FORENSIC BIOLOGY SECTION

CASEWORK
TEST METHODS

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

FOREWORD

The Forensic Biology Section in the Laboratory Division of the Indiana State Police (ISP) conducts tests on various body fluids, body fluid stains, and human hair for criminal justice agencies. DNA analysis is performed as needed on the various biological materials. The Laboratory Division reserves the right to evaluate and prioritize the items submitted and limit the total number to expedite service and to manage available resources. The accuracy and specificity of test results are ensured by running known controls with each set of tests.

The analysts of the Forensic Biology Section shall have a minimum of a baccalaureate or an advanced degree in a natural science or a closely related field. Analysts shall have successfully completed college course work covering the subject areas of genetics, biochemistry, molecular biology, and statistics. All analysts undergo a formalized training program covering forensic techniques and instrumentation. Successful completion of the Training Program is required before analysis of evidence is performed. Additionally, all analysts participate in external proficiency testing from an accredited vendor.

A technician shall follow the same guidelines and procedures as an analyst in all methods in which they are authorized.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
TABLE OF CONTENTS

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

1. Serology Methods:

1.1. Scope: This test method is designed for the guidance of laboratory personnel who assist investigations where body fluids or stains occur as physical evidence in a variety of alleged criminal activities. The scope of this type of evidence includes but is not limited to the following: identification of human blood, semen, and human hair. This test method may be expanded or altered as techniques and/or new genetic analyses are found applicable and validated.

1.1.1. Trace evidence including but not limited to hair, fibers, glass, or paint may be examined. Trace evidence shall be retained in the original packaging unless value is immediately apparent. Appropriate notes shall be taken on the trace evidence collected. When required, hairs shall be evaluated for the possibility of human origin and the potential for future analysis.

1.1.2. A search is made of submitted items for the presence of biological material.

1.1.3. Previously retained items may be released at the prosecutor's request. The request shall be documented in the case record. A valid court order shall circumvent this procedure.

1.2. Precautions/Limitations:

1.2.1. Evidence

1.2.1.1. Liquid items such as blood standards shall be refrigerated not frozen. Stained materials may be frozen, refrigerated or stored at room temperature in a dry environment. Items to be analyzed for latent prints should not be frozen or refrigerated.

1.2.1.2. Stains that are still moist shall be air dried in a secure area before submission to the laboratory.

1.2.1.3. Clean paper or cardboard containers shall be used to package dried evidence. Plastic containers shall not be used for packaging because they hold moisture which often leads to putrefaction of biological materials. Exceptions to this rule would include blood tubes, body tissue samples and condoms. Each item should be placed in separate containers from the point of collection.

1.2.1.4. If multiple items are placed in one container at time of collection, they may be submitted in the single, original package.

1.2.1.5. Specific examinations performed on items of serological evidence shall be determined by laboratory personnel.

1.3. Related Information:

1.3.1. Appendix 1 Flow Charts

1.3.2. Work Sheet Manual

1.4. Instruments:

1.4.1. Balances – An analytical balance used for preparation of analytical reagents and buffers.

1.4.2. Centrifuges – A serofuge capable of operating at 3,400 rpm and a microcentrifuge capable of greater than 15,000 rcf, are used for separation of solid components from fluids.

1.4.3. Alternate light source (ALS) – A light source to aid in the location of stains and trace evidence by use of various wavelengths of light.

1.4.4. Crosslinker - Preprogrammed ultraviolet exposure unit which is factory set to ~120,000 microjoules per cm^2.

1.4.5. <u>Digital camera</u> – Used to document evidence and/or the packaging.

1.4.6. <u>Microscopes</u> – A light microscope with magnification up to 400X, a stereoscope for general screening of items, and a phase contrast microscope for the identification of spermatozoa.

1.4.7. <u>Miscellaneous Laboratory Equipment</u> – Supportive laboratory equipment including ovens, incubators, pipettors, rotators, stirring/heating plates, vortex mixers, vacuum pumps, UV lights, and refrigerators/freezers for storing of reagents, buffers, and evidence.

1.4.8. <u>pH meter</u> – An instrument capable of manual or automatic temperature compensation and reading +/- 0.01 pH units. Used for preparation of buffers and reagents.

1.5. Reagents/Materials: See Reagent Preparation Manual for instructions and logs.

1.5.1. Alpha Naphthyl Phosphate Solution
1.5.2. Acid phosphatase Acetate Buffer
1.5.3. Acid phosphatase Dye Solution
1.5.4. Glucose, saturated
1.5.5. HemDirect Hemoglobin Cassettes
1.5.6. HemDirect Hemoglobin Buffer
1.5.7. 3% H_2O_2
1.5.8. Phenolphthalein Stock Solution
1.5.9. Phenolphthalein Working Solution
1.5.10. 10% Sodium Hydroxide

1.6. Hazards/Safety:

1.6.1. All chemicals shall be handled in a safe method as referenced in the specific SDS.

1.6.2. Preparation of the Phenolphthalein Stock Solution shall be performed in a chemical fume hood.

1.6.3. Universal Precautions shall be in use whenever biological materials are being handled.

1.6.4. Extreme caution shall be used when handling liquid body fluid samples.

1.6.5. Biological waste shall be disposed of in the appropriate waste receptacle.

1.7. Reference Materials/Controls/Calibration Checks:

1.7.1. HemDirect Hemoglobin tests shall be performance checked before use in casework.

1.7.1.1. Each new lot of HemDirect Hemoglobin Tests shall be analyzed with a 1:10 dilution of blood dried on filter paper or a 1:500 dilution of liquid human blood. A positive result as described in the HemDirect test method shall be obtained before use in casework. The worksheet used to record the results shall be retained.

1.7.1.2. If the lot fails, the quality control test it may be retested. If it fails a second time the lot shall not be used for casework. The Technical Leader shall be informed within one business day of the failure.

1.7.2. Running known controls with each test ensures the accuracy and specificity of the test results. See the specific test procedure for the appropriate controls to be run.

1.8. Procedures/Instructions:

1.8.1. Examination Procedure

1.8.1.1. **Standards for Comparison**

1.8.1.1.1. Appropriate standards, such as whole blood or buccal swabs, from those individuals involved should be submitted along with the unknown materials to be analyzed.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

1.8.1.1.2. The whole blood standard should be submitted in a purple top tube that contains ethylene-diamine-tetra-acetic acid (EDTA). Prior to examination, while in the custody of the ISP Laboratory, this tube shall be refrigerated.

1.8.1.1.3. A stain card may be prepared from at least one whole blood standard from each individual.

1.8.1.1.4. Buccal swab standards containing multiple swabs from an individual may be labeled and treated as one item.

1.8.1.1.5. Oral swabs in a sexual assault kit with no identified standard may be used as a standard if a single source profile is obtained.

1.8.1.1.6. The analyst's notes shall indicate the presence of hair standards. In sexual assault kits where a blood or buccal swab standard is present the hair standards do not need to be opened or examined, just noted.

1.8.1.1.7. DNA profiles shall be developed from suspect standards in all cases and from victim and elimination standards in crimes against person cases. DNA profiles may be developed for victim and elimination standards in property crimes; however it is not required if no unknown profiles are developed for comparison. If any analysis is performed (e.g. extraction through quantification), then the DNA profile should be generated.

1.8.1.1.8. In cases where a secondary standard is needed, the item to be used as a secondary standard shall be clearly identified as such in the item description section of the case report.

    1.8.1.1.8.1. The use of a blood stained article of clothing may be appropriate if stains from the person wearing the clothing can be definitively identified (i.e., blood from around the wound area).

    1.8.1.1.8.2. In the case of a missing person, personal items such as toothbrush, hairbrush, razor or other item may be submitted.

    1.8.1.1.8.3. When a contributor requests items to be used as secondary standards during the submission process, it should be indicated on the Request for Laboratory Examination form.

    1.8.1.1.8.4. Items collected as surreptitious standards or from trash pulls to be used for comparison shall be clearly identified within the body of the report. If a statistic is calculated, it shall be reported as "purported to belong to" or "contributor of the DNA profile from item #."

        1.8.1.1.8.4.1. If items within the trash pull generate profiles from different individuals of the same sex as the person of interest, no assumptions of profile ownership shall be done and no statistics shall be calculated.

1.8.1.2. **Trace Evidence**

1.8.1.2.1. A clean piece of paper shall be used under each item as it is examined. The exam paper should be retained in the original package. After returning the item to its package, fold the exam paper, tape it closed (making sure all trace evidence is secure within the bundle) and place it in the package with the item.

1.8.1.2.2. Items may be tape lifted to collect evidence. Where tape lifting is not appropriate, tweezing and/or scraping may be substituted. Items involving multiple examination

requests shall only be tape lifted or scraped after consultation with analysts from the other disciplines.

1.8.1.2.3. All collected trace evidence shall be stored with original item unless value is immediately apparent.

1.8.1.2.4. Analysts shall document in the notes the presence of hair, fibers, plant material, glass, paint, etc. Such trace evidence shall be reported out only when it is deemed to have value. In those cases where the material appears to be extraneous debris of no significance, the trace statement may be omitted from the report.

1.8.1.2.5. Hair combings need not be opened or examined, just their presence noted. If opened, the approximate number and quality of hairs shall be documented.

1.8.1.2.6. In cases where there is other DNA evidence of investigative value that has been tested and profiles obtained, no DNA testing of the collected hair shall be performed without the approval of a Biology Unit Supervisor or Laboratory Manager.

1.8.1.2.7. When appropriate, examination of the hair collected shall be performed to determine if hair consistent with human origin is present.

1.8.1.2.8. Human hairs can be submitted for DNA analysis when beneficial.

1.8.1.3. **Dried Stains**

1.8.1.3.1. Visual Inspection – A visual inspection of the item shall be conducted to determine the location, if present, of unknown stains. An ultraviolet light or an alternate light source may be used to help locate stains.

1.8.1.3.2. Confirmatory testing for semen and blood/human blood is at the discretion of the analyst.

1.8.1.4. **Stain Evaluation – Sexual Assault kits**

1.8.1.4.1. No serological testing shall be performed on samples within a kit.

1.8.1.4.2. Exceptions require notification to a supervisor with documentation in the case record.

1.8.1.4.3. Acid Phosphatase testing and sperm searches may be done at the request of a Prosecutor with proper justification and documentation in the case record.

1.8.1.5. **Stain Evaluation – All other**

1.8.1.5.1. After attempting to identify the stain, the stain shall be evaluated for further testing.

1.8.1.5.2. If no biological material is identified on the item, no further testing shall be performed, except for instances where touch DNA or handler/wearer analysis is appropriate.

1.8.1.5.3. If the stain is found to be biological, the analyst shall determine if DNA testing is performed based on an evaluation of the stain. Things that may be considered include, but are not limited to, the amount, size, concentration, location, and condition of the stain.

1.8.1.6. **Collection and labeling of swabs (or other items such as shoes, sheets, etc.)**

1.8.1.6.1. Each swab in a submitted item shall have a unique identifier if serological testing is performed on any of the swabs for that item. If different testing results are obtained between swabs in the same item, each result will be reported.

1.8.1.6.2. Swabs may be used as a sampling method for DNA analysis. If no serological testing is performed and the swabs are being treated as one sample (i.e., portions of all

swabs are combined together for DNA analysis), the swabs do not need to be distinguished in the report or described as "combined".

1.8.1.6.2.1. If the swabs are being treated differently for DNA analysis (i.e., one swab is tested while the other is not), the samples shall have unique identifiers and be reported separately.

1.8.2. Sampling of items that may contain unknown or dangerous drugs

1.8.2.1. Caution should be exercised when opening and examining evidence with unknown powders and liquids or known dangerous powders or liquids. Every effort should be made to prevent exposure to potentially hazardous materials. If there is any concern or knowledge about a specific hazard, analysts may use a fume hood for processing.

1.8.2.2. Narcan (or similar product) should be available and in a clearly marked location during analysis.

1.8.3. Phenolphthalein (Kastle-Meyer Reagent), Presumptive Test for Blood

1.8.3.1. **Principle:** Phenolphthalein is an oxidizable organic molecule, which can be oxidized by free hydroxyl ions liberated by peroxidase-like action. The heme group of hemoglobin possesses a peroxidase-like activity, which may catalyze the breakdown of hydrogen peroxide to form free hydroxyl radicals. Phenolphthalin (reduced form) is oxidized by the free hydroxyl ions to phenolphthalein (oxidized form), producing a pink color.

Phenolphthalein (abbreviated Phe on controlled worksheets) is a presumptive test for the presence of blood. Confirmatory testing shall be performed for conclusive identification. Phenolphthalein has been shown to give false positives or weak reactions with various oxidizing agents, plant material, etc.

1.8.3.2. **Procedure**

1.8.3.2.1. Rub the suspected bloodstain with a piece of filter paper or a cotton swab or make a small cutting of the suspected bloodstain and place on white filter paper or in a white spot plate well.

1.8.3.2.2. Add one to three drops of the phenolphthalein working solution to the stain.

1.8.3.2.3. Wait 10-15 seconds, assuring no pink color develops at this time.

1.8.3.2.4. Add one to three drops of 3% Hydrogen Peroxide.

1.8.3.2.5. Upon addition of the Hydrogen Peroxide an immediate pink color is indicative of the possible presence of blood.

1.8.3.3. Reagents shall be tested with positive and negative controls and the results recorded in the case notes. This shall be performed and recorded daily when in use and prior to use on evidence. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.4. HemDirect Hemoglobin Test, Confirmatory Test for Human Blood

1.8.4.1. **Principle:** Human blood reacts with an anti-human hemoglobin antibody, which forms a red line in the "test" region of the HemDirect cassette.

1.8.4.2. **Procedure**

1.8.4.2.1. Place a ~2mm² cutting, a few flakes, a few drops of liquid blood diluted to 1:500, or ~1/8 of a swab in the tube of supplied extraction buffer. The cutting shall remain in the buffer solution for 5 minutes. A maximum extraction time of 10 minutes may be utilized for inhibited samples.

1.8.4.2.2. Add three drops of the extraction buffer/sample mix with the provided pipette to the sample well of the cassette.

