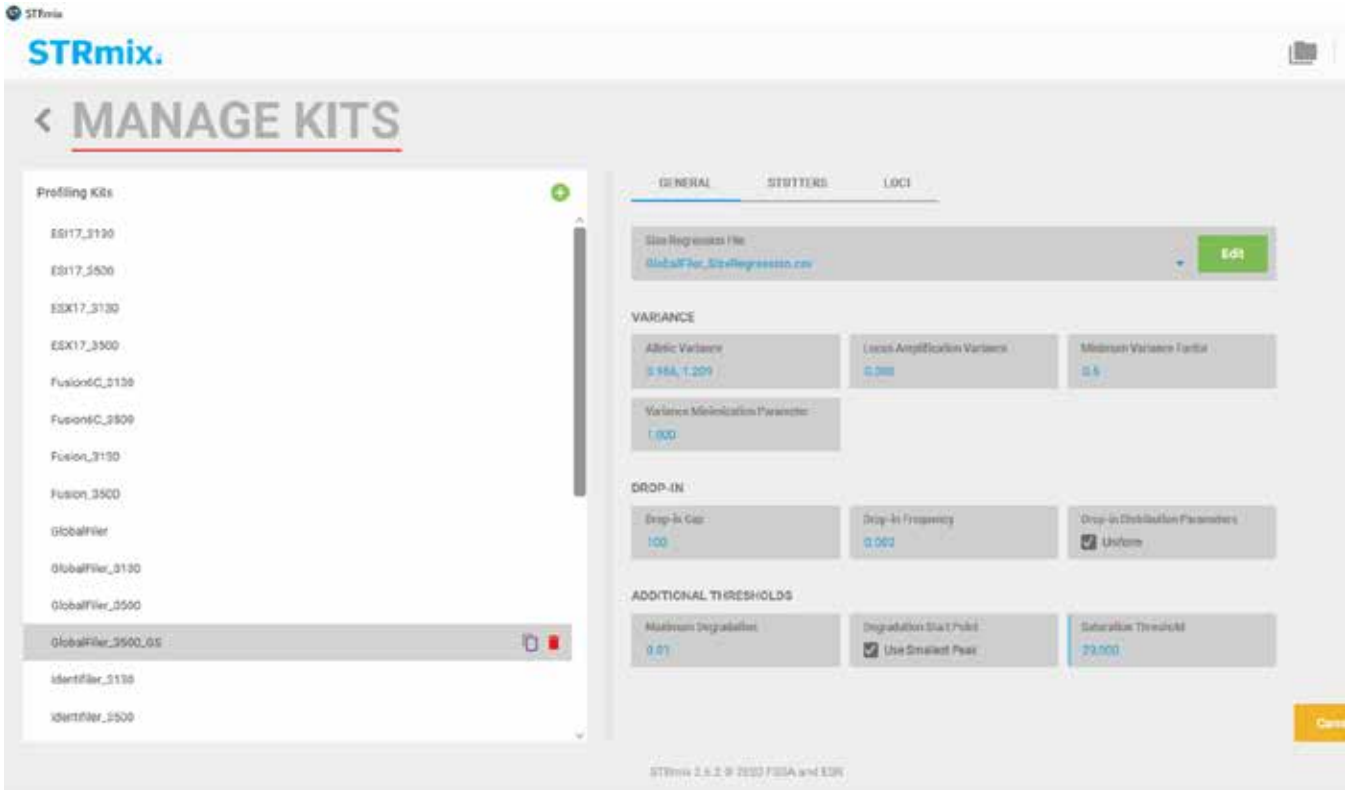
Additional Guidelines on Interpretation and Comparison:

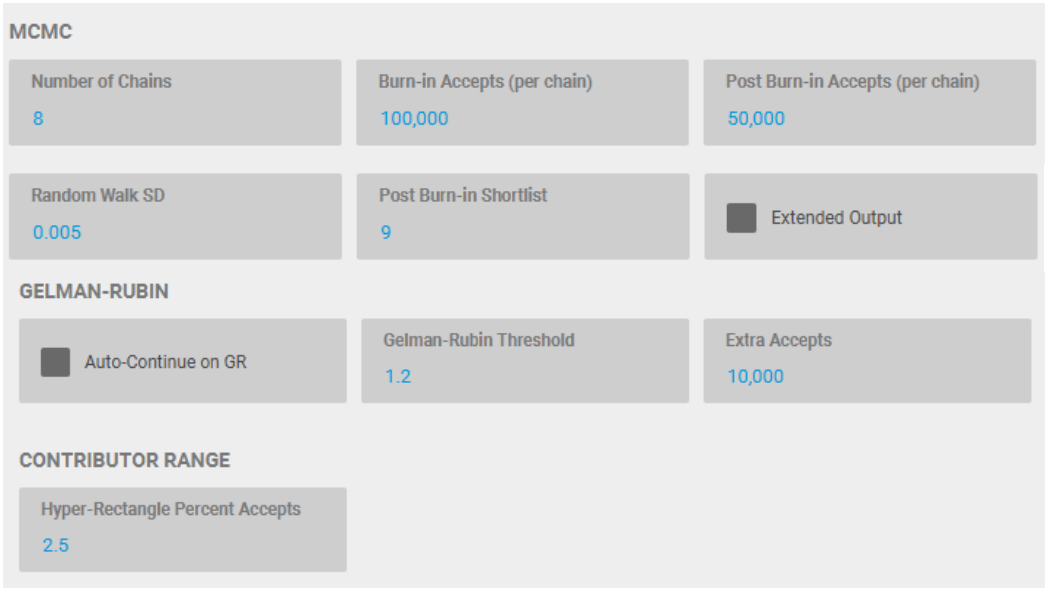
Non-numeric values such as OL, < or > are not permitted within the STRmix™ input files.

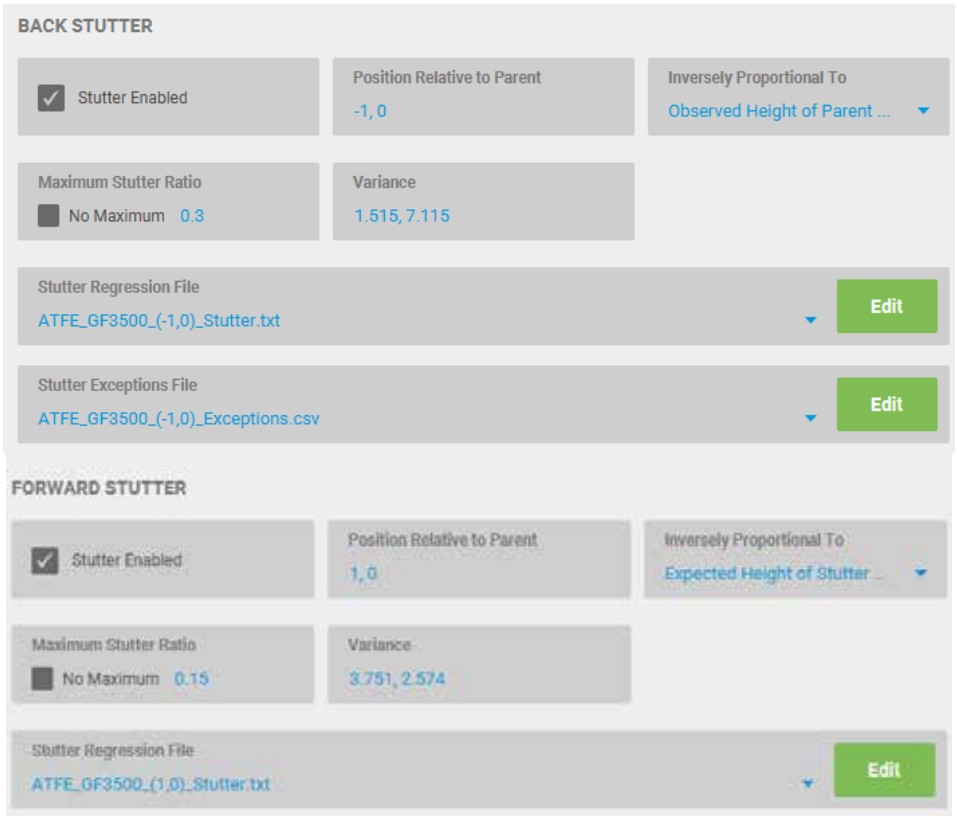
Unambiguous alleles including those that are rare should appear in the corresponding input file as their actual allelic size designation, for example D21 30.1. If alleles fall more than two repeat units off the end of the ladder, they are unable to be sized accurately or labelled correctly. These loci should be removed from the deconvolution using the *Ignore Locus* option. If an LR is calculated, the POI should be manually compared at this locus to ensure this is not an exclusion.