1.8.4.2.3. A positive result may be recorded as soon as red lines are observed in both the "C" (control) and "T" (test) regions.

1.8.4.2.4. Negative results (a red line in only the "C" region; no line in the "T" region) should be confirmed after 10 minutes.

1.8.4.2.5. If a line in the "C" region does not appear after 10 minutes, test results are invalid and shall be repeated.

Each new lot of HemDirect kits shall be performance checked before use. Additional testing of reagents with positive and negative controls before use on evidence is not required. Lot numbers of reagents and the result of the internal positive control shall be recorded.

1.8.5. Acid Phosphatase, Presumptive Test for Semen

1.8.5.1. **Principle:** Seminal fluid is typically found to contain acid phosphatase in levels 400 -1000 times those found in other body fluids. This test uses the ability of acid phosphatase to hydrolyze a phosphate ester substrate to release a free alcohol, which in turn reacts with a diazonium salt to give a brilliantly colored end product.

Acid Phosphatase (abbreviated AP on controlled worksheets) is a presumptive test for the presence of semen. Conclusive identification of semen can only be done by performing confirmatory testing. Possible sources of acid phosphatase other than semen include vaginal secretions, urine, sweat or fecal material.

1.8.5.2. **Procedure:** Prepare substrate and dye solution immediately prior to testing. These solutions will be stable for 1-2 hours at room temperature. Alternatively, use previously aliquoted and frozen solutions.

1.8.5.2.1. Weigh out about 20 µg of alpha naphthyl phosphate and place in shell vial or small test tube.

1.8.5.2.2. Weigh out about 20 µg of fast blue or fast red dye (shall be in diazonium salt form) and place in second shell vial or small test tube.

1.8.5.2.3. Add about 1 ml of acetate buffer to each and mix.

1.8.5.2.4. Cut a small (approximately 2 mm x 2 mm) portion of the stained material and place it on a small piece of filter paper, in a white ceramic spot plate well or in a small test tube. An extract can be used.

1.8.5.2.5. Wet the cutting with the alpha naphthyl phosphate solution. Wait about 1 minute.

1.8.5.2.6. Add 1-2 drops of the dye solution to the cutting.

1.8.5.2.7. The development of color (depending on dye used) in less than 15 seconds is indicative of the presence of acid phosphatase. Color soaked into the filter paper under the sample is also indicative of a positive test.

1.8.5.2.8. A slow development of color could be an indication of acid phosphatase from sources other than semen. This may be indicated on the worksheet as a slight color change (SCC). A SCC result shall be reported as a negative result.

1.8.5.2.9. The interpretation of the results shall be reported by the analyst.

1.8.5.3. Reagents shall be tested with positive and negative controls and the results recorded in the case notes. This shall be performed and recorded daily when in use and prior to use on evidence. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.6. Extraction of Body Fluid Stains

1.8.6.1. **Principle:** Evidentiary stains can be extracted and the extracts used for body fluid stain identification.

1.8.6.2. **Procedure**

1.8.6.2.1. Prepare samples (and backgrounds, if used) for testing by eluting a cutting of the stain (approximately 3 mm by 3 mm) in an appropriate amount of distilled water in a microcentrifuge tube overnight in the refrigerator. Alternatively, the sample can be eluted in a 37°C oven for a minimum of 30 minutes or at room temperature for a minimum of 1 hour.

1.8.6.2.2. After incubation, the cutting should be removed, placed into the cap with holes for drainage or a spinex basket, and the extract spun down.

1.8.7. Confirmation of Seminal Material

1.8.7.1. **Principle:** The visual identification of spermatozoa is a scientifically accepted method of positively identifying the presence of seminal material.

1.8.7.1.1. The observations should be rated using the following system:

POS (+) Only a few sperm or sperm heads on the entire slide.
1+ One sperm or sperm head per several fields of view.
2+ One sperm or sperm head in about half of all fields of view.
3+ One sperm or sperm head in most fields of view.
4+ Several sperm or sperm heads in most fields of view.

1.8.8. Phase Contrast Microscopy for the Visual Observation of Spermatozoa

1.8.8.1. **Principle:** Phase contrast microscopy is used to differentiate the cellular appearance. Spermatozoa are round to ovoid with an acrosomal cap, midpiece, and tail. When the tail and midpiece are not present, the distinctive flattened profile of turned spermatozoa should be observed.

1.8.8.2. **Procedure**

1.8.8.2.1. Place ~10 µl of the pellet from the bottom of a previously extracted sample on a microscope slide and cover.

1.8.8.2.2. Alternatively, a small (1 mm x 1 mm) cutting may be eluted directly onto the slide with a drop of distilled water, agitating with forceps. Remove the cutting and cover the extract with a cover slip.

1.8.9. Christmas Tree Stain (Kernechtrot-Picroindigocarmine Differential Stain) (Optional procedure to help visualize spermatozoa)

1.8.9.1. **Principle:** The Christmas tree stain is used to assist in scanning slides for the presence of spermatozoa. Red and green colored stains are utilized to enhance the appearance of spermatozoa and epithelial cells in biological samples.

1.8.9.2. **Procedure**

1.8.9.2.1. Pipette ~5-10 µl of the pellet from the bottom of extracted sample tube and place onto microscope slide. Previously prepared slides from the evidence collection kit may go directly to heat fix and staining procedures.

1.8.9.2.2. Alternatively, a small (1 mm x 1 mm) cutting may be eluted directly onto the slide with a drop of distilled water, agitating with forceps. Remove the cutting and cover the extract with a cover slip.

1.8.9.2.3. Also prepare a slide from a 1:50 dilution of a sperm positive semen stain to use as a positive control for the staining procedure.

1.8.9.2.4. Heat fix the sample. Pre-heat a hot plate to approximately 200°C. Place the prepared slides on the hot plate for approximately 30 seconds. Ensure that the sample is dry. Alternatively, a lower temperature with a longer time can be used and adjusted, as needed, to adequately fix the sample (e.g. 100 – 140°C heated for 5 – 20 minutes).

1.8.9.2.5. After the slide has cooled, place on a level surface and add several drops of the SERI Christmas Tree Stain A (red stain) to the slide. Stain for about 10 to 15 minutes.

1.8.9.2.6. Rinse stain from the slide by gently running distilled water over the slide.

1.8.9.2.7. Return slide to a level surface and add several drops of the SERI Christmas Tree Stain B (green stain) to the slide. Stain for about 10 seconds.

1.8.9.2.8. Rinse stain from the slide by gently running 95 to 100% ethanol over the slide.

1.8.9.2.9. Allow slide to air dry or dry in 56°C oven. After drying, slide is ready to view microscopically applying bright field procedure.

1.8.9.3. Reagents shall be tested with a positive control and the results recorded in the case notes. This shall be performed and recorded with each batch of staining. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.9.4. **Interpretation**

1.8.9.4.1. Spermatozoa will appear as small red/pink structures and have the following staining characteristics:

Acrosomal cap	clear to pink
Post acrosomal cap	dark pink to red
Midpiece	green to blue
Tail	green to blue

1.8.9.4.1.1. Nucleated epithelial cells will appear as green rhomboid-like structures with a red/pink nucleus. The cell's appearance can vary in shape, often being folded or overlapping and are normally larger than the spermatozoa heads.

1.8.9.4.2. **NOTE**: The smear slides contained in evidence collection kits shall be viewed using phase contrast microscopy before proceeding to the Christmas tree stain procedure.

1.8.10. Visual Observation of Human Hair

1.8.10.1. **Principle:** Before DNA analysis can be performed on hair collected as evidence, a determination of possible human origin and the identification of the root shall be performed.

1.8.10.2. **Procedure**

1.8.10.2.1.   When the unknown hairs are on a tape lift, the lift should be examined using a stereomicroscope to eliminate obvious hair fragments, fibers, and animal hairs. All hairs of possible human origin with sufficient root material should be removed from the lift and placed in a paper bindle. A wet mount should be prepared when further examination is required to determine human origin and/or presence of root material. Unknown hairs are placed on a microscope slide with a drop of distilled water, covered with a cover slip, and examined with a light microscope. The number and general appearance of the human hairs should be noted when practical. If additional hairs/fibers are present in the original package, they should be placed in a separate bindle and retained in the package.

1.8.10.2.2.   Observation should begin with the presence of scales (imbricate in humans and some animals). Human hairs demonstrate a wide variety in color and pigment distribution among individuals. The color in the cortex should be fairly uniform throughout the length and breadth of the hair. An exception would be dyed or bleached hairs, which could demonstrate a sudden or gradual color change at the dye line. (Animal guard hairs demonstrate a characteristic color banding). The medulla (the amorphous central core) can be continuous, discontinuous, fragmentary, or not present in human hair. Medullary index should be one-third or less the total diameter of the hair. There is considerable variation in the general appearance of human hairs depending on the body area. In general, there is less variation in diameter along the length in human hairs than in animal hairs. The appearance of the root is very important both in determining origin and in determining the area to sample for DNA. The appearance of a large skin tag is not necessarily an indication of success in extraction of DNA.

1.8.10.2.3.   Examination documentation shall be of sufficient detail so that an equally qualified analyst is provided enough information in the case notes so as to reach the same conclusion. In the case of hair, when a "human hair" conclusion is reached the notes shall include observations regarding scale type, medulla, coloring/pigment distribution and any other pertinent observations.

1.8.11.   Crosslinker Procedure

1.8.11.1. Consumables (pipette tips, microtubes, columns, etc.) may be exposed in the crosslinker for 2 to 15 minutes before use in casework. Time in excess of 15 minutes shall have the approval of the Technical Leader documented in the case record.

1.8.11.2. The use of the crosslinker may reduce the presence of amplifiable DNA.

1.9. Records:

1.9.1.   If a worksheet is utilized, the examination of sexual assault kits and suspect collection kits shall be recorded on an approved worksheet.

1.9.2.   Results of testing of body fluids shall be recorded on the worksheets designed for that purpose, or alternatively be recorded in the case notes within the description of the item as long as all required controls are documented appropriately.

1.9.3.   All photos and electronically created documentation shall be stored as part of the case record. See Appendix 9 for instructions.

1.9.4. The technical review of the case record shall be recorded on the worksheet provided for that purpose.

1.10. Interpretations of Results:

1.10.1. The quality and quantity of a sample shall determine which tests are performed. Conclusions reached are based on the actual tests performed in each case. See specific tests for interpretation guidelines.

1.10.2. The analyst shall report out any identified body fluid stains present and list the number of samples tested for DNA in the report.

1.11. References:

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1.11.13. Instructions for use: SERATEC® HemDirect (Cat No: HBF07). Rev: 02.2016

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

2. DNA METHODS:

2.1. Scope: This test method is designed for the guidance of Laboratory personnel who assist investigations where the specificity of DNA testing is needed. Evidence amenable to DNA testing includes blood, seminal fluid, saliva, bone, tissue, and hair. This manual may be expanded or altered as new techniques and/or genetic systems are found applicable.

2.1.1. The DNA is first extracted from the biological sample, quantified, amplified to produce many fluorescently tagged copies of specific regions of the DNA and finally processed to separate and detect a DNA profile(s). The analyst compares the DNA profile identified in the sample with the DNA profile from a reference standard from an individual to determine similarities or dissimilarities. In addition, the analyst may provide likelihood ratio statistic to give weight to the strength of an inclusion or exclusion.

2.1.2. Return of Evidence

2.1.2.1. Stain cards from whole blood samples, cuttings from body fluid stains, appropriate DNA extracts, and other selected DNA evidence may rarely be retained by the laboratory for any possible future testing.

2.1.2.2. All extracts are considered work product. Extracts should only be retained if needed for pending additional analysis, such as Y-STR, NGS or outsourced analysis. Extracts shall be discarded when analysis is complete.

2.1.2.3. Subitems or extracts may be retained in long-term storage with supervisor permission if case circumstances indicate it would be beneficial. Special storage conditions may be required for long-term retention of extracts.

2.1.2.3.1. Retained extract tubes shall be labeled with the case number and unique sample designation.

2.1.2.4. Any retained items may be released at the prosecutor's request. The request shall be documented in the case record. A valid court order shall circumvent this procedure.

2.2. Precautions/Limitations:

2.2.1. Evidence

2.2.1.1. Liquid items such as blood standards shall be refrigerated.

2.2.1.2. Stains which are still moist shall be air dried in a secure area before submission to the Laboratory.

2.2.1.3. Clean paper or cardboard containers shall be used to package dried evidence. Plastic containers shall not be used for packaging because they hold moisture which often leads to putrefaction of biological materials. Exceptions to this rule would include blood tubes, body tissue samples and condoms.

2.2.1.4. Specific examinations performed on items for DNA analysis shall be determined by laboratory personnel.

2.3. Related Information:

2.3.1. Worksheet Manual

2.3.2. [Appendix 6](#) PowerPlex® Fusion 6C Stutter Table

2.4. Instruments:

2.4.1. <u>Applied Biosystems® 3500 Genetic Analyzer Capillary Electrophoresis Instrument</u> – Simultaneously separates and detects multiple amplified DNA samples by size by capillary electrophoresis using fluorescent tagged primers.

2.4.2. <u>Applied Biosystems® 7500 Real Time PCR System</u> – Measures accumulation of amplification product over time to determine total DNA present by the use of a fluorescent reporter molecule.

2.4.3. <u>Autoclave</u> - An instrument that uses heat and steam to sterilize solutions, contaminated equipment and waste.

2.4.4. <u>Balance</u> - An analytical balance used for preparation of analytical reagents and buffers.

2.4.5. <u>BIOMEK NX^P Automation Platform</u> – Robotic liquid handling system.

2.4.6. <u>BIOMEK 3000 Automation Platform</u> – Robotic liquid handling system.

2.4.7. <u>Centrifuges</u> - A microcentrifuge capable of operating at greater than 15,000 rcf and a clinical serofuge capable of operating at a maximum of 3,400 rpm; used for the purification of DNA in evidence samples and the separation of solid components from liquids, respectively.

2.4.8. <u>Crosslinker</u> - Preprogrammed ultraviolet exposure is factory set to 120,000 microjoules per cm^2. Preset ultraviolet time exposure is factory set to 2 minutes.