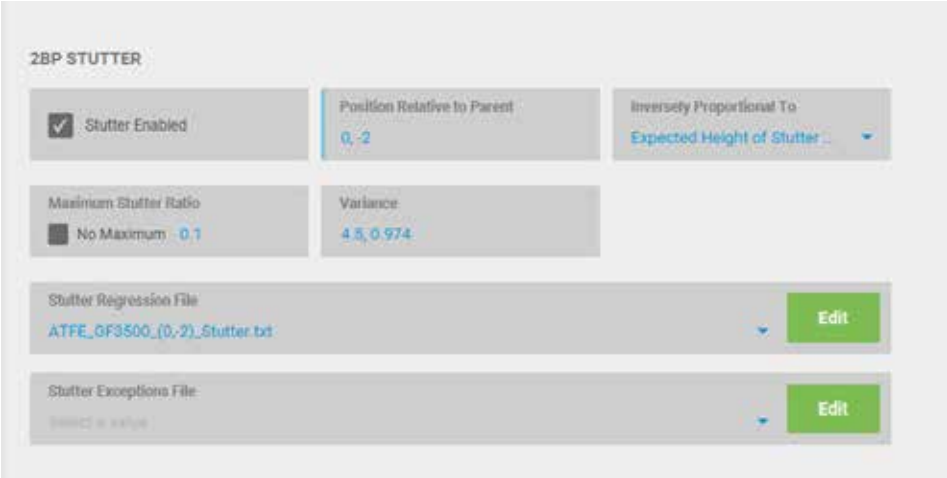
If multiple amplifications of the same extract are performed, the concordance of the genotyping results should be evaluated. In general, both samples will require STRmix™ deconvolution, unless one has significantly more genetic information than the other. Only the most informative profile shall be analyzed further.

Appendix

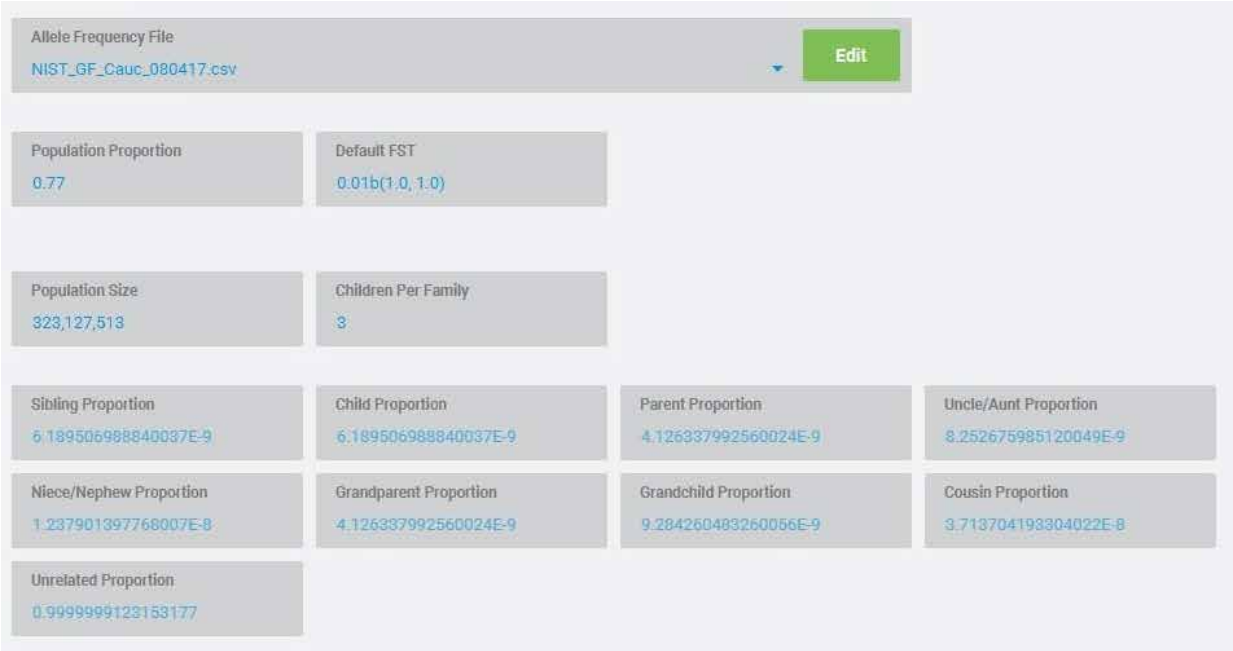

STRmix.
MANAGE KITS
Profiling Kits
GENERAL
STUTTERS
LOCI
Edit
VARIANCE
DROP-IN
ADDITIONAL THRESHOLDS
Uniform
Use Smallest Peak

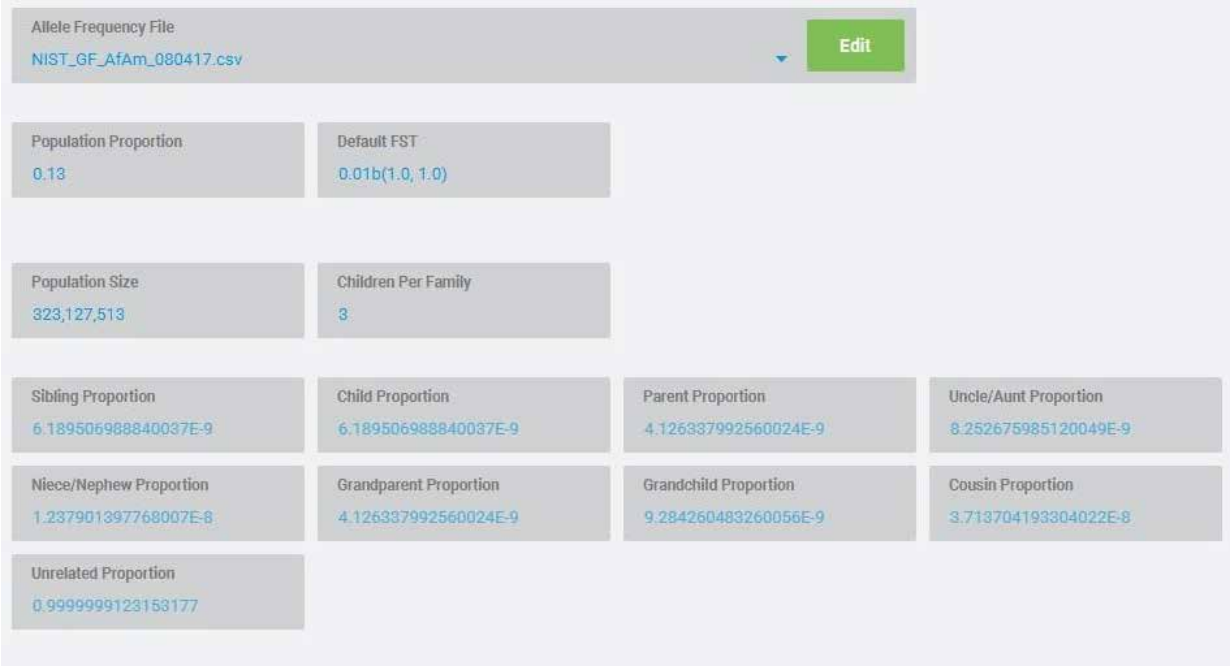

MCMC
Number of Chains
8
Burn-in Accepts (per chain)
100,000
Post Burn-in Accepts (per chain)
50,000
Random Walk SD
0.005
Post Burn-in Shortlist
9
Extended Output
GELMAN-RUBIN
Auto-Continue on GR
Gelman-Rubin Threshold
1.2
Extra Accepts
10,000
CONTRIBUTOR RANGE
Hyper-Rectangle Percent Accepts
2.5

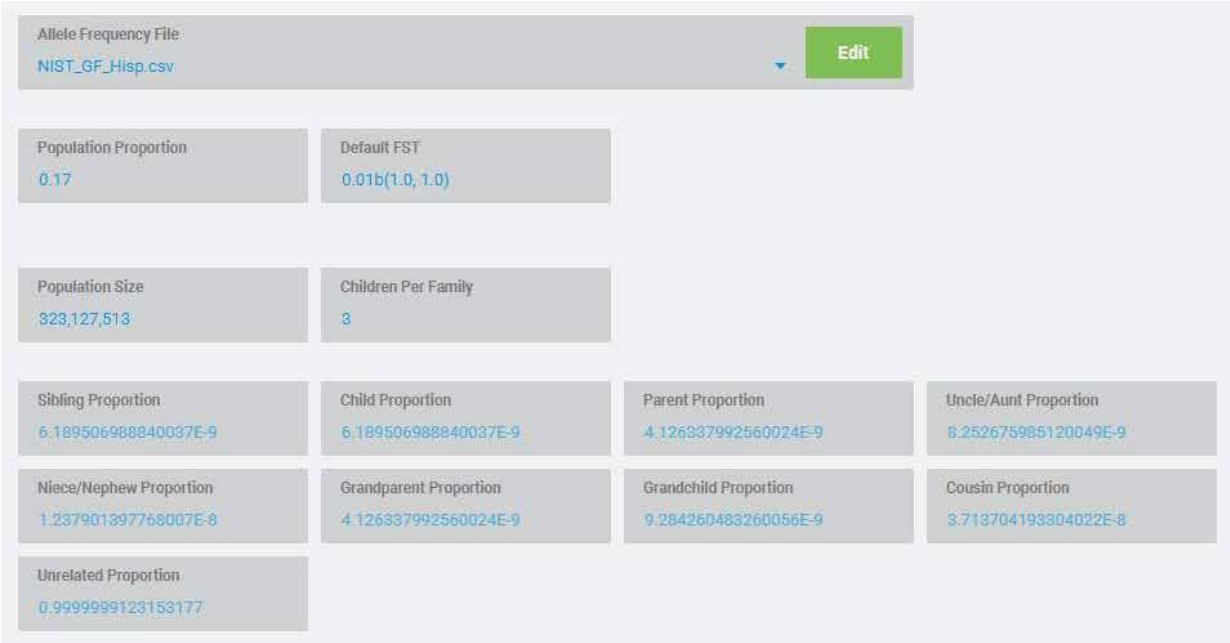

BACK STUTTER
Stutter Enabled
Position Relative to Parent
-1, 0
Inversely Proportional To
Observed Height of Parent ...
Maximum Stutter Ratio
No Maximum 0.3
Variance
1.515, 7.115
Stutter Regression File
ATFE_GF3500_(-1,0)_Stutter.txt
Edit
Stutter Exceptions File
ATFE_GF3500_(-1,0)_Exceptions.csv
Edit
FORWARD STUTTER
Stutter Enabled
Position Relative to Parent
1, 0
Inversely Proportional To
Expected Height of Stutter ...
Maximum Stutter Ratio
No Maximum 0.15
Variance
3.751, 2.574
Stutter Regression File
ATFE_GF3500_(1,0)_Stutter.txt
Edit


2BP STUTTER
Stutter Enabled
Position Relative to Parent
0, -2
Inversely Proportional To
Expected Height of Stutter ...
Maximum Stutter Ratio
No Maximum 0.1
Variance
4.5, 0.974
Stutter Regression File
ATFE_GF3500_(0,-2)_Stutter.txt
Edit
Stutter Exceptions File
Edit


DOUBLE BACK STUTTER
Stutter Enabled
Position Relative to Parent
-2, 0
Inversely Proportional To
Expected Height of Stutter ...
Maximum Stutter Ratio
No Maximum 0.1
Variance
2.558, 9.411
Stutter Regression File
ATFE_GF3500_(-2,0)_Stutter.txt
Edit
Stutter Exceptions File
Edit

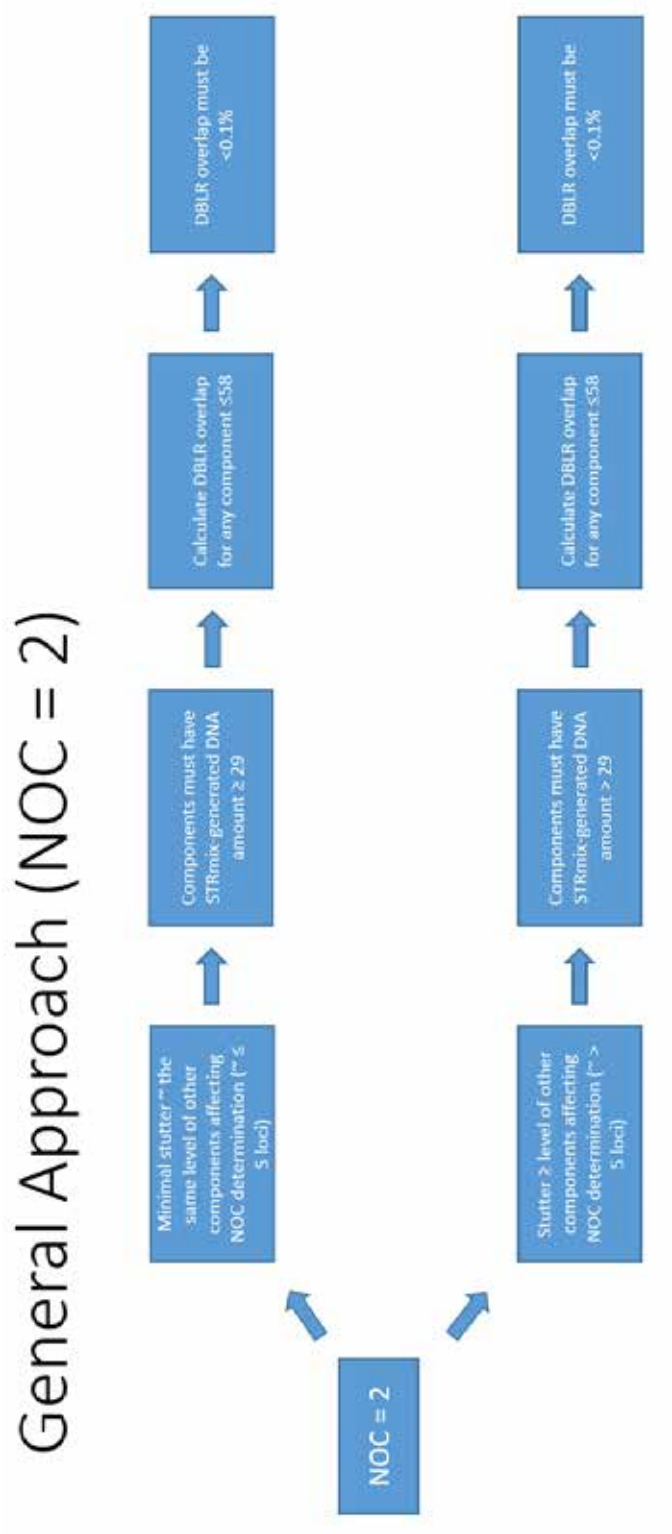

Allele Frequency File
NIST_GF_Cauc_080417.csv
Edit
Population Proportion
0.77
Default FST
0.01b(1.0, 1.0)
Population Size
323,127,513
Children Per Family
3
Sibling Proportion
6.189506988840037E-9
Child Proportion
6.189506988840037E-9
Parent Proportion
4.126337992560024E-9
Uncle/Aunt Proportion
8.252675985120049E-9
Niece/Nephew Proportion
1.2379013977680O7E-8
Grandparent Proportion
4.126337992560024E-9
Grandchild Proportion
9.284260483260056E-9
Cousin Proportion
3.713704193304022E-8
Unrelated Proportion
0.9999999123153177