2.4.9. <u>Laminar Flow Hood</u> – An air purifying biohazard cabinet that maintains a nominal inflow velocity of 80 fpm which prevents contaminants from entering or escaping the work area.

2.4.10. <u>Maxwell® 16</u> – Robotic extraction system.

2.4.11. <u>Microscope</u> - A light microscope with magnification up to 400X, a stereoscope for general screening of items, and a phase contrast microscope for the identification of spermatozoa.

2.4.12. <u>Miscellaneous Laboratory Equipment</u> - Supportive laboratory equipment including ovens, incubators, pipettors, rotators, stirring/heating plates, vortex mixers, vacuum pumps, UV lights, and refrigerators/freezers for storing of reagents, buffers and evidence.

2.4.13. <u>pH Meter</u> – An instrument capable of manual or automatic temperature compensation and reading +/- 0.01 pH units. Used for preparation of buffers and reagents.

2.4.14. <u>Thermal cycler</u> - An instrument that can be programmed to rapidly cycle between high and low temperatures. This process is used to make many fluorescently tagged copies of specific regions on a DNA strand(s).

2.4.15. <u>Water Purification System</u> - An apparatus that routes water through a series of filtering devices to produce high quality, uncontaminated water used in buffer preparation and DNA typing methods.

2.5. Reagents/Materials: See Reagent Preparation Manual for instructions and logs where appropriate. Reagents critical to the DNA analysis process are listed in the Critical Reagent Manual.

2.5.1. Bone Demineralization Buffer
2.5.2. Digest/Wash Buffer
2.5.3. DTT 0.39M
2.5.4. DTT 1M
2.5.5. EDTA 0.5M
2.5.6. Nuclease Free Water (NFH$_2$O)
2.5.7. Phenol/Chloroform/Isoamyl Alcohol
2.5.8. Proteinase K (Pro K) 10 mg/ml
2.5.9. Proteinase K (Pro K) 18 mg/ml
2.5.10. Proteinase K (Pro K) 20mg/ml

2.5.11. Sarkosyl 20% w/v

2.5.12. SDS 20% w/v

2.5.13. Stain Extraction Buffer Stock Solution

2.5.14. Stain Extraction Buffer with DTT

2.5.15. Stain Extraction Buffer for Automation

2.5.16. TE $^{-4}$

2.5.17. TRIS/EDTA/NaCl Solution

2.5.18. TRIS-HCl pH 7.5, 1M

2.5.19. TRIS-HCl pH 8.0, 1M

2.6. Hazards/Safety:

2.6.1. All chemicals shall be handled in a safe method as referenced in the specific Safety Data Sheets (SDS).

2.6.2. The addition of phenol/chloroform/isoamyl alcohol during extraction, vortexing of the extracts, and transfer to Microcon® shall be performed in a chemical fume hood.

2.6.3. The manual preparation of samples for electrophoresis by the addition of Hi-Di™ formamide shall be confined to a chemical fume hood. **Caution:** Formamide is an irritant and teratogen; therefore, universal precautions and a fume hood shall be utilized when manually working with formamide to avoid inhalation and contact with the skin.

2.6.4. The manual handling of lysis buffer should be confined to a chemical fume hood.

2.6.5. Universal Precautions shall be in use whenever biological materials are being handled.

2.6.6. Extreme caution shall be used when handling liquid body fluid samples.

2.6.7. Biological waste shall be disposed of in the appropriate waste receptacle.

2.7. Reference Materials/Controls/Calibration Checks:

2.7.1. The accuracy and specificity of test results are ensured by running known DNA controls and reagent controls at the same time as evidence samples. See the specific test for the appropriate controls to be run and the interpretation of the results.

2.8. Procedures/Instructions:

2.8.1. Examination Procedure

2.8.1.1. **Standards for Comparison**

2.8.1.1.1. Appropriate standards, preferably whole blood or buccal swabs, from individuals involved, should be submitted along with the questioned materials.

2.8.1.1.2. Whole blood standards should be submitted in a purple top tube that contains ethylene-diamine-tetra-acetic acid (EDTA). This tube shall be refrigerated.

2.8.1.1.3. Alternately a swabbing taken from the inside cheek of an individual may be collected, dried, and placed in a sealed envelope to be used as a standard.

2.8.1.1.4. When supplied, a secondary standard shall be clearly marked as such and treated as a standard.

2.8.1.1.4.1. Surreptitious standards or items collected from trash pulls shall be clearly marked and may be extracted separately from both unknowns and standards, depending on the sample type.

2.8.1.2. **DNA Analysis**

2.8.1.2.1. If more than one swab from an area/stain is present within an item the analyst must indicate whether a single swab, multiple swabs, or a combined sample of two or more swabs are being analyzed in the case notes. Combined samples in which not all samples within the item were combined shall be tracked in the case notes by a unique identifier.

    2.8.1.2.1.1. If an analyst is sampling an item by swabbing and the swabs are combined into one extract, this can be documented in the notes and report as a single sample.

    2.8.1.2.1.2. If an analyst is sampling an item by swabbing and the swabs are extracted separately, these are considered separate samples. They shall be tracked in the case notes by a unique identifier and the number of samples tested shall be indicated in the report.

2.8.1.2.2. Sexual assault kit processing will depend on case scenario and requires analyst discretion. In order to expedite traditional SAK workflow, an optional tier-based process is allowed for situations with a female victim and a single male assailant.

2.8.1.2.3. Sexual Assault kits (female victim/single male assailant): A minimum of four samples/body areas may be chosen for the first tier of analysis based on the priority list below. If foreign alleles at eight or more loci are not obtained from first tier testing, the remaining samples present in the kit shall be tested in a second tier of testing. Analysts may choose to test all samples in the first tier. Not testing additional samples if no informative results are obtained requires permission of a supervisor with documentation in the case record.

    2.8.1.2.3.1. If the kit contains the samples listed below, these samples shall be selected for testing utilizing differential extraction methods. Regular extractions may be utilized for these samples for cases in which only digital penetration or fondling is indicated. Supervisor notification is required in these instances. If meaningful results are not obtained and quantification results indicate a differential extraction could be beneficial, the analyst should repeat the extraction as a differential.

        2.8.1.2.3.1.1. Either vaginal/cervical swabs or vaginal wash (choice to pick one is only for first tier priority testing. If all samples are required to be tested, both shall be tested, if present.)

        2.8.1.2.3.1.2. Anal/rectal swabs

        2.8.1.2.3.1.3. External genital swabs

    2.8.1.2.3.2. Additional samples shall be chosen based on the following suggested priority list developed based on internal studies of success rates by sample type. Selection of samples is dependent on case information provided and other samples that are present in the kit or already being tested.

        2.8.1.2.3.2.1. Breast swabs

        2.8.1.2.3.2.2. Dried secretions/bitemark swabs (including other body area swabs)

        2.8.1.2.3.2.3. Internal genital swabs

2.8.1.2.3.2.4. Underwear

2.8.1.2.3.2.5. Speculum swabs

2.8.1.2.3.2.6. Bra

2.8.1.2.3.2.7. Oral swabs

2.8.1.2.3.3. Differential extractions should be performed on samples considered intimate or most likely to contain seminal material (e.g., internal genital swabs, oral swabs, or underwear). If a sample is selected that is likely targeting skin cells and not seminal material (e.g., dried secretions/bitemark swabs or fingernail scrapings), the analyst may choose to perform a regular extraction.

2.8.1.2.3.3.1. The analyst may choose to perform a regular extraction for any samples tested as part of a second tier of analysis (as directed above), regardless of sample type.

2.8.1.2.3.3.2. If a sperm fraction is amplified, the corresponding non-sperm fraction may be amplified regardless of the FTMR and amount of male DNA present; however, amplification of the non-sperm fraction is not required when the corresponding sperm fraction has sufficient male DNA.

2.8.1.2.3.4. Swabs (up to 4) from each selected envelope/body area shall be combined for one extraction and evaluated for the presence of male DNA. The total sample must be completely covered by the specified reagent volume without needing to be adjusted/increased. Supervisor notification is required for multiple extractions of swabs from a single envelope/body area.

2.8.1.2.3.5. Representative areas from underwear should be extracted as one extract. Analyst discretion (based upon case scenario and visual examination) may be used to sample multiple areas (and perform multiple extractions) from a single pair of underwear.

2.8.1.2.3.6. Smear slides, fingernail scrapings, debris, hairs, clothing other than undergarments and other items not listed above are never required but may be extracted at analyst's discretion, depending on the contents and results of other items in a kit.

2.8.1.2.3.7. Case results should be carefully evaluated to determine if additional testing or technologies could provide meaningful information. For example, if only non-intimate samples demonstrate foreign alleles, any intimate samples that qualify should be analyzed for Y-STRs.

2.8.1.2.4. Underwear submitted outside of a kit shall be evaluated for stains as appropriate. No serological testing is required if no staining is observed; however, at a minimum, DNA analysis shall be performed on a sample of the crotch area.

2.8.1.2.5. A quantification procedure shall be performed to estimate the quantity of extracted DNA, human and male.

2.8.1.2.6. To be used in a comparison with evidentiary samples, any standard (primary, secondary, or trash pull standard) shall demonstrate a single source profile. Any exception to this rule shall only be made with approval of the Technical Leader and documented in the case record.

2.8.1.2.7. A portion of the amplified DNA is removed and used in the typing method. The remaining amplified DNA, considered work product, shall be destroyed after the case has been administratively reviewed.

2.8.1.2.8. The amount of sample available for testing, the recoverability of DNA from the sample, and the quality of the DNA shall affect which tests are performed. The conclusions reached are based upon the analysis performed.

2.8.1.3. **General Rules**

2.8.1.3.1. If one sample/cutting needs additional processing after quantitation (clean-up or concentration) and another sample/cutting from the same item has an appropriate quantity for analysis without additional processing; it shall be left to the analyst's discretion as to whether or not both samples or just the one sample will be typed.

2.8.1.3.2. Multiple reagent blanks (RB) may be extracted as part of a sample batch. Any samples proceeding for additional testing such as Y-STR analysis shall have more than one reagent blank if there are two or more samples associated with the blank. All reagent blanks shall at a minimum be carried through quantification. Amplification shall be performed on the reagent blank demonstrating the highest small autosomal quantification value. If no small autosomal value is detected, the highest large autosomal or Y value shall be analyzed. One reagent blank may be reserved without additional treatment to be used with those samples in the batch not requiring additional treatment. A batch is defined as all samples before a reagent blank(s) with no intervening reagent blanks.

2.8.1.3.3. Any additional treatment to a sample shall be done to the corresponding reagent blank. This would include but not be limited to additional Microcon® purifications, use of Centri-sep columns, amplifying more than 5 μl, or increased injection parameters. Reagent blanks shall not be diluted unless performed during the Biomek robotic processing of samples.

2.8.1.3.4. The extraction of unknown and reference samples shall be performed separately (separated by time and/or space). This shall include a separate reagent blank for reference and unknown samples and, when practical, use of separate extraction worksheets, separate columns of the same extraction worksheet, or clear indication of which samples are unknown and which are references.

2.8.1.3.4.1. An assumed reference standard (for any type of case) may be included within the same extraction batch as the associated unknowns on the large scale (Beckman) automated platform only as long as:

2.8.1.3.4.1.1. All pre-robot processing steps were performed separately (separated by time and/or space); AND

2.8.1.3.4.1.2. There is no additional evidence within the batch where the reference cannot be assumed. (For example: If sexual assault kit samples are submitted to the robot along with a penile swab from the suspect, the

assumed victim standard cannot be included on that robot batch plate.)

2.8.1.3.5. Extraction and typing of background samples is often not beneficial, and is therefore not required, but may be done at the discretion of the DNA analyst to aid in the interpretation of case sample results. If a background sample is extracted, it shall be typed if DNA was detected during the quantification step. The background sample shall be concentrated only if the sample it is the background for is concentrated.

2.8.1.3.6. If during sampling for extraction, an evidence swab or cutting is dropped on the floor or other unclean area, a background swab of the area the evidence touched shall be collected and processed. The DNA results obtained from the background swab shall be utilized to evaluate the results from the evidence. The Technical Leader shall provide guidance on interpreting the data if DNA results are obtained from the background swab.

2.8.2. Crosslinker Procedure

2.8.2.1. Consumables (pipette tips, microtubes, columns, etc.) may be exposed in the crosslinker for 2 to 15 minutes before use in casework. Time in excess of 15 minutes shall have the approval of the Technical Leader documented in the case record.

2.8.2.2. The use of the crosslinker may reduce the presence of amplifiable DNA.

2.8.3. Sampling Protocols

2.8.3.1. Sample size may vary from indicated values depending on many factors including but not limited to total sample available and serology test results.

2.8.3.2. The analyst should consume only as much of the available sample as is needed. Generally, not more than half of likely body fluid samples (e.g., cigarette butts, blood or semen) will be used for testing.

2.8.3.3. In order to obtain usable results, epithelial cell swabs should be consumed. The laboratory shall receive permission to consume samples from the prosecutor or investigator prior to analysis of epithelial cell swabs or samples. This may be done at the time of submission, or the analyst may request it after sample evaluation. No analysis shall be performed on epithelial cell swabs or samples without permission to consume documented in the case record.

2.8.3.4. **Sampling Suggestions for Regular Extraction**

2.8.3.4.1. **Blood:** Sample approximately 3 X 3 mm from a blood stain or 3 µl of whole blood.

2.8.3.4.2. **Envelope**: Sample approximately 1 X 1 cm of a gummed envelope flap or stamp. Cut sample into smaller pieces before placing in extraction tube. Alternatively, may swab gummed area or wash with three 50 µl rinses and place each rinse into the extraction tube.

2.8.3.4.3. **Cigarette**: Sample ½ to all of paper approximately 5 mm down from end or ½ of the paper and filter approximately 5 mm down. Filter not to be used when extracting with Maxwell® 16. Cut sample into smaller pieces before placing in extraction tube.