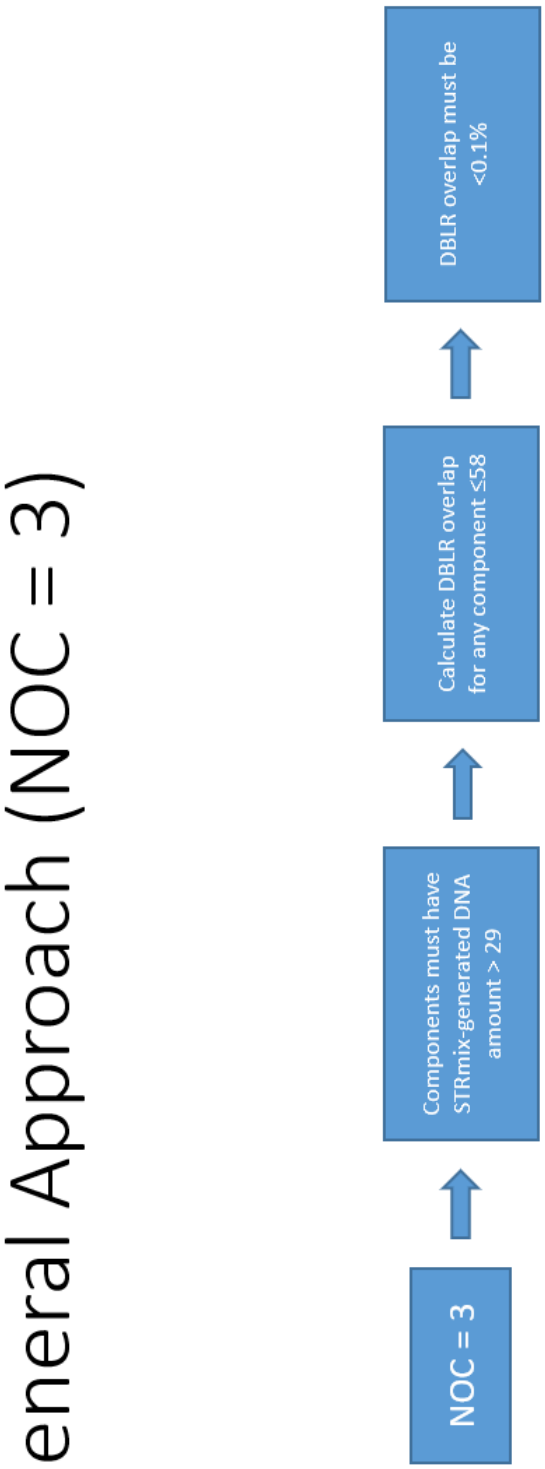

Allele Frequency File
NIST_GF_AfAm_080417.csv
Edit
Population Proportion
0.13
Default FST
0.01b(1.0, 1.0)
Population Size
323,127,513
Children Per Family
3
Sibling Proportion
6.189506988840037E-9
Child Proportion
6.189506988840037E-9
Parent Proportion
4.126337992560024E-9
Uncle/Aunt Proportion
8.252675985120049E-9
Niece/Nephew Proportion
1.2379013977680O7E-8
Grandparent Proportion
4.126337992560024E-9
Grandchild Proportion
9.284260483260056E-9
Cousin Proportion
3.713704193304022E-8
Unrelated Proportion
0.9999999123153177


Allele Frequency File
NIST_GF_Hisp.csv
Edit
Population Proportion
0.17
Default FST
0.01b(1.0, 1.0)
Population Size
323,127,513
Children Per Family
3
Sibling Proportion
6.1895069888400037E-9
Child Proportion
6.1895069888400037E-9
Parent Proportion
4.126337992560024E-9
Uncle/Aunt Proportion
8.2526759851200049E-9
Niece/Nephew Proportion
1.2379013977680007E-8
Grandparent Proportion
4.126337992560024E-9
Grandchild Proportion
9.284260483260056E-9
Cousin Proportion
3.713704193304022E-8
Unrelated Proportion
0.9999999123153177

General Approach:

- These are ***work instructions.***.
- Apparent NOC based on maximum alleles per locus (*above and below AT*), balance, stutter, etc.
- Minimum loci *guidelines* for mixtures:
    - Apparent 2 person mixture: 50% of STR loci or 11 loci,
    - Apparent 3 or more person mixture; 75% of STR loci or 16 loci.
- DNA amounts per component:
    - Component must have a STRmix-generated DNA amount of >29 or ≥29 depending on the peak heights (see the following slides).
    - For 3130/v2.4 decons – any DNA amount <58, decon with v2.6 for DNA amounts and DBLR evaluation only (not for LR calculation)
- DBLR analysis:
    - At a minimum, any component with a STRmix-generated DNA amount of 58 or below must have the Hp and Hd true overlap analysis performed. If the overlap is ≥0.1%, then the component is not suitable for comparison purposes.
    - For two and three person mixtures, if only one distinct allele is observed for a component, a DBLR overlap analysis should be performed for that component if the DNA amount is less than 150. In these situations, the STRmix analysis demonstrates greater range. It is important that components with only one distinct allele observed produce intuitive results, including the DNA amount. If the results are not intuitive, then the STRmix analysis may be re-run and/or DBLR analysis can be performed on the component in question (even if the DNA amount is greater than 150).
    - If the Degradation Max has been increased to allow STRmix™ to better model a DNA profile with one or more components that display severe degradation, the threshold for when the DBLR overlap analysis should be performed may need to be increased to ensure data would be expected to be detected at sufficient loci. In general, the DBLR overlap analysis should be performed on a component displaying severe degradation (~ 0.01) with a template amount less than 150 for two person mixtures and 250 for three person mixtures.


General Approach (NOC = 2)
NOC = 2
Minimal stutter ~ the same level of other components affecting NOC determination (~ ≤ 5 loci)
Components must have STRmix-generated DNA amount ≥ 29
Calculate DBLR overlap for any component ≤58
DBLR overlap must be <0.1%
Stutter ≥ level of other components affecting NOC determination (~ > 5 loci)
Components must have STRmix-generated DNA amount > 29
Calculate DBLR overlap for any component ≤58
DBLR overlap must be <0.1%


General Approach (NOC = 3)
NOC = 3
Components must have STRmix-generated DNA amount > 29
Calculate DBLR overlap for any component ≤58
DBLR overlap must be <0.1%

General Approach for NOC = 4 or 5

Follow previously established parameters for pulling a major component

The major component contains no more than three contributors.

There is no indication that the mixture contains more than a total of five contributors.

The lowest contributor to the major component has a Mixture Proportion of at least 20%, as determined by STRmix™.

The lowest contributor to the major component has a mixture proportion at least 3 times greater than the next highest component, as determined by STRmix™. NOTE: we are using the mixture proportion vs DNA amount for this parameter due to the binning of DNA amounts

Each of the contributors to the major component has a Template Amount of at least 87 RFU, as determined by STRmix™.

3130 / STRmix™ v2.4 Data

3130 and 3500 data was used during the evaluation

3130/v2.4 DNA amounts were compared to 3130/v2.6 DNA amounts and were found to be similar (expected variation observed due to binning with v2.6)

Because of this, the same red flag of a DNA amount of 58 or less is used to indicate when the DBLR Tool evaluation will be performed

The parameters for this approach were based on the v2.6 DNA amounts which uses binning. Therefore, v2.4 data must be re-analyzed using v2.6. Because 3130 data was not validated using v2.6, the decon generated from 3130 data decon'ed using v2.6 is only valid for the determination of suitability for comparison and not LR calculation.

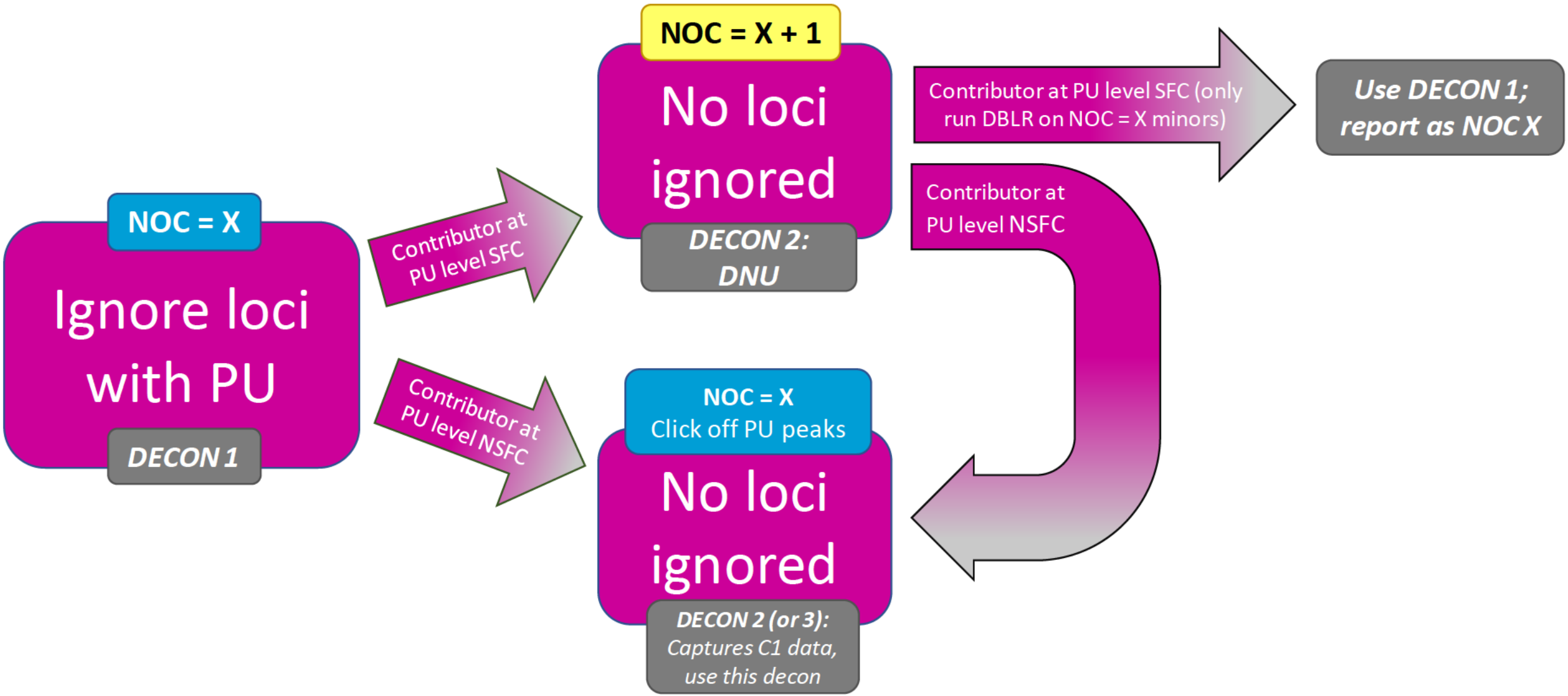
How to analyze pull up: Starting Criteria

- Click off all OL/OMR pull up
- For Single Source only: In-bin pull up peaks can be clicked off prior to initial decon
  - Decon as NOC = 1
- If pull up present is at/near the level of C1:
  - A decon should be run at the apparent NOC to verify if pull up is a weighted genotype for C1. If so, these loci should always be ignored for the final decon.
  - Ultimately, SFC weighted genotypes should NEVER include possible pull up, regardless of contributor position.
- If all autosomal peaks for a given contributor are possibly pull up:
  - This contributor is NSFC regardless of template/DBLR.
  - A decon must have been run as a NOC that includes this contributor.

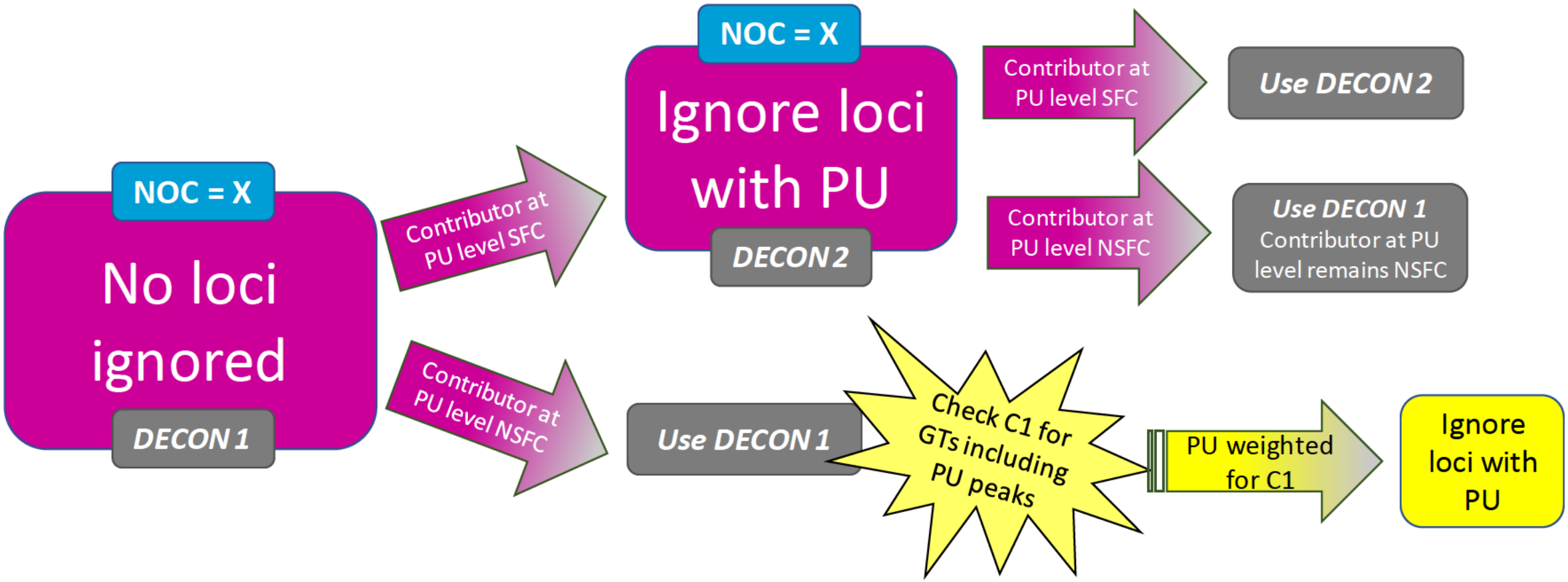
How to analyze pull up: Additional Info

- C1 alleles from ignored loci may be able to be entered into CODIS with CODIS admin initials
  - Leave ignored loci out unless needed for NDIS
- Decons should still always meet expectations.
  - With high C1s and low-level minor contributors, increased accepts are more likely to be needed.
- At the end of the workflow, if your decon ignores ≥ 6 loci (in general), you may consider reporting a separate decon for C1 and C2.
  - Discuss with Todd.
- This workflow does not apply to other artifacts
  - Non-human, soil peaks, etc