2.8.3.4.4. **Epithelial Swabs**: The entire (swab/surface/area) used to collect epithelial cells should be consumed. The total sample must be completely covered by the specified reagent volume without needing to be adjusted/increased.

2.8.3.4.5. **Hair:** Rinse root end of hair in 100% ethanol and then dH_2O before sampling 1 cm of root end. If desired a second 1 cm of hair may be extracted separately as a sample blank.

2.8.3.4.6. **Tissue:** Cut a piece of tissue approximately 3 X 3 mm. If tissue is immersed in liquid nitrogen, it shall also be crushed into a fine powder. Transfer the sample to a microcentrifuge tube. Larger samples than indicated may be used, as needed, with the amounts of stain extraction buffer with DTT and Pro K increased proportionally.

2.8.3.4.7. **Firearms:** Swab the grip, slide/sight, and trigger areas with separate swabs consumed for individual extractions. If magazine is present, an additional single swab should be utilized to swab the loading area and end where the magazine is handled. Additional areas may be sampled depending on the type of firearm.

2.8.3.4.8. **M-Vac filters:** For regular extraction (organic, Maxwell® and Biomek) and differential extraction for Biomek submission: Half of the filter should be used per extraction tube due to liquid volume. If not already cut, cut the filter in half. Cut that half in three strips, stack them (reduces static) and cut a few more times the other direction. Place all of the pieces in a microcentrifuge tube (required for differential extraction) or lyse and spin basket for incubation. Use of tweezers and a static gun is recommended. For differential extraction on Maxwell® or organic: Up to two thirds of the filter may be used due to greater liquid volume.

If it is indicated that a funnel was used with the M-Vac concentration the sample should be only in the center of the filter. Cut out about 1.5 cm diameter circle from the center of the filter using static gun and tweezers. Further cut the center sample into smaller pieces and test as above. This will consume the sample.

2.8.3.4.9. **Combining extracts:**

2.8.3.4.9.1. Two or more swabs/samples may be extracted in separate extraction tubes, when using an organic extraction, combining at step 2.8.8.2. Multiple completed automation extracts may be combined before concentrating at step 2.8.8.2. The number and volume of the associated RBs shall be the same as the samples.

2.8.3.5. **Sampling for Semen (Differential Extraction)**

2.8.3.5.1. **Swabs**: A sample from at least one swab contained in an envelope shall be taken and combined into one tube. Samples from multiple swabs may be placed in one tube. The total sample addition must be covered by the specified reagent volume without needing to be adjusted/increased.

2.8.3.5.2. **Clothing**: Sample approximately a 5 X 5 mm cutting.

2.8.3.5.3. **Smear Slides**: Use a moistened swab and swab entire smear, including underside of coverslip. Sample swab as above.

2.8.3.5.4. **Vaginal Wash**: Spin down vaginal wash and collect pellet on one or more swabs. Sample swab(s) as above.

2.8.3.6. **Bone, Teeth and Fingernails**

2.8.3.6.1. Sample cleaning/preparation methods (e.g., scraping, heating, etc.) that best address the variability of sample quality may be used, as necessary.

2.8.3.6.1.1. Bone marrow and soft tissue may be extracted using the Maxwell® protocol, however bone powder is only suitable for organic, demineralization or QIAquick extractions.

2.8.3.6.2. Remove and separate any soft tissue adhering to sample with a sterilized scalpel or other appropriate tool.

2.8.3.6.3. Immerse and agitate at least 1 cubic inch of bone in cold distilled water.

2.8.3.6.3.1. A small brush or toothbrush may be used to gently remove any dirt/debris from the outer surfaces and exposed inner surfaces of sample.

2.8.3.6.3.2. Small bone samples with difficult-to-reach surfaces may be cleaned by sealing the sample in a 50 ml conical tube containing distilled water, then vortexing briefly, changing out the solution, and repeating, as necessary.

2.8.3.6.3.3. If necessary, the specimen can be further cleaned by immersion in ethanol and ethyl ether for about 15 minutes each.

2.8.3.6.3.4. If surface staining is present, it is recommended to remove the outer table of bone by scraping or sanding.

2.8.3.6.4. Rinse sample in distilled water and allow the sample to dry.

2.8.3.6.5. Pulverize sample into a fine powder by manual mechanical crushing or by use of a freezer/mill. Bone samples may require multiple freezing/crushing steps. Larger samples (e.g., bones and adult teeth) may need to be initially reduced in size to accommodate the method of pulverization. Note: any medical/dental /restorative work on a bone or tooth sample shall be removed prior to pulverization.

2.8.3.6.5.1. **Manual Mechanical Crushing with Tissue Pulverizer**

2.8.3.6.5.1.1. Larger samples may initially need to be reduced to ~0.5 in. fragments or smaller to accommodate the physical size of the pulverizer.

2.8.3.6.5.1.2. Crush bone sample using the pulverizer and mallet. Optionally, sample may be immersed in liquid nitrogen for ~1–10 minutes to aid in crushing.

2.8.3.6.5.2. **SPEX SamplePrep® 6770 Freezer/Mill**

2.8.3.6.5.2.1. Sample must initially be reduced to ~0.25 in. fragments (width of a pencil eraser) or smaller to accommodate the requirements of the mill. Larger fragments will decrease pulverization efficiency and may damage the mill.

2.8.3.6.5.2.2. Insert a sterilized blunt end cap into a sterilized grinding cylinder.

2.8.3.6.5.2.3. Add the sample to the cylinder. Do not add sample in excess of 1/3 total volume of the cylinder.

2.8.3.6.5.2.4. Insert a steel impactor into the cylinder and seal the cylinder with a sterilized flanged end cap.

2.8.3.6.5.2.5. Slowly add liquid nitrogen to the freezer/mill until it reaches the fill mark. An initial amount of liquid nitrogen will quickly boil off as the tank cools. When the liquid settles, add additional liquid nitrogen up to the fill mark. Initial cooling of the instrument will consume

~4–5 L of liquid nitrogen, and each sample pulverization will require an additional ~1 L liquid nitrogen.

2.8.3.6.5.2.5.1. Always handle liquid nitrogen containers or any item exposed to liquid nitrogen (including grinding cylinders) with cryogenic gloves.

2.8.3.6.5.2.6. Load assembled sample vial into the freezer/mill chamber (blunt end first). Optionally, up to two additional samples may also be prepared in vials and placed in the pre-cooling chamber above the freezer/mill chamber.

2.8.3.6.5.2.7. Close the freezer/mill and pulverize the sample using the following protocol: Cycles: 4, Pre-cooling: 10 min, Run: 2 min, Cool: 2 min, CPS (cycles per second): 10. Regularly check the control panel on the freezer/mill to ensure that sufficient liquid nitrogen is remaining in the tank. If "LOW LN LEVEL" is displayed, pause the protocol by pressing "PAUSE", open the freezer/mill, and add liquid nitrogen to the fill mark, then close the freezer/mill and resume the protocol.

2.8.3.6.5.2.8. When the program is complete, open freezer/mill and remove vial from freezer/mill chamber. To conserve liquid nitrogen, any subsequent samples should be immediately loaded into the mill and pulverized as described above. If additional samples were loaded in the pre-cooling chamber during the initial sample run, they may be pulverized with the following protocol (omitting the pre-cooling phase): Cycles: 4, Pre-cooling: 0 min, Run: 2 min, Cool: 2 min, CPS: 10.

2.8.3.6.5.2.9. Allow vial to warm up for ~5–10 minutes before using the cap extractor tool to open the vial. If the sample was "sticky" or "tacky" prior to pulverization, it may be necessary to open the vial earlier to aid removal. Transfer the contents of the vial into a sterilized weigh boat and separate the impactor from the sample. A sterilized spatula may be used to aid sample removal.

2.8.3.6.6. Transfer the pulverized sample into one or more tubes. Manual mechanical crushing typically generates small sample fragments accompanied by some powder, while a freezer/mill typically converts an entire sample to a very fine powder.

2.8.3.6.7. For a regular organic extraction, fill one or more microcentrifuge tubes approximately halfway with powder. The amounts of extraction reagents may be increased proportionately as needed for a regular organic extraction. A sample size of approximately 0.5 grams of powder is recommended for the bone demineralization procedure.

2.8.4. Regular Organic Extraction with Microcon® Concentration Of All Sample Types Including Blood, Hair, Tissue And Bone. (Microcon® 100 or Fast Flow)

2.8.4.1. Place the sample in a 1.5 ml microcentrifuge tube. Qiagen® Investigator® Lyse&Spin tubes shall not be used. Each group of samples being extracted shall include a reagent blank as the last sample in the batch.

2.8.4.2.   To the sample add 300 µl of stain extraction buffer with DTT and 7.5 µl Proteinase K (Pro K) solution (10 mg/ml). Vortex and spin briefly in a microcentrifuge or tap the tube to force the cutting into the extraction fluid. Analyst may increase the amounts of stain extraction buffer with DTT and Pro K proportionally for larger samples. The same treatment shall be performed to any associated reagent blanks.

2.8.4.3.   Incubate the tube at 56°C overnight (18 hours minimum/24 hours maximum). An extraction time of no less than 2 hours may be used for standards (blood or buccal swabs). A shortened extraction time may only be used with the approval of the Technical Leader (documented in case record) for any other sample types.

2.8.4.4.   Spin briefly in a microcentrifuge to force the condensate into the bottom of the tube. For hairs, bones, or tissue it may be preferable to transfer supernatant to a clean microcentrifuge tube before proceeding to 2.8.4.6.Using a wooden applicator stick, remove the cutting and proceed to 2.8.4.6 or

2.8.4.5.   Transfer the cutting into a spin basket insert. Place the basket insert into the tube containing the stain extract. Spin in a microcentrifuge at approximately 14,000 rcf (relative centrifugal force) for about 5 minutes. Remove and discard the basket insert.

2.8.4.6.   In a fume hood, add 300 µl of phenol/chloroform/isoamyl alcohol to which buffer has been added (p/c/i) to the stain extract and vortex the mixture at low speed (in the fume hood) to attain a milky emulsion.

2.8.4.7.   Spin in a microcentrifuge until completely separated (for at least 3 minutes) at approximately 14,000 rcf. Multiple p/c/i extractions may be performed to further clean up an extract if desired. If larger volumes are used in step 2.8.4.2, equivalent volumes of p/c/i shall be used. The same treatment shall be performed to any associated reagent blanks.

2.8.4.8.   See 2.8.8 for Microcon® procedure.

2.8.5.   Demineralization and Organic Extraction of Bone, Teeth and Fingernail Samples with Amicon® Ultra-4 30 kDa Concentration

2.8.5.1.   Place the sample in a 50 ml conical or other appropriately sized container. Each group of samples being extracted shall include a reagent blank as the last sample in the batch.

2.8.5.2.   To the sample add 4.5 ml of bone extraction buffer and 300 µl Proteinase K (Pro K) solution (20 mg/ml). Vortex and spin briefly to force the sample into the extraction fluid. The same treatment shall be performed to any associated reagent blanks.

2.8.5.2.1.   An alternative method including the addition of 2.0 ml Lysis Buffer containing Guanidinium Thiocyanate found in the Maxwell® DNA IQ™ Casework Kit to the demineralization buffer may be considered at the analyst's discretion.

2.8.5.3.   Incubate the samples in an orbital shaker at 56°C and 150 rpm overnight (18 hours minimum/24 hours maximum). with an addition of an extra 300 µl Proteinase K (Pro K) solution (20 mg/ml) during the last 1-8 hours of incubation. A shortened extraction time may only be used with the approval of the Technical Leader (documented in case record).

2.8.5.4.   Spin samples briefly at high speed. It may be preferable to transfer supernatant to a clean tube before proceeding to 2.8.5.5.

2.8.5.5.   In a fume hood, add an equal volume (e.g., 5.1 ml for the standard demineralization protocol or 7.1 ml for the alternative protocol) of phenol/chloroform/isoamyl alcohol to

which buffer has been added (p/c/i) to the extract and vortex the mixture (in the fume hood) to attain a milky emulsion.

2.8.5.6.   Spin in a centrifuge until completely separated (for about 5 minutes at maximum speed).

2.8.5.7.   In a fume hood, transfer approximately one-half to one-third of the aqueous layer to an Amicon® Ultra-4 30 kDa column (do not UV crosslink Amicon® Ultra-4 columns).

2.8.5.8.   Place the Amicon® Ultra-4 columns in the centrifuge with one filter facing the outer wall. Centrifuge samples at max speed (up to 7500 x g (rcf) on a fixed angle rotor). Continue spinning until approximately 250 µl of sample remains.

    2.8.5.8.1. Note: Do not allow columns to become completely dry. Spin times may be adjusted per sample to address variability.

2.8.5.9.   In a fume hood, discard the flow through and add the remaining aqueous layer to the column.

2.8.5.10.   Repeat 2.8.5.8 until approximately 250 µl of sample remains. Discard the flow through.

2.8.5.11.   Wash sample with 2 ml of nuclease free water. Spin (maintaining column orientation) at max speed until approximately 100 µl of sample remains.

2.8.5.12.   Transfer the sample to a clean microcentrifuge tube.

2.8.5.13.   Wash the column with an additional 100 µl of nuclease free water by pipetting up and down. Combine with extract.

2.8.5.14.   Store refrigerated or proceed to QIAquick® purification.

2.8.6. QIAquick® Purification for Bone, Teeth and Fingernail Samples

2.8.6.1.   QIAquick® purification may be performed at any time. The same treatment shall be performed on the associated reagent blank (or a portion of) for each sample batch.

2.8.6.2.   Optionally, UV crosslink columns for 15 minutes.

2.8.6.3.   Add 5 volumes of Buffer PB to 1 volume of sample (example for 200 µl of sample add 1000 µl Buffer PB). Place up to 750 µl into the spin column.

2.8.6.4.   Spin 30 seconds at 17,900 x g (rcf); 13,000 rpm. If liquid remains in column, spin another 30 seconds.

2.8.6.5.   Discard the flow through.

2.8.6.6.   Repeat until all sample has been applied to the column.

2.8.6.7.   Wash the sample by adding 750 µl of Buffer PE.

2.8.6.8.   Spin 30 seconds at 17,900 x g (rcf); 13,000 rpm. If liquid remains in column, spin another 30 seconds.

2.8.6.9.   Discard the flow through.

2.8.6.10.   Spin 1 minute at 17,900 x g (rcf); 13,000 rpm.

2.8.6.11.   Place the column into a clean microcentrifuge tube (not provided in kit). To elute DNA, add 30-100 µl Buffer EB to the center of the membrane and let stand at least 1 minute.

2.8.6.12.   Spin 1 minute at 17,900 x g (rcf); 13,000 rpm. Discard the column.

2.8.6.13.   Extracts can be stored refrigerated or frozen prior to quantification/amplification. Prior to use after storage, the samples shall be vortexed and spun briefly in a microcentrifuge.

2.8.7. Differential Organic Extraction with Microcon® Concentration Of Semen Stains (Microcon® 100 or Fast Flow)

2.8.7.1.   Place the sample in a 1.5 ml microcentrifuge tube. Qiagen® Investigator® Lyse&Spin baskets shall not be used for differential extractions. Each group of samples being extracted shall include reagent blanks (sperm and non-sperm fraction) as the last samples in the batch.

2.8.7.2.   To the sample add:
   400 µl Tris/EDTA/NaCl
   25 µl 20% Sarkosyl
   75 µl NFH_2O
   5 µl Pro K (10 mg/ml)

2.8.7.3.   Vortex and spin briefly in a microcentrifuge or tap the tube to force the cutting into the extraction fluid.

2.8.7.4.   Incubate at 37°C for 2 hours.

2.8.7.5.   Spin briefly in a microcentrifuge to force the condensate into the bottom of the tube.

2.8.7.6.   Using a wooden applicator stick, remove the cutting and spin in a microcentrifuge for about 5 minutes at approximately 14,000 rcf.

2.8.7.7.   Alternately transfer the cutting into a spin basket insert. Place the basket insert into the tube containing the stain extract. Spin in a microcentrifuge at approximately 14,000 rcf for about 5 minutes. Remove and discard the basket insert.

2.8.7.8.   While being very careful to not disturb any pelleted material, remove the supernatant fluid from the extract and place it into a new, labeled tube. This supernatant is the non-sperm fraction. Analysis of the non-sperm fraction resumes at 2.8.7.15. The pellet remaining in the tube is the sperm fraction.

2.8.7.9.   Wash the sperm pellet by resuspending it in 500 µl digest/wash buffer, vortexing the suspension briefly, and spinning the tube in a microcentrifuge at approximately 14,000 rcf for about 5 minutes. Remove and discard the supernatant fluid, being careful not to disturb the pellet.

2.8.7.10.   Repeat 2.8.7.9 two additional times for a total of three washes of the sperm pellet.

2.8.7.11.   To the tube containing the washed pellet add:
   150 µl Tris/EDTA/NaCl
   50 µl 20% Sarkosyl
   150 µl NFH_2O
   10 µl Pro K (10 mg/ml)
   40 µl 0.39M DTT

2.8.7.12.   Close the tube cap, vortex and spin briefly in a microcentrifuge to force all fluid and material to the bottom of the tube.

2.8.7.13.   Incubate at 37°C for 2 hours.

2.8.7.14.   Spin briefly in a microcentrifuge to force the condensate into the bottom of the tube.

2.8.7.15.   In the fume hood, add 400 µl phenol/chloroform/isoamyl alcohol to which buffer has been added (p/c/i) to both the tube containing the sperm pellet and to the tube containing the non-sperm fraction. Vortex the mixture briefly at low speed (in the fume hood) to attain a milky emulsion.

2.8.7.16. Spin in a microcentrifuge until completely separated (for at least 3 minutes) at approximately 14,000 rcf. Multiple p/c/i extractions may be performed to further clean up an extract if desired. The same treatment shall be performed to any associated reagent blanks.

2.8.7.17. See 2.8.8 for Microcon® procedure.

2.8.8. Microcon® Concentration of Extracted DNA (Microcon® 100 or Fast Flow)

2.8.8.1. To a Microcon® Concentrator add 100 µl TE^{-4}.

2.8.8.2. For Microcon® concentration immediately after organic extraction, transfer the aqueous phase (top layer) to the concentrator in the fume hood. Avoid pipetting organic solvent from the tube into the concentrator. For Microcon® concentration of other DNA extracts, transfer the extract(s) to the concentrator.

2.8.8.3. Close the cap on the concentrator and spin in a microcentrifuge at 500 rcf for at least 10 minutes. Microcon® 100 may be spun up to 1,200 rcf.

2.8.8.4. Optional Step: Carefully remove the concentrator unit from the assembly and discard the fluid from the filtrate cup. Return the concentrator to the top of the filtrate cup.

2.8.8.5. Remove the cap and add 200 µl TE^{-4} to the concentrator. Replace the cap and spin the assembly in a microcentrifuge at 500 rcf for at least 10 minutes. Microcon® 100 may be spun up to 1,200 rcf.

2.8.8.6. Remove the cap and add a measured volume of TE^{-4} (40 µl is the recommended amount however, varied amounts can be added in some circumstances) to the concentrator. Remove the concentrator from the filtrate cup and carefully invert the concentrator into a new, labeled retentate cup. Discard the filtrate cup.

2.8.8.7. Spin the concentrator in a microcentrifuge at 1,000 rcf for at least 3 minutes. The Microcon® 100 should be spun 500-1,200 rcf for at least 5 minutes.

2.8.8.8. Discard the concentrator. If desired, the sample can be brought up with additional water by adding it to the retentate cup. Cap the retentate cup.

2.8.8.9. Extracts can be stored refrigerated or frozen prior to quantification/amplification. Prior to use after storage the samples shall be vortexed and spun briefly in a microcentrifuge.

2.8.8.10. Multiple Microcon® concentration steps may be performed to further clean up or concentrate any extract. The same treatment shall be performed on the associated reagent blank (or a portion of) for each sample batch.

2.8.9. Centri-sep Clean-Up of Extracts

2.8.9.1. Clean-up of extracts with Centri-sep columns may be performed at any time. The same treatment shall be performed on the associated reagent blank (or a portion of) for each sample batch.

2.8.9.2. Remove the top column cap and reconstitute the column by adding 800 µl of nuclease free water (NFH$_2$O). Leave the column end stopper in place so the column can stand up by itself. Replace the column cap and hydrate the gel by shaking and inverting the column or vortexing briefly.

2.8.9.3. Allow the column to hydrate for at least 30 minutes at room temperature. Reconstituted columns may be refrigerated overnight. Allow to warm up to room temperature before proceeding.

2.8.9.4. Remove the air bubbles from the column gel by inverting the column and sharply tapping the column, allowing the gel to slurry to the opposite end of the column. Stand the column up and allow the gel to settle while in a microtube rack.

2.8.9.5. First remove the column cap, and then remove the column end stopper from the bottom.

2.8.9.6. Allow the excess column fluid to drain into a 2 ml wash tube. If the fluid does not begin to flow immediately through the column apply gentle pressure to the top of the column to force the fluid to start through the column filter. This can be accomplished by snapping the cap on briefly. Discard this fluid.

2.8.9.7. Spin the column and wash tube in a microcentrifuge at 750 rcf for 2 minutes. **NOTE:** It is important to keep track of the position on the column using the orientation mark molded into the column.

2.8.9.8. Optionally: discard eluate and rinse column with 150-200 µl NFH$_2$O and spin for 2 minutes at 750 rcf.

2.8.9.9. Blot any drop at the end of the column. Discard the wash tube. Do not allow the gel material to dry excessively.

2.8.9.10. Transfer the sample extract to the top of the column, without disturbing the gel surface. Keep track of amount of sample added to column (less than 50 µl is recommended).

2.8.9.11. Place the column into the sample collection tube (1.5 ml) and place both into the microcentrifuge. Maintain proper column orientation. The highest point of the gel media in the column should always point toward the outside of the rotor. Spin the column and the collection tube at 750 rcf for 2 minutes.

2.8.9.12. Discard the spin column and cap the sample collection tube.

2.8.9.13. Extracts can be stored refrigerated or frozen prior to quantification/amplification. Prior to use after storage, the samples shall be vortexed and spun briefly in a microcentrifuge.

2.8.10. Maxwell® Protocol

2.8.10.1. **Maxwell® Preparation of Samples for Any Cellular Material (except bone powder):**

2.8.10.1.1. For each sample, combine 350 µl Incubation Buffer with 10 µl Pro K (18 mg/ml) and 40 µl DTT (1M). Alternatively, Stain Extraction Buffer for Automation (SEBA) may be substituted for Incubation Buffer. *Example: for 16 samples, combine 5,600 µl Incubation Buffer (or SEBA) with 160 µl Pro K and 640 µl DTT.*

2.8.10.1.2. Place sample at the bottom of a 1.5 ml microcentrifuge tube or a Qiagen® Investigator® Lyse&Spin basket with 2 ml collection tube. Each group of samples being extracted shall include a reagent blank as the last sample in the batch. A batch can be no larger than the capacity of the Maxwell Unit being utilized. For a Maxwell® 16 or FSC it is 15 samples (plus 1 reagent blank). For a Maxwell® 48 it is 47 samples (plus 1 reagent blank). The reagent blank associated with each batch shall be run in the same Maxwell® run as the associated samples.

2.8.10.1.3. To the sample add 400 µl Master Mix. Vortex and spin briefly in a microcentrifuge or tap the tube to force the cutting into the extraction fluid.

2.8.10.1.4. Incubate the samples at 56°C for at least one hour. Alternatively, samples may be incubated overnight.

2.8.10.1.5. **For samples lysed in a 1.5 ml microcentrifuge tube:**

2.8.10.1.5.1. Spin briefly in a microcentrifuge to force the condensate into the bottom of the tube. (For hairs it may be preferable to transfer supernatant to a clean microcentrifuge tube before proceeding).

2.8.10.1.5.2. Add 200 µl of Lysis Buffer to each sample.

2.8.10.1.5.3. Vortex samples briefly and spin down.

2.8.10.1.5.4. Using a wooden applicator stick, remove the cutting and proceed to 2.8.10.1.7 or

2.8.10.1.5.5. Transfer the cutting into a spin basket insert. Place the basket insert into the tube containing the stain extract. *Optionally, the Lysis Buffer may also be transferred to the spin basket.* Spin in a microcentrifuge at approximately 14,000 rcf for about 5 minutes. Remove and discard the basket insert.

2.8.10.1.6. **For samples lysed in a Qiagen® Investigator® Lyse&Spin basket:**

2.8.10.1.6.1. Spin in a microcentrifuge at 10,000 – 14,000 rcf for at least 1 minute. Remove and discard the basket insert.

2.8.10.1.6.2. Add 200 µl of Lysis Buffer to each sample.

2.8.10.1.7. Save the extract until ready for automated DNA extraction. Do not refrigerate or freeze sample. Leave processed sample at room temperature (~22-25°C) overnight, if necessary.

2.8.10.1.8. Proceed to 2.8.10.3, 2.8.10.4 or 2.8.10.5 for Maxwell® automated extraction.

2.8.10.2. **Maxwell® Preparation of Samples for Differential Extractions:**

2.8.10.2.1. For each sample, combine 400 µl Tris/EDTA/NaCl, 10 µl 20% Sarkosyl, 90 µl NFH$_2$O and 5 µl Proteinase K (10 mg/ml).

2.8.10.2.2. Place the sample in a 1.5 ml microcentrifuge tube. Qiagen® Investigator® Lyse&Spin baskets shall not be used for differential extractions. Each group of samples being extracted shall include reagent blanks (sperm and non-sperm fraction) as the last samples in the batch. A batch can be no larger than the capacity of the Maxwell Unit being utilized. For a Maxwell® 16 or FSC it is 15 samples (plus 1 reagent blank). For a Maxwell® 48 it is 47 samples (plus 1 reagent blank). The reagent blank associated with each batch shall be run in the same Maxwell® run as the associated samples.

2.8.10.2.3. To the sample add 505 µl of the Master Mix. Vortex and spin briefly in a microcentrifuge or tap the tube to force the cutting into the extraction fluid.

2.8.10.2.4. Incubate the sample at 37°C for 2 hours.

2.8.10.2.5. Spin briefly in a microcentrifuge to force the condensate into the bottom of the tube.

2.8.10.2.6. Using a wooden applicator stick, remove the cutting and spin in a microcentrifuge for about 5 minutes at approximately 14,000 rcf.

2.8.10.2.7. Alternately transfer the cutting into a spin basket insert. Place the basket insert into the tube containing the stain extract. Spin in a microcentrifuge at approximately 14,000 rcf for about 5 minutes. Remove and discard the basket insert.

2.8.10.2.8. While being very careful to not disturb any pelleted material, remove the supernatant fluid from the extract and place it into a new, labeled tube. This supernatant is the non-sperm fraction. Analysis of the non-sperm fraction resumes at 2.8.10.2.11. The pellet remaining in the tube is the sperm fraction.

2.8.10.2.9. Wash the sperm pellet by resuspending it in 500 µl digest/wash buffer, vortexing the suspension briefly, and spinning the tube in a microcentrifuge at approximately 14,000 rcf for about 5 minutes. Remove and discard the supernatant fluid, being careful not to disturb the pellet.

2.8.10.2.10. Repeat step 2.8.10.2.9 two times for a total of three washes of the sperm pellet.

2.8.10.2.11. Lysis buffer step

    2.8.10.2.11.1. For each sample pair (sperm and non-sperm fraction) combine 600 µl of Lysis Buffer with 6 µl of 1M DTT.

    2.8.10.2.11.2. Add 400 µl of the Lysis Buffer master mix to each sperm fraction sample and 200 µl to each non-sperm fraction sample.

2.8.10.2.12. Vortex sample and spin down briefly.

2.8.10.2.13. Save until ready for automated DNA extraction. Do not refrigerate or freeze sample. Leave processed sample at room temperature (~22-25°C) overnight, if necessary.