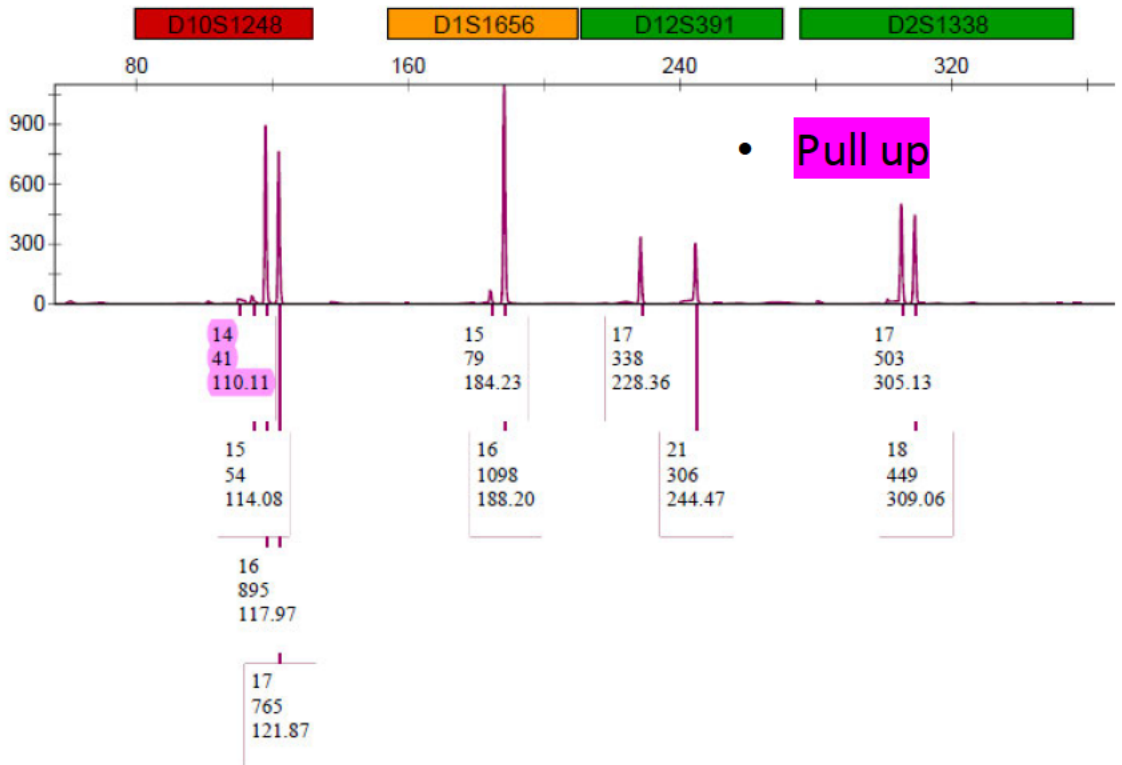
Scenario I: Pull up (PU) is the only indication of an additional contributor. *Non-pull-up contributors = X*



Scenario II: Pull up is present but does not add a contributor. *Non-pull-up contributors = X*



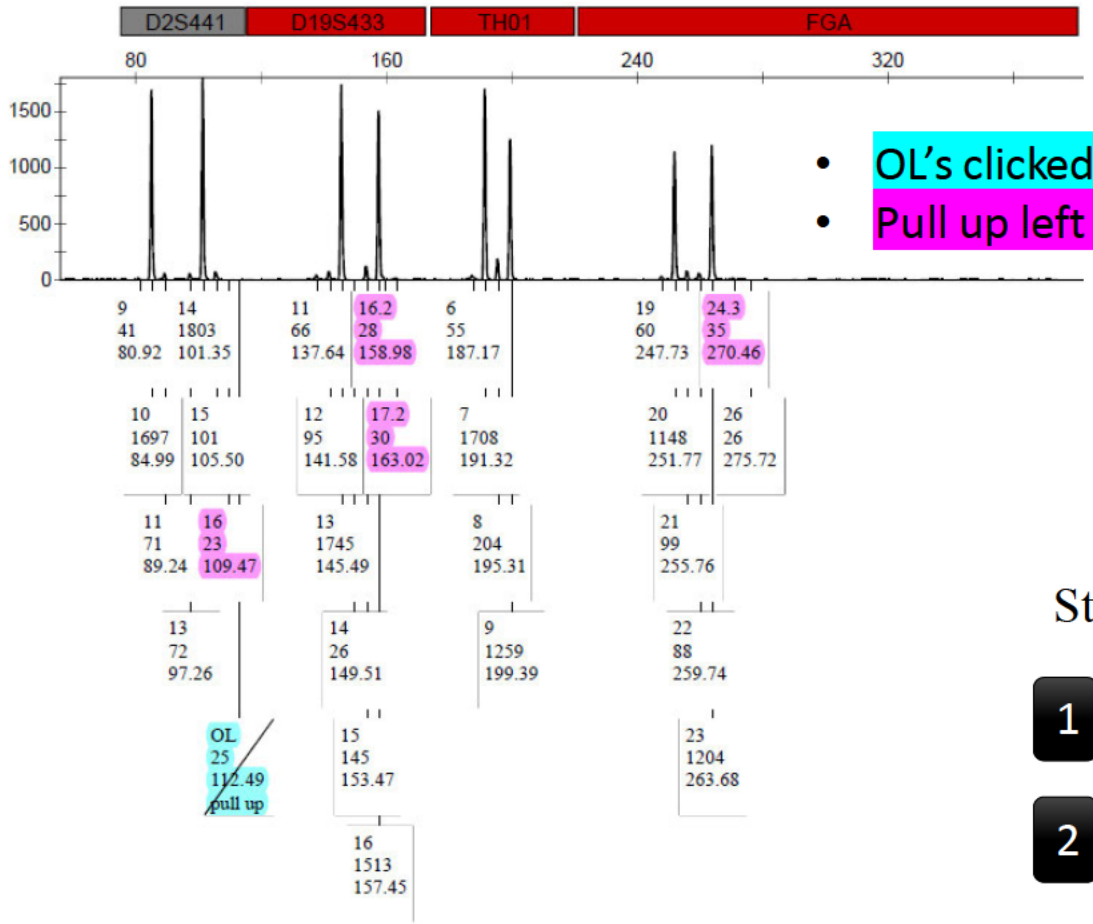
Example 1: NOC = 1, PU is the only indication of a 2nd



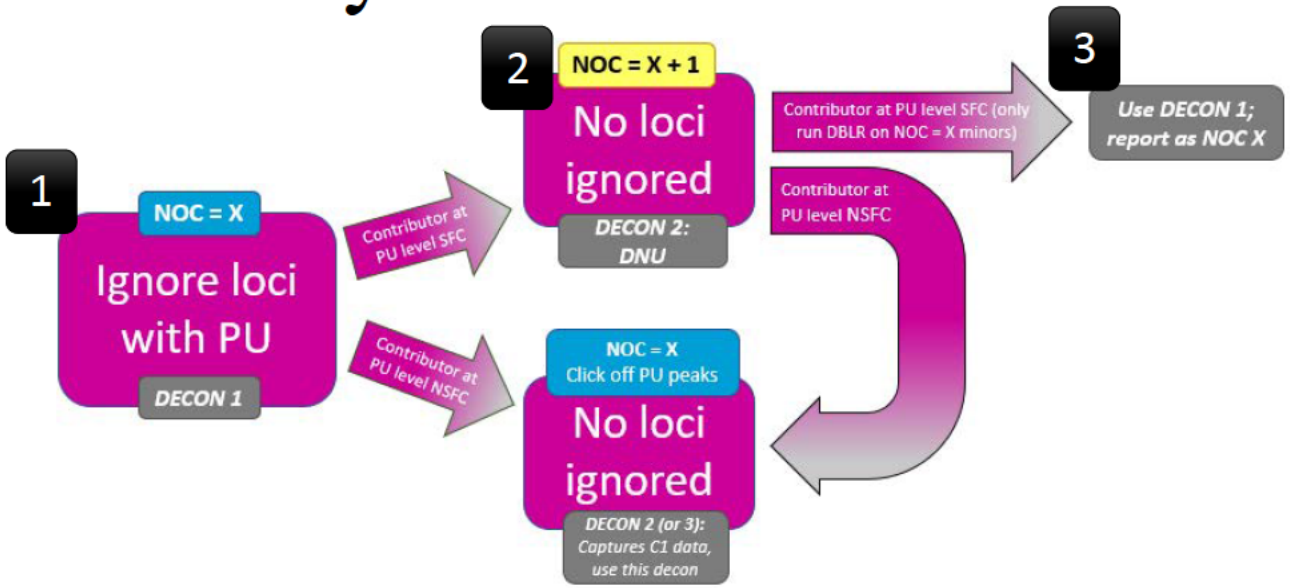
Steps: No workflow needed

1. In-bin pull up peaks can be clicked off prior to initial decon
2. Click off pull up, run as NOC = 1

Example 2: NOC = 2, PU is the only indication of a 3rd



- OL's clicked off
- Pull up left in



Steps: Use "Scenario I" workflow

1. Decon as NOC = 2, ignore D2S441, D19, FGA
   - Results: C1, C2 SFC
2. Decon as NOC = 3, no loci ignored
   - Results: C1, C2 SFC.
   - C3 template = 33, this contributor will not be reported, no need to run DBLR.
3. Use Decon 1