2.8.10.2.14. Proceed to 2.8.10.3, 2.8.10.4 or 2.8.10.5 Maxwell® automated extraction.

2.8.10.3. **Maxwell® 16 Instrument Automated DNA Purification:**

2.8.10.3.1. Place the number of cartridges to be used into the cartridge preparation rack. Each cartridge corresponds to one sample. Place each cartridge into the rack. Hold the cartridge firmly and remove the seal.

2.8.10.3.2. Place one plunger into well #8 of each cartridge.

2.8.10.3.3. Transfer the entire sample into well #1.

2.8.10.3.4. Place 0.5 ml elution tubes (properly labeled) into the elution tube slot. Add 50 µl of Elution Buffer.

2.8.10.3.5. Turn on the Maxwell® 16 Instrument. Verify "LEV" and "FNSC" modes are displayed.

2.8.10.3.6. Scroll to "Run" on the Menu screen and press the "Run/Stop" button.

2.8.10.3.7. Open the door when prompted to do so on the LCD display. Press the "Run/Stop" button to extend the platform.

2.8.10.3.8. Place the cartridge rack into the Maxwell® 16 Instrument.

2.8.10.3.9. Press the "Run/Stop" button. The platform will retract. Close the door.

2.8.10.3.10. Upon method completion, open the instrument door. The plungers should be located in Well #8 at the end of the run. Check to make sure that all of the plungers have been removed from the magnetic rod assembly. If the plungers have not been removed, push them down gently by hand and remove them from the magnetic rod assembly.

2.8.10.3.11. Press the "Run/Stop" button to extend the platform.

2.8.10.3.12. Remove the elution tubes from the heated elution tube slots, as soon as possible and close the top on each tube. (Leaving the heated elution tubes in the instrument for an extended period of time can result in evaporation of the sample.) Samples can be stored at 4°C or frozen. Prior to use after storage, the samples shall be vortexed briefly and spun in a centrifuge for about 5 seconds.

2.8.10.3.13. Remove cartridges and plungers from the instrument platform and discard.

2.8.10.3.14. When completed, clean the Maxwell® 16 Instrument and cartridge rack with 70% ethanol and turn off machine.

2.8.10.4. **Maxwell ® FSC Instrument Automated DNA Purification:**

2.8.10.4.1. Place the number of cartridges to be used into the cartridge preparation rack - one per sample. Ensure front and back of each cartridge is pushed down and snapped in. Remove the seals.

2.8.10.4.2. Place one plunger into well #8 of each cartridge (closest well to elution tube).

2.8.10.4.3. Transfer entire sample into well #1 of respective cartridge (furthest well from elution tube).

2.8.10.4.4. Place an appropriately labeled 0.5ml elution tube into the elution tube slot associated with each sample, respectively. Ensure the elution tube is pressed all the way down into the holding well. Add 50µl of Elution Buffer to each elution tube.

2.8.10.4.5. DO NOT WEAR GLOVES WHEN TOUCHING THE TABLET! Use the power button on the far left top edge of the tablet to wake up the tablet.

2.8.10.4.6. Swipe up on the tablet screen to select "Analyst" account on the tablet. Touch "Sign In" (no password necessary).

2.8.10.4.7. Double tap the Maxwell® FSC icon on the tablet to open the instrument software. The instrument will perform a self-test and ask to start a sanitization cycle. This will take approximately 1 minute. Touch "Sanitize" to run the sanitization cycle. Each time the software is opened, the sanitization cycle needs to be run. If you do not run it upon opening of software it will force you to run before your samples are processed. Optionally this sanitization cycle may be performed between sample runs. After removing cartridge rack, from the home screen touch "Sanitize" then "Start".

2.8.10.4.8. To begin a sample run select "Start" from the Maxwell® software home screen.

2.8.10.4.9. Using the barcode scanner attached to the instrument, scan the QR code at the <u>top</u> of the kit label of the Maxwell® FSC DNA IQ™ Casework Kit. Alternatively, you may touch the appropriate kit listed on the screen. This will highlight the appropriate kit on the screen. Touch "Proceed" on the right side of the highlighted line.

2.8.10.4.10. Touch "Proceed" at the bottom center of the screen. If the software window needs to be minimized to allow the proceed button to be visible, double tap the top center of the software window and move, as necessary.

2.8.10.4.11. "The door will now open" box will appear. Touch "OK".

2.8.10.4.12. Load cartridge rack with cartridges, plungers, samples and **open** elution tubes. Touch "Start".

2.8.10.4.13. If a run needs to be aborted, touch "Abort" then "Open Door". A report will populate the screen. Touch the home icon in the upper left corner. To remove plungers, run a clean-up as described in 2.8.10.4.16.

2.8.10.4.13.1. If a run is aborted by the analyst or by the instrument, Technical Leader notification is required.

2.8.10.4.14. When the run is complete touch "Open Door". A report will populate the screen. Wait for the report to populate, then touch the home icon in the upper left corner.

2.8.10.4.15. If all plungers have been properly ejected from the bar into the cartridges, proceed to 2.8.10.4.17. If plungers have not properly ejected from the bar, you MUST run a clean-up. Do not remove cartridge rack from instrument.

2.8.10.4.16. To run a clean-up, touch "Settings" from the home screen. Then select "Clean Up" and touch "Start". A report will populate the screen when finished. Touch the home icon in the upper left corner. You will then need to open the instrument door with the door icon (rectangle) in the upper right corner next to the question mark.

2.8.10.4.17. Remove cartridge rack from instrument.

2.8.10.4.18. Touch the door icon in the upper right to close the instrument door.

2.8.10.4.19. Close all elution tubes and remove from cartridge rack.

2.8.10.4.20. Remove all plungers from cartridges and dispose.

2.8.10.4.21. Remove all cartridges from cartridge rack and dispose.

2.8.10.4.22. Clean Maxwell® instrument and cartridge rack with 70% ethanol – never bleach! Tablet screens may be wiped with microfiber screen cleaning cloths.

2.8.10.4.23. Close out of the Maxwell® FSC software using the X icon in the upper left corner then touch "Yes".

2.8.10.4.24. Using the Windows icon in the lower left corner of the tablet, sign out of the tablet by touching the person icon in the circle then "Sign out".

2.8.10.4.25. Tablet will go to sleep after 1 hour. DO NOT turn off the instrument.

2.8.10.5. **Maxwell ® RSC 48 Instrument Automated DNA Purification:**

2.8.10.5.1. Place the number of cartridges to be used into the cartridge preparation rack - one per sample. Ensure front and back of each cartridge is pushed down and snapped in. Remove the seal.

2.8.10.5.2. Place one plunger into well #8 of each cartridge (closest well to elution tube).

2.8.10.5.3. Transfer entire sample into well #1 of respective cartridge (furthest well from elution tube).

2.8.10.5.4. Place an appropriately labeled 0.5ml elution tube into the elution tube slot associated with each sample, respectively. Ensure the elution tube is pressed all the way down into the holding well. Add 50μl of Elution Buffer to each elution tube.

2.8.10.5.5. DO NOT WEAR GLOVES WHEN TOUCHING THE TABLET! Use the power button on the far left top edge of the tablet to wake up the tablet.

2.8.10.5.6. Swipe up on the tablet screen to select "Analyst" account on the tablet. Touch "Sign In" (no password necessary).

2.8.10.5.7. Double tap the Maxwell® RSC 48 icon on the tablet to open the instrument software. The instrument will perform a self-test and ask to start a sanitization cycle. This will take approximately 1 minute. Touch "Sanitize" to run the sanitization cycle. Each time the software is opened, the sanitization cycle needs to be run. If you do not run it upon opening of software it will force you to run before your samples are processed. Optionally this sanitization cycle may be performed between sample runs. After removing cartridge racks, from the home screen touch "Sanitize" then "Start".

2.8.10.5.8. To begin a sample run, select "Start" from the Maxwell® software home screen.

2.8.10.5.9. Using the barcode scanner attached to the instrument, scan the QR code at the top of the kit label of the Maxwell® FSC DNA IQ™ Casework Kit. Alternatively, you may touch the appropriate kit listed on the screen. This will highlight the appropriate kit on the screen. Touch "Proceed" on the right side of the highlighted line.

2.8.10.5.10. "The door will now open" box will appear. Touch "OK".

2.8.10.5.11. Load cartridge rack with cartridges, plungers, samples and elution tubes. Rack labeled "Front" must go in the front and rack labeled "Back" must go in the back of the instrument. Touch "Start". The instrument will automatically detect placement of the sample cartridges, if plungers are present, if elution tubes are present and if elution tubes are opened. If anything is wrong an error will pop up and ask you to take a closer look at your sample preparation before proceeding.

2.8.10.5.12. If a run needs to be aborted, touch "Abort" then "Open Door". A report will populate the screen. Touch the home icon in the upper left corner. To remove plungers, run a clean-up as described in 2.8.11.5.15.

    2.8.10.5.12.1. If a run is aborted by the analyst or by the instrument, Technical Leader notification is required.

2.8.10.5.13. When the run is complete touch "Open Door". A report will populate the screen. Wait for the report to populate, then touch the home icon in the upper left corner.

2.8.10.5.14. If all plungers have been properly ejected from the bar into the cartridges, proceed to 2.8.10.5.16. If plungers have not properly ejected from the bar, you MUST run a clean-up. Do not remove cartridge rack from instrument.

2.8.10.5.15. To run a clean-up, touch "Settings" from the home screen. Then select "Clean Up" and touch "Start". A report will populate the screen when finished. Touch the home icon in the upper left corner. You will then need to open the instrument door with the door icon (rectangle) in the upper right corner next to the question mark.

2.8.10.5.16. Remove cartridge rack from instrument.

2.8.10.5.17. Touch the door icon in the upper right to close the instrument door.

2.8.10.5.18. Close all elution tubes and remove from cartridge rack.

2.8.10.5.19. Remove all plungers from cartridges and dispose.

2.8.10.5.20. Remove all cartridges from cartridge rack and dispose.

2.8.10.5.21. Clean Maxwell® instrument and cartridge rack with 70% ethanol – never bleach! Tablet screens may be wiped with a microfiber screen cleaning cloth.

2.8.10.5.22. Close out of the Maxwell® RSC 48 software using the X icon in the upper left corner then touch "Yes".

2.8.10.5.23. Using the Windows icon in the lower left corner of the tablet, sign out of the tablet by touching the person icon in the circle then "Sign out".

2.8.10.5.24. Tablet will go to sleep after 1 hour. DO NOT turn off the instrument.

2.8.11. Rehydration of a Sample after Storage

2.8.11.1. Evaluate the stored sample and associated reagent blank for approximate volume.

2.8.11.2. If enough liquid is present in the sample and the reagent blank, they can be used for amplification.

2.8.12.2.1 The sample and reagent blank shall be quantified and treated as normal.

2.8.11.3. If the volume is low or none, determine if the sample and/or the reagent blank extract was concentrated and consumed or almost consumed from the case record.

2.8.11.3.1. If the reagent blank was concentrated and consumed after the issue of the 2009 DNA QAS audit document no further testing should be done.

2.8.11.3.2. If the case was completed before the issue of the 2009 DNA QAS audit document and the reagent blank was consumed consult with the Technical Leader about reagent blanks.

2.8.12.4 If the volume is low and the loss of volume is from dehydration, the sample can be rehydrated.

2.8.12.4.1 To the sample add 400 µl of TE^{-4}.

2.8.12.4.2 Add TE^{-4} to the reagent blank as needed. The volume added should not exceed the least amount added to any associated sample.

2.8.12.4.3 The final volume of the reagent blank should not exceed the volume of any associated sample.

2.8.12.4.4 If the reagent blank did not dehydrate, an additional reagent blank with the same volume of TE^{-4} as added to the sample shall be created and tested with the sample.

2.8.12.4.5 Briefly vortex the samples.

2.8.12.4.6 Incubate at least 2 hours to 24 hours at 4°C, vortexing periodically.

2.8.12.4.7 Concentrate the sample and associated reagent blank(s) to approximately 50 µl each with a Microcon®.

2.8.12.4.8 Quantify the sample and reagent blank(s) after rehydration.

2.8.13 Quantifiler® Trio DNA Quantification

2.8.13.1 Introduction

2.8.13.1.1 The Applied Biosystems® Quantifiler® Trio DNA Quantification Kit utilizes multi-copy target loci to simultaneously quantify an 80 bp small autosomal target (sm. auto), a 214 bp large autosomal target (lg. auto), and a 75 bp male Y-chromosome target (Y) to enable estimation of the amount of human DNA, male DNA, and level of degradation. The Internal PCR Control (IPC) enables inhibitor detection in the sample extract prior to STR amplification.

2.8.13.1.2 Each assay target consists of PCR primers and TaqMan® probes labeled with both a reporter dye and non-fluorescent quencher. During PCR, the TaqMan® probes anneal to a specific location between the targeted forward and reverse primers. Taq DNA polymerase cleaves the hybridized probes separating the reporter dye from the quencher, resulting in increased fluorescence.

2.8.13.1.3 The 7500 Real-Time PCR System measures the fluorescent signal of all four target reporter signals (VIC®, ABY®, FAM™, and JUN®) as well as a passive reference dye (Mustang Purple®) to help normalize the signal between samples. The signals for each sample are compared to that of a standard curve of known quantity to estimate DNA concentrations.

2.8.13.1.4 The Quantifiler® Trio kit shall be stored at -15 to -25°C upon receipt. All kit components shall be maintained together in the kit sleeve.

2.8.13.1.5 Allow the entire kit to thaw prior to first use after which all components shall be stored at 2 to 8°C.

2.8.13.1.6 The Quantifiler® Trio Primer Mix and the Quantifiler® THP PCR Reaction Mix shall be protected from excessive exposure to light.

2.8.13.1.7 The preparation of the quantification plate shall be performed in the PCR amplification set-up area.

2.8.13.2 Standard Curve Preparation (may be set up in the PCR Amplification set-up area**)**

2.8.13.2.1 Label four microcentrifuge tubes "Std 1" through "Std 4".

2.8.13.2.2 Dispense the appropriate volume of Quantifiler® THP Dilution Buffer to each tube using the appropriate option from the table below.

2.8.13.2.3 Briefly vortex the Quantifiler® THP DNA Standard [100 ng/µl stock].

2.8.13.2.4 Add the calculated amount of Quantifiler® THP DNA Standard to the tube labeled "Std 1" and mix thoroughly.

2.8.13.2.5 Using a new pipette tip, add 4 µl of "Std 1" to "Std 2" and mix thoroughly.

2.8.13.2.6 Complete the dilution series by adding 4 µl of "Std 2" to "Std 3" and "Std 3 to "Std 4", mix thoroughly and use a new pipette tip with each step.

2.8.13.2.7 The standard curve is stable for up to 2 weeks when stored at 2 to 8°C.

Standard	Concentration (ng/µl)	Dilution Buffer	DNA Standard	Dilution Factor
Std 1	50	15 µl dilution buffer (Option 1) 25 µl dilution buffer (Option 2) 35 µl dilution buffer (Option 3)	15 µl DNA stock (Option 1) 25 µl DNA stock (Option 2) 35 µl DNA stock (Option 3)	2X
Std 2	2.0	96 µl dilution buffer	4 µl Std 1	25X
Std 3	0.08	96 µl dilution buffer	4 µl Std 2	25X
Std 4	0.0032	96 µl dilution buffer	4 µl Std 3	25X
Note:	Option 1 – sufficient volume for 6 quant plates; Option 2 – sufficient volume for 11 quant plates; Option 3 – Sufficient volume for 16 quant plates			

2.8.13.3 Quantification Reaction Preparation

2.8.13.3.1   Each quantification plate shall contain a standard curve series run in duplicate in column 12 and a Non-Template Control (NTC) in well H11.

2.8.13.3.2   Determine the number of samples to be quantified, including standards and NTC, and add ~3 reactions to compensate for the loss that occurs during pipetting.

2.8.13.3.3   Using the Quantifiler® Trio Plate Record Worksheet, calculate the required amount of each component of the master mix.

PCR Master Mix Component	Volume per Reaction (µl)
Quantifiler® Trio Primer Mix	8
Quantifiler® THP PCR Reaction Mix	10
Total Volume	18

2.8.13.3.4   Vortex the Primer Mix 3 to 5 seconds, then centrifuge briefly.

2.8.13.3.5   Gently vortex the PCR Reaction Mix.

2.8.13.3.6   Pipette the required volume of each component into a microcentrifuge tube. Total volumes exceeding 1300 µl may be split into 2 tubes equally.

2.8.13.3.7   Vortex the Master Mix 3 to 5 seconds, then centrifuge briefly.

2.8.13.3.8   Dispense 18 µl of the Master Mix into each reaction well of a 96-well optical plate.

2.8.13.3.9   Add 2 µl of sample, standard, or Non-Template Control (water or dilution buffer) to the appropriate wells for a total reaction volume of 20 µl.

2.8.13.3.10   Seal the reaction plate with an Optical Adhesive Cover using the cover applicator.

2.8.13.3.11   Centrifuge at 3000 rpm for approximately 10 seconds or spin using a salad spinner to remove any bubbles and force samples into the bottom of each well.

2.8.13.3.12   Load the plate on the 7500 Real-Time PCR instrument ensuring proper orientation.

2.8.13.4   Quantification Experiment Setup

2.8.13.4.1   Turn on the computer.

2.8.13.4.2   Turn on the 7500 Real-Time PCR instrument.

2.8.13.4.3   Open the HID Real-Time PCR Analysis Software v1.2.

2.8.13.4.4   Select the **Quantifiler® Trio** icon on the Home screen.

2.8.13.4.5   Enter the experiment name: First case on plate_date_plate# (i.e., 00A1234_01Jan16_01).

2.8.13.4.6   Ensure the following experiment properties are selected:

Instrument: 7500 (96 Wells)
Experiment Type: Quantitation – HID Standard Curve
Reagents: TaqMan® Reagents
Ramp speed: Standard (~1 hour to complete a run)

2.8.13.4.7   Select **Plate Setup** from the left navigational panel.

2.8.13.4.8   Sample names may be entered manually or imported from a DNA Workbook into the HID Software. All samples shall be uniquely identified by case number and sample identifier.

   2.8.13.4.8.1   Sample Name manual entry:

   2.8.13.4.8.1.1   Select **Add New Sample** to enter each sample name.

   2.8.13.4.8.1.2   Sample Type shall be designated as "Unknown" for casework samples.

   2.8.13.4.8.1.3   Select the **Assign Targets and Samples** tab to enter the sample information into each well. Unused wells shall be left blank.

   2.8.13.4.8.1.4   Verify that the standard curve and NTC information is correct.

   2.8.13.4.8.2   Sample Name import:

   2.8.13.4.8.2.1   In the DNA Workbook, navigate to the "Quant" tab, ensure that the sample information is filled out, and click **Create Quant Import File**. Copy the .txt file to the "IMPORT" folder on the 7500 Real-Time computer desktop.

   2.8.13.4.8.2.2   In the HID Software, select **File → Import**, navigate to and select the .txt file that was created by the workbook.

   2.8.13.4.8.2.3   The associated samples and standards will populate within their respective wells. Verify that the samples, standard curve, and NTC information is correct.

2.8.13.4.9   Assay targets, standard curve quantities, assay parameters, and export criteria are automatically assigned when using the Quantifiler® Trio template.

2.8.13.4.10   Save the plate document to the "CASEWORK" folder on the D: drive.

2.8.13.4.11   Click   START RUN ▷

2.8.13.4.12   The Quantifiler® Trio assay will take ~1 hour.

2.8.13.5   **HID Real-Time PCR Analysis Software set-up See Appendix 2**

2.8.13.6   **Experiment Analysis**

2.8.13.6.1   Analyze the experiment by clicking   Analyze

2.8.13.6.2   Evaluate the slope, Y-intercept, and R^2 values of all three standard curves as described under **2.8.13.7 Interpretation Guidelines.**

   2.8.13.6.2.1   Up to 2 points from each curve may be deleted by right-clicking on the well, selecting **omit,** and selecting the appropriate target(s) or well. The results must be reanalyzed to incorporate the changes.

   2.8.13.6.2.2   Omission of more than 2 points in any standard curve requires documented approval by a supervisor and notification to the Technical Leader.
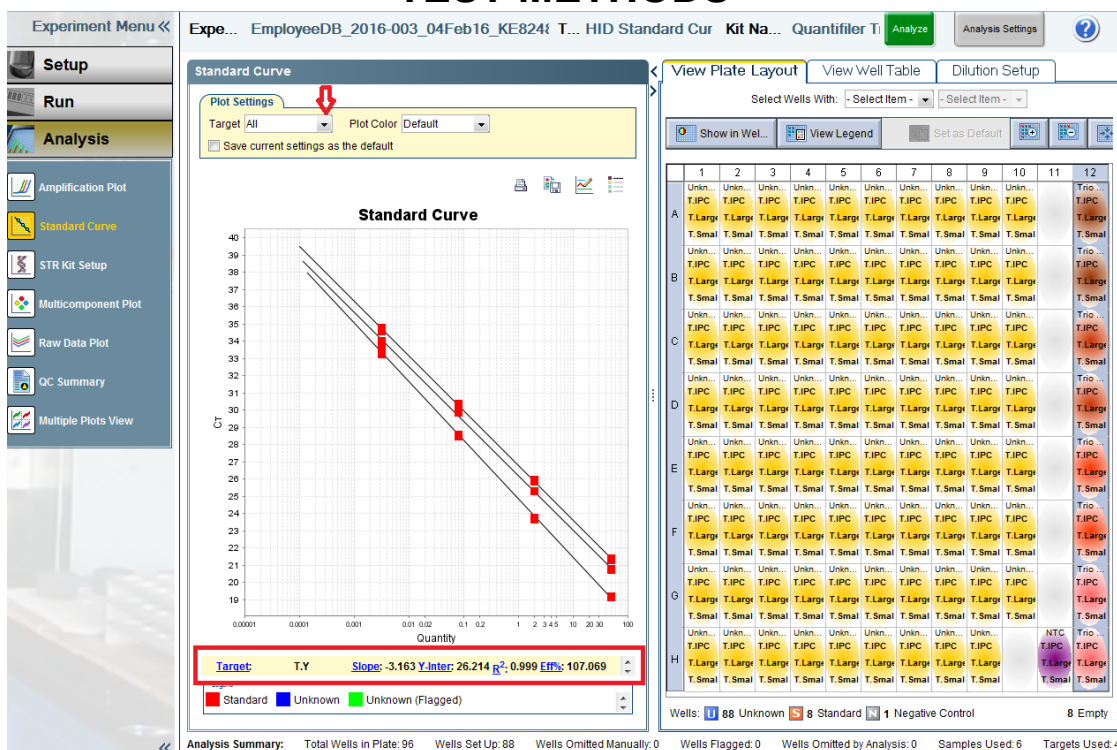
2.8.13.6.3 Export the .txt data file to the "CASEWORK" folder on the D: drive using the previously established export properties.

2.8.13.6.4 Import the .txt data file to the DNA Workbook **Quant** tab.

2.8.13.6.5 View and retain the **Quant Results** and **Quant Std Curve** tabs. The case notes shall contain the quantification values, IPC C_T values, degradation indices, and a graph containing all three standard curves with slope, R^2, and y-intercept values for each curve.

2.8.13.6.6 The .eds run file and .txt data file shall be saved to each associated laboratory case number and request folder located in the analyst's folders on the server.

2.8.13.6.7 Quantification values from the small autosomal target shall be used for amplification of autosomal STRs.

2.8.13.6.8 Quantification values from the male target shall be used for amplification of Y-STRs.

2.8.13.6.9 The quantification plate shall be discarded after the analysis is complete.

2.8.13.7 Interpretation Guidelines

2.8.13.7.1 **Standard Curve**: Evaluate the Small Autosomal, Large Autosomal, and Y standard curves individually or simultaneously.

2.8.13.7.1.1 **Slope:** Indicates the PCR amplification efficiency for the assay. A slope of -3.3 indicates 100% amplification efficiency. The analyst shall obtain documented Technical Leader approval to use data when a slope is outside the acceptable range. Scientific rounding rules to one decimal place apply.

Target	Acceptable slope range	Average slope
Large Autosomal	-3.1 to -3.7	-3.4
Small Autosomal	-3.0 to -3.6	-3.3
Y	-3.0 to -3.6	-3.3

2.8.13.7.1.2 **Y-intercept:** The expected C_T value for a 1 ng/µl sample. Y-intercept averages may vary by instrument, kit lot, and pipetting differences. The analyst shall obtain documented Technical Leader approval to use data when a Y-intercept value is outside the acceptable range. Scientific rounding rules to one decimal place apply.

Target	Acceptable Y-intercept range (C_T)
Large Autosomal	23.0 to 27.0
Small Autosomal	25.0 to 29.0
Y	25.0 to 29.0

2.8.13.7.1.3 **R^2 value:** A measure of the closeness of fit between the standard curve regression line and the individual C_T data points of the standard curve. A value of 1.00 indicates a perfect fit between the regression line and the data points. This value shall be ≥ 0.980. If the R^2 value is <0.98 the experiment is inconclusive and shall be repeated with notification to the Technical Leader documented in the case record. Scientific rounding rules to two decimal places apply.

2.8.13.7.2 Internal PCR Control (IPC): Differentiates between a sample containing no human DNA and reactions affected by the presence of PCR inhibitors, assay setup, and chemistry or instrument failure.

2.8.13.7.2.1 An elevated IPC C_T value (relative to quantitation standards) is an indication of the presence of an inhibitor or high concentration of human template DNA.

2.8.13.7.2.2 If inhibition is suspected, the analyst may re-Microcon® and/or Centri-sep the sample prior to amplification and typing of the sample.

2.8.13.7.2.3 If high concentration of human DNA is present, the analyst may make dilutions of the sample and re-quantify.

2.8.13.7.2.4 The analyst shall make reasonable efforts to rectify samples in which the IPC is undetected. If a DNA profile is not developed from a sample with an undetected IPC, it shall be reported as inconclusive.

Autosomal or Y targets	IPC	Interpretation	Suggestions / Options

Undetected	Detected	Negative result; No human DNA	No further analysis of sample
Undetected	Undetected	Invalid result; possible inhibition	Re-Microcon® and/or Centri-sep; Re-extract; or dilute and re-quantify
Detected (Low C_T)	Undetected or Elevated	Inconclusive IPC result	Dilute and re-quantify (optional)
Detected (High C_T)	Undetected or Elevated	Partial PCR Inhibition	Re-Microcon® and/or Centri-sep; Re-extract; or dilute and re-quantify

2.8.13.7.3 **Non-Template Control (NTC):** Contains PCR reagents with water or dilution buffer in place of template DNA and is therefore expected to be undetected (i.e., value of 0.00) for all three quantification values. However, background fluorescence or contamination may occur resulting in quantification values with one or more targets.

2.8.13.7.3.1 A NTC demonstrating quantification value(s) ≥0.001 ng/µl requires documented notification to the Technical Leader and shall be considered a failed control. The quantification shall be repeated.

2.8.13.7.3.2 A NTC demonstrating quantification value(s) <0.001 ng/µl may be due to background fluorescence. The analyst may proceed with amplification and typing of the samples using caution with interpretation of any profiles obtained from the samples.

2.8.13.7.4 **Dynamic range:** The quantitation standard curve ranges from 50 to 0.0032 ng/µl. Quantitation values are most reliable when they fall near the middle of the standard curve and less reliable as they approach the ends and beyond.

2.8.13.7.4.1 If the quantity of small autosomal DNA in a sample is <0.01 ng/µl, no additional autosomal STR analysis of that sample shall be performed.

2.8.13.7.4.2 If the quantity of male DNA in a sample is <0.006 ng/µl, no additional Y-STR analysis of that sample shall be performed.

2.8.13.7.4.3 The analyst should make a reasonable effort to target <50 ng/µl during extraction. If a sample quantifies >50 ng/µl and is suitable for amplification, a dilution shall be made and quantified.