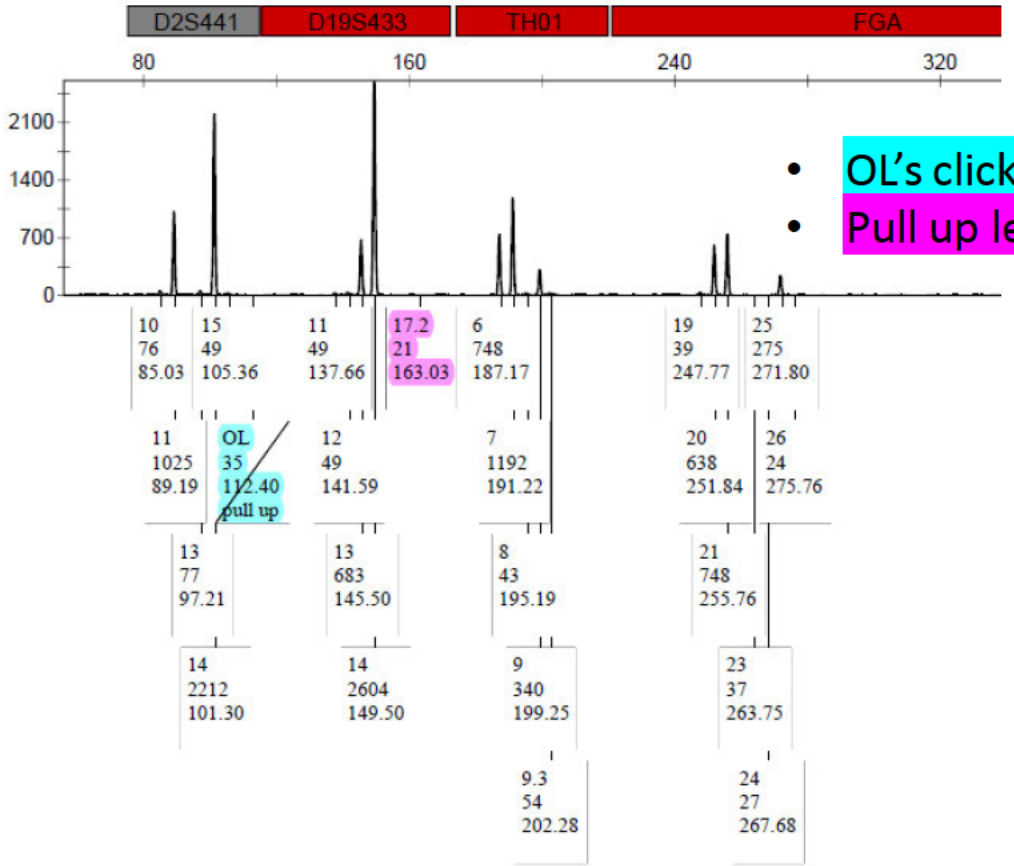
1

1	2
2164	109
95%	5%

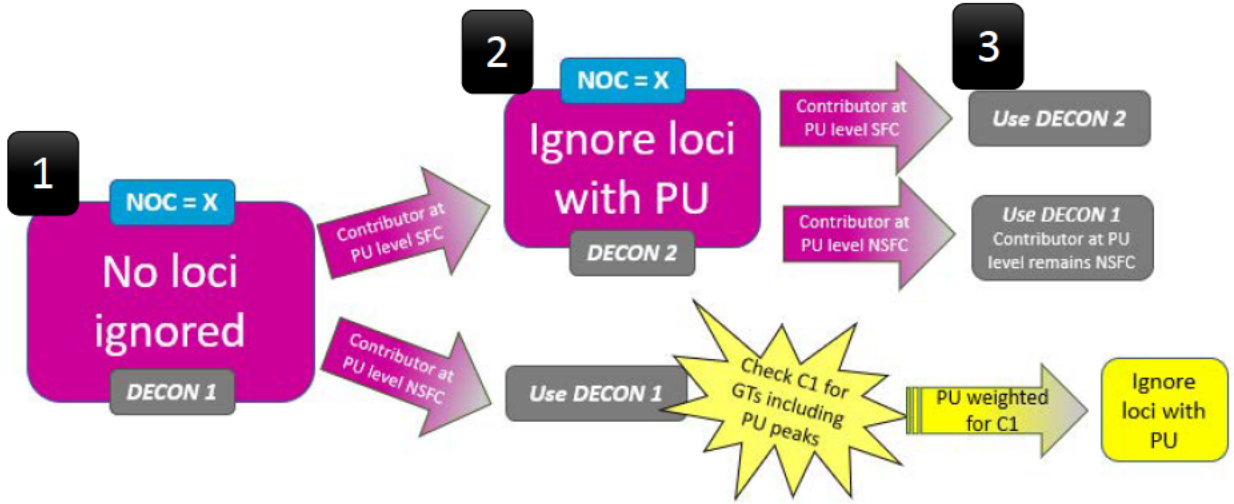
2

1	2
2146	91
95%	4%

Example 3: NOC = 3, PU is a weighted genotype for C3



- OL's clicked off
- Pull up left in



Steps: Use "Scenario II" workflow

1. Decon as NOC = 3, no loci ignored
   - Results: C1, C2, C3 SFC.
   - Pull-up highly weighted for C3
2. Decon as NOC = 3, ignored D19
   - Results: C1, C2, C3 SFC.
3. Use decon 2

1

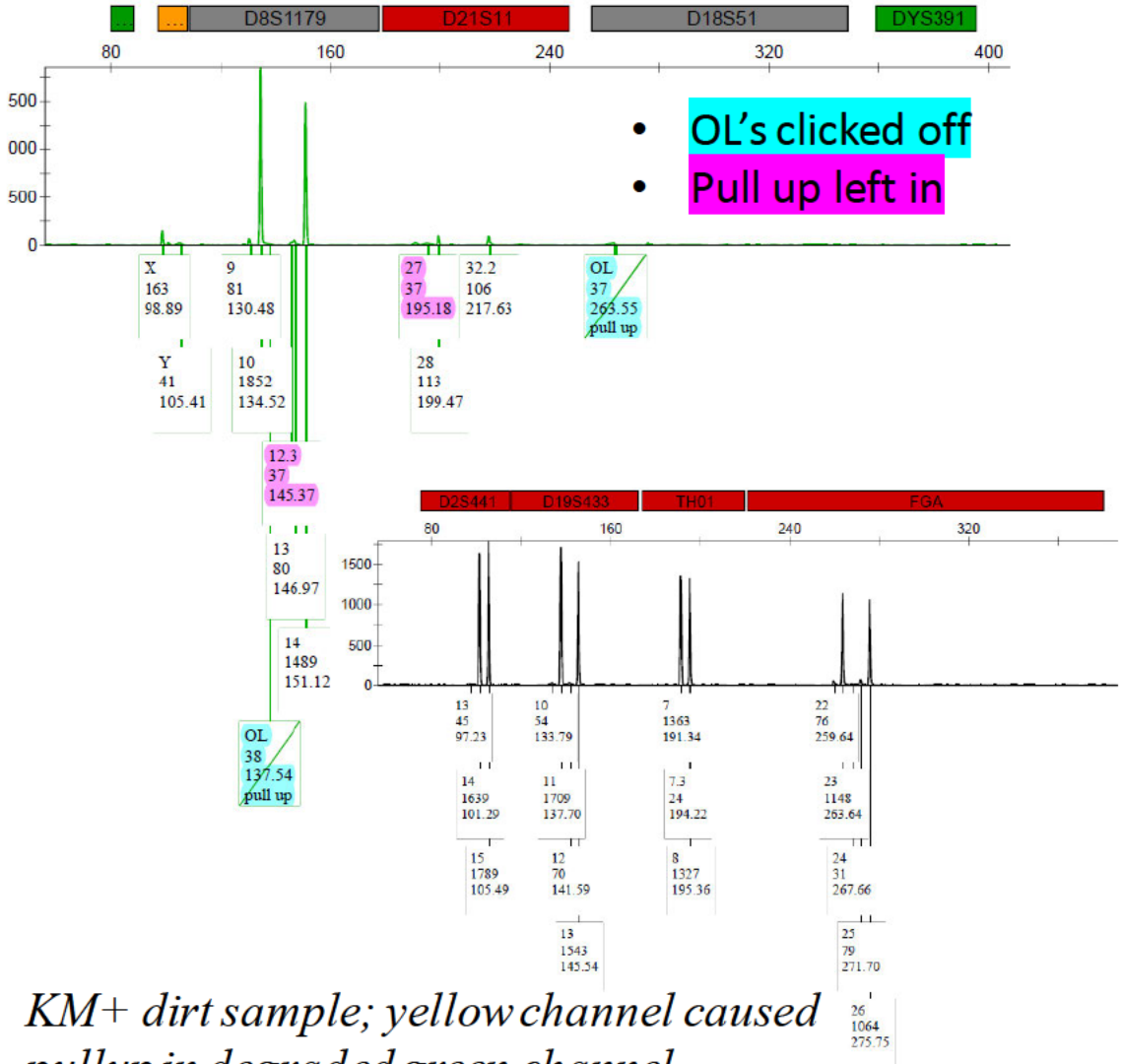
1	2	3
1479	906	54
61%	37%	2%

D19S433	11, 17.2	98.10%
	12, 17.2	0.87%

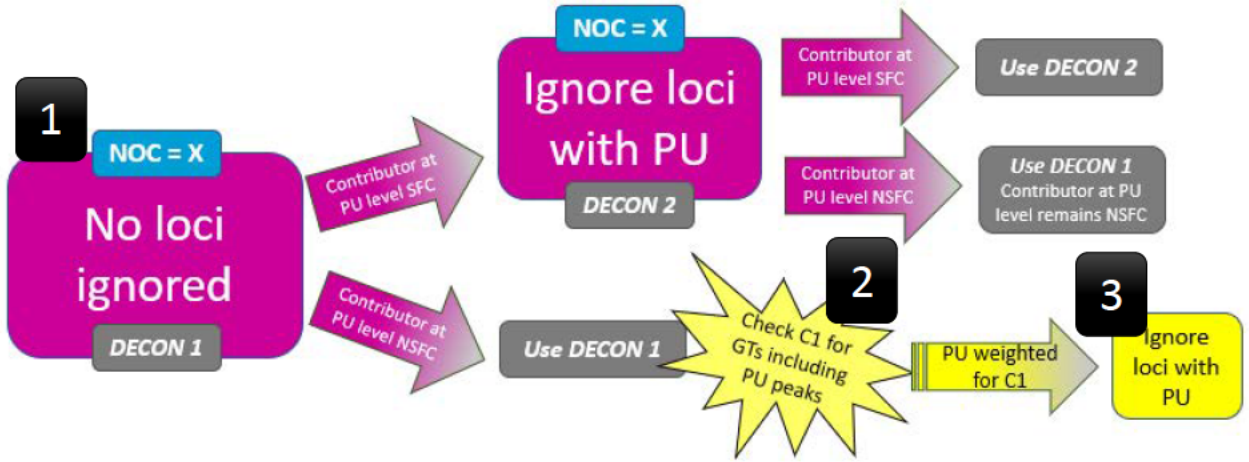
2

1	2	3
1548	939	73
60%	37%	3%

Example 4: NOC = 2, PU is a weighted genotype for C1



KM+ dirt sample; yellow channel caused pullup in degraded green channel



Steps: Use "Scenario II" workflow

1. Decon as NOC = 2, no loci ignored
   - Results: C1 SFC, but…
2. PU weighted for C1 at D8 and D21. C2 NSFC (DBLR)
3. Decon as NOC = 2, ignore D8 and D21
   - Results: C1 SFC, C2 NSFC
   - Use decon 2

1. Scope

This protocol is to be used when examining an exhibit of evidence or a crime scene for the presence of blood and is only a presumptive test. A presumptive test is one that is highly sensitive but not specific to the body fluid in question. The BLUESTAR® test is a presumptive test for the presence of hemoglobin, a component of the red blood cells in blood. The BLUESTAR® test is based on the peroxidase activity of the iron in heme or heme derivatives, which catalyzes the oxidation of the BLUESTAR® reagent. A positive reaction is observed when the colorless BLUESTAR® solution is oxidized and produces a chemiluminescent blue color. Other substances such as plant peroxidases, chemical oxidants, and some metal ions may also cause a positive reaction; therefore, a positive BLUESTAR® test only indicates the presence of blood and does not confirm the presence of blood.

2. References

2.1. Manufacturer's insert for the BLUESTAR® Forensic test kit, May 2014.
2.2. R.E. Gaensslen, Identification of blood, in: Sourcebook in Forensic Serology, Immunology and Biochemistry, U.S. Department of Justice, Washington, D.C., 1983, pp. 73-133.
2.3. R.C. Shaler, Modern forensic biology, in: R. Saferstein, Forensic Science Handbook, second ed., Prentice Hall, Upper Saddle River, 2002, pp. 531-535.

3. Equipment

3.1. Disposable gloves
3.2. Eye protection
3.3. Lab coat
3.4. Sterile swabs
3.5. Sterile water
3.6. Presumptive blood test kit (BLUESTAR® Forensic tablets (reagent and catalyst) – E.g. BLUESTAR Forensic: Product #BL-FOR-TAB4)
3.7. Known positive control (dried blood)
3.8. Known negative control
3.9. Spray bottle with adjustable spray nozzle
3.10. Scissors
3.11. Forceps
3.12. 70% ethanol or alcohol wipes
3.13. 10% bleach solution
3.14. Bench paper

4. Safety/Quality Assurance

4.1. Any utensils used to cut or manipulate swabs or other types of evidence must be cleaned between uses with 10% bleach solution followed by 70% ethanol or alcohol wipes.
4.2. Disposable gloves shall be worn when handling kit reagents and evidence.
4.3. Record the lot number of each reagent used in notes. Do not use the reagents after the expiration date.
4.4. Best results are obtained within 3 hours of combining the BLUESTAR® tablets with water.
4.5. At a minimum, each day the reagents are to be used, a negative control and positive control must be tested and documented in the analyst's notes, along with the manufacturer, lot number, and expiration date. If either of the controls fail, a different lot of reagents shall be used.
4.6. This test is only a presumptive test. It is not human specific. Other means to confirm the presence of human blood are available.
4.7. Lab coat and eye protection must be worn at all times while performing this procedure.
4.8. When practical, only one item of evidence shall be open at a time.
4.9. The laboratory bench surface shall be cleaned before and after use with 10% bleach solution or other sanitizing agent and may be followed by 70% ethanol. Fresh bench paper shall then be placed on the surface prior to examination.
4.10. Minor deviations from the protocol may be made at the analyst's discretion based on the analyst's training and experience and shall be indicated in the analyst's notes. Significant deviations from the protocol must be approved by the DNA Technical Leader.

5. Reagent Preparation

5.1. Add 125 ml of sterile water to a spray bottle and add one beige reagent tablet and one white catalyst tablet from the BLUESTAR® tablet packet (if more than 125 ml is required, the solution may be scaled-up; one pair of tablets for every 125 ml of sterile water).
5.2. Allow the tablets to dissolve for 1-2 minutes and gently swirl the spray bottle to mix (do not invert the bottle).

6. Procedure

6.1. Darken the environment as much as possible.
6.1.1. Indoors: Turn off lights and block outside light from windows.
6.1.2. Outdoors: If possible, wait until night or block out as much light as possible.

6.2. Adjust the spray nozzle for finest spray (mist) possible and spray the test area with the prepared reagent at a distance of ~2 feet in a side to side motion (over-spraying will not produce better results and may dilute the sample).

6.3. An immediate faint to intense blue colored luminescence indicates a positive reaction. No luminescence indicates a negative reaction. Interpretation of results may be made immediately (within 5 seconds). Luminescence should persist for 30 seconds to 1 minute.

6.3.1. Positive Result: Visualization of a luminescent blue color

6.3.2. Negative Result: No luminescence

6.4. Record results by noting the luminescence or lack of luminescence and the conclusion of the test.

6.5. A cutting or swabbing of an unstained area adjacent to the stained area may be tested as a substrate control at the analyst's discretion.

6.6. A positive BLUESTAR® result, alone, cannot be reported as an indication of the presence of blood. This result must be followed by a positive phenolphthalein test result in order to report the indication of the presence of blood.

6.7. A negative result can be reported following the "Results of testing for the presence of blood" section within ATF-LS-FB34 Reporting the Results of Forensic Biology Analysis using STRmix™.