2.8.13.7.5 **Female To Male Ratio (FTMR):** In instances where it is valuable to identify a male contributor from a female contributor (i.e., sexual assault cases), the FTMR can assist in prioritizing samples and determining which are suitable for autosomal STR analysis and which are suitable for Y-STR analysis. The female DNA contribution is determined by subtracting the quantity of male DNA from the quantity of small autosomal DNA (equation: [sm. auto-Y]/Y). The selection of samples to continue DNA analysis may be prioritized at analyst discretion by using the FTMR and quantity of male DNA input as described below. Priority 1 samples and Priority 2 and 3 samples with male DNA concentrations greater than 0.006 ng/µl should be amplified if no other samples have provided informative results.

2.8.13.7.5.1 Priority 1: Samples with FTMR ≤5:1 are generally expected to yield interpretable information from male contributor with autosomal STR analysis.

2.8.13.7.5.2   Priority 2: Samples with FTMR between 5:1 and 20:1 may yield interpretable information from the minor male contributor with autosomal STR analysis if the concentration of male DNA is greater than 0.006 ng/μl. If the male DNA concentration is less than 0.006 ng/μl, no further analysis should be performed.

2.8.13.7.5.3   Priority 3: Samples with FTMR between 20:1 and 50:1 are generally expected to yield minimal information from the minor male contributor with autosomal STR analysis if the concentration of male DNA is greater than 0.006 ng/μl. If the male DNA concentration is less than 0.006 ng/μl, no further analysis shall be performed.

2.8.13.7.5.4   Priority 4: Samples with FTMR >50:1 are generally not expected to yield interpretable information from the minor male contributor with autosomal STR analysis, therefore no further autosomal analysis shall be performed Y-STR analysis may be recommended if the concentration of male DNA is greater than 0.006 ng/μl.

2.8.13.7.6   Degradation Index: A value to assist in the identification of degradation prior to amplification. The degradation index is calculated by dividing the small autosomal by the large autosomal quant values. An index >1 may indicate that the analyst should increase the template DNA for autosomal STR amplification to generate interpretable data from the larger molecular weight loci.

2.8.13.7.7   Reagent blank: Should not have associated DNA and is therefore expected to be undetected (i.e., value of 0.00) for all three quantification values. However, background fluorescence or contamination may occur resulting in quantification values with one or more targets.

2.8.13.7.7.1   A reagent blank that demonstrates quantification value(s) ≥0.001 ng/μl requires documented notification to the Technical Leader. It shall be considered a failed control unless re-quantification and/or amplification demonstrate no contamination. It is recommended that the analyst evaluate the results for possible sample switching and requantify if that is suspected.

2.8.13.7.7.1.1   If the reagent blank is amplified and no peaks above analytical threshold are observed in the electropherogram, the associated samples may be interpreted with caution.

2.8.13.7.7.2   A reagent blank that demonstrates quantification value(s) <0.001 ng/μl may be due to background fluorescence or low level contamination.

2.8.13.7.7.2.1   If all associated samples fail to demonstrate a sufficient quantity of DNA for amplification (i.e., <0.01 ng/μl for autosomal STRs; <0.006 ng/μl for Y-STRs), they shall be reported as such.

2.8.13.7.7.2.2   If the reagent blank is amplified and no peaks above analytical threshold are observed in the electropherogram, the associated samples may be interpreted normally.

2.8.13.7.7.3   A reagent blank that demonstrates peaks in the electropherogram shall be considered a failed control.

2.8.13.7.7.4   Any sample associated with a failed reagent blank, but not amplified due to an insufficient quantity of DNA (i.e., <0.01 ng/μl for autosomal STRs; <0.006

ng/µl for Y-STRs) shall be reported as failed to demonstrate a sufficient quantity of (male) DNA for further/autosomal STR analysis.

2.8.13.7.7.5   Samples associated with a failed reagent blank shall not be submitted for Y-STR analysis.

2.8.14 PowerPlex® Fusion 6C System (Applied Biosystems® 3500 Series Genetic Analyzer)

2.8.14.1   Introduction

2.8.14.1.1   The Promega PowerPlex® Fusion 6C System is a 27-locus multiplex that amplifies 23 autosomal short tandem repeat (STR) loci (CSF1PO, FGA, Penta D, Penta E, SE33, TH01, TPOX, vWA, D1S1656, D2S1338, D2S441, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, and D22S1045), two gender determining loci (Amelogenin and DYS391), and two additional rapidly mutating Y-STR loci (DYS570 and DYS576) in a single reaction. Separation and detection occurs using capillary electrophoresis with overlapping loci visualized simultaneously by using PCR primers labeled with six different fluorescent tags (FL-6C, JOE-6C, TMR-6C, CXR-6C, TOM-6C, and WEN-6C).

2.8.14.1.2   The Applied Biosystems® 3500 Series Genetic Analyzer utilizes electrokinetic injection of DNA molecules into polymer-filled capillaries which separates the DNA fragments by size. The fluorescent tag labeled primers incorporated into amplification products are responsive to the frequency of the 20mW solid state laser. Upon excitation, the fluorophores are raised to a higher energy level and when they return to their normal energy level a fluorescent signal is emitted. This signal is detected by a CCD (charge-coupled device) camera within the 3500 Genetic Analyzer which converts the signal to a computer image where it is visualized as a peak on the electropherogram.

2.8.14.1.3   The data produced by the 3500 Genetic Analyzer is analyzed with GeneMapper™ ID-X Software which results in peaks labeled with their allele designation. The allele designation for each sample is accomplished through the use of an internal lane standard (ILS). The ILS contains 21 fragments of known length and is injected with each sample. The sample DNA fragments are compared to the ILS to determine the base pair size and the software assigns the allele designation by comparing the sizes to an allelic ladder.

2.8.14.1.4   The PowerPlex® Fusion 6C System locus-specific and allelic ladder information is available at *www.promega.com/protocols/* in the PowerPlex® Fusion 6C System Technical Manual TMD045.

2.8.14.1.5   All PowerPlex® Fusion 6C components should be stored at -10°C to -30°C upon receipt in the appropriate laboratory locations. The PowerPlex® Fusion 6C Allelic Ladder is packaged separately for shipping and should be stored in the post-amplification kit. Once thawed all components are stable at 2-10°C for 6 months. Do not refreeze.

2.8.14.1.6   The PowerPlex® Fusion 6C 5X Primer Pair Mix, PowerPlex® Fusion 6C Allelic Ladder Mix, and WEN ILS 500 shall be protected from excessive exposure to light.

2.8.14.1.7   The preparation of the amplification reactions shall be performed in the PCR amplification set-up area.

2.8.14.2   PowerPlex® Fusion 6C Amplification Set-Up

2.8.14.2.1   For initial use, thaw the Amplification Grade Water, PowerPlex® Fusion 6C 5X Master Mix, and PowerPlex® Fusion 6C 5X Primer Pair Mix. Centrifuge briefly to bring contents to the bottom, then vortex to mix. Do not centrifuge after vortexing as this may cause the primers/dNTPs to be concentrated at the bottom of the tube. The Master Mix and Primer Pair Mix shall be stored at 2-10°C after first use.

2.8.14.2.2   Determine the number of samples to be amplified including controls. Add ~3 reactions to this number to compensate for the loss that occurs during reagent transfer.

2.8.14.2.3   Place one 0.2 ml PCR reaction tube for each sample into a rack and label appropriately. A 96-well plate may be used.

2.8.14.2.3.1   If utilizing a 96-well plate for amplification, well locations where allelic ladder will be utilized for capillary electrophoresis shall be left empty during amplification.

2.8.14.2.4   The recommended amplification target range is 1.0 to 2.0 ng of template DNA. Amplification of greater than 2.0 ng of template DNA may result in increased artifacts and interfere with interpretation. Only samples with concentration less than 0.066 ng/µl may target less than 1.0 ng template DNA and shall be interpreted with caution.

2.8.14.2.5   For samples that will amplify 5 µl of template DNA, use the current version of the **DNA Analysis Workbook** to calculate the required amount of each component of the PCR master mix. Multiply the volume per reaction by the total number of reactions to obtain the final volume.

PCR Master Mix Component	Volume per Reaction (µl)
Amplification Grade or Nuclease Free H_2O	10
PowerPlex® Fusion 6C 5X Master Mix	5
PowerPlex® Fusion 6C 5X Primer Pair Mix	5
Total Volume	20

2.8.14.2.6   Combine the calculated volume of each component into a 1.5 ml tube and vortex ~5 seconds.

2.8.14.2.7   Pipette 20 µl of the PCR master mix to each sample tube or well.

2.8.14.2.8   Add 5 µl of sample, reagent blank, positive amplification control, or negative amplification control to the appropriate tube or well.

2.8.14.2.8.1   For the positive amplification control, dilute the 2800M DNA standard supplied with the PowerPlex® Fusion 6C kit, or other approved DNA standard, to 0.2-0.4 ng/µl and record the final concentration on the amplification worksheet. A positive control shall be included in each thermal cycler. The diluted positive control should be stored at 2-10°C.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

2.8.14.2.8.2   Amplification Grade H_2O or NF H_2O shall be used as a negative amplification control included with each thermal cycler and will be the last sample in the batch.

2.8.14.2.9   For samples requiring more than 5 µl of template DNA, use the current version of the **DNA Analysis Workbook** worksheet to calculate the required amount of water and template DNA for individual samples. Prepare the PCR Master Mix without water. Multiply the volume per reaction by the total number of reactions to obtain the final volume.

PCR Master Mix Component	Volume per Reaction (µl)
PowerPlex® Fusion 6C 5X Master Mix	5
PowerPlex® Fusion 6C 5X Primer Pair Mix	5
Total Volume	10

2.8.14.2.10   Combine the calculated volume of each component into a 1.5 ml tube and vortex ~5 seconds.

2.8.14.2.11   Pipette 10 µl of the PCR master mix to each sample tube or well.

2.8.14.2.12   Add the calculated volume of Amplification Grade H_2O or NF H_2O (0-10 µl) to each sample tube or well.

2.8.14.2.13   Add the calculated volume of sample, reagent blank, positive amplification control, or negative amplification control (5-15 µl) to each sample tube or well.

2.8.14.2.14   For samples amplified in a 96-well plate, seal the plate with adhesive foil. Press the foil well using a roller tool.

2.8.14.3   **PowerPlex® Fusion 6C Amplification**

2.8.14.3.1   Place the tubes or 96-well plate in a Veriti™ Thermal Cycler.

2.8.14.3.2   Select the **Fusion 6C** protocol with the following amplification procedure. Approximately 1 hour cycling time.

1 Cycle	29 Cycles	1 Cycle	Hold
96°C	96°C		
1 minute	5 seconds		
		60°C	60°C
		1 minute	10 minutes
			4°C
	(All ramp rates set to 100%)		∞
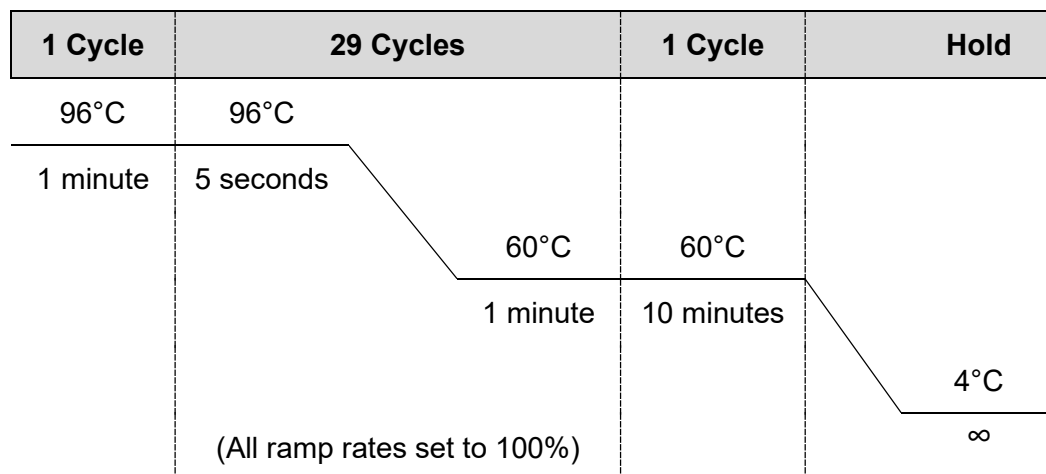
2.8.14.3.3 Remove samples after the amplification process is completed and proceed with capillary electrophoresis or store the amplified samples at -10°C to -30°C. Alternatively, amplified samples may be stored at 2-10°C if they are to be used within 2 days.

2.8.14.3.3.1 Any remaining amplification products shall be discarded after the case has been administratively reviewed.

2.8.14.4 **Instrument setup for Applied Biosystems® 3500 Genetic Analyzer - Data Collection Software version 2.0** see Appendix 3.

2.8.15 **Applied Biosystems® 3500 Series Genetic Analyzer - Data Collection Software version 2.0 - PowerPlex® Fusion 6C Electrophoresis**

2.8.15.1 **Sample Preparation**

2.8.15.1.1 **Note:** The quality of formamide is critical for the successful detection of a DNA profile. Deionized formamide shall be used that has a conductivity of less than 100µS/cm, such as Hi-Di™ Formamide. The formamide shall be frozen in aliquots at -20°C and the remainder of each aliquot shall be discarded after it is thawed. Multiple freeze-thaw cycles or long-term storage at 4°C may cause breakdown of the formamide which can create ions that compete with DNA during injection. This will cause lower peak heights and decreased sensitivity.

2.8.15.1.2 **Caution:** Formamide is an irritant and teratogen; therefore, universal precautions and a fume hood shall be utilized when manually working with formamide to avoid inhalation and contact with the skin.

2.8.15.1.3 For initial use, thaw the WEN ILS 500, PowerPlex® Fusion 6C allelic ladder, and an aliquot of Hi-Di™ Formamide. When thawed, centrifuge briefly to bring contents to the bottom, then vortex to mix. The ILS and allelic ladder shall be stored at 2-10°C after first use.

2.8.15.1.4 Determine the number of samples to be injected, including allelic ladders and controls (reagent blanks, positive control, and amplification blank). Add ~3 reactions to compensate for the loss that occurs during pipetting.

2.8.15.1.5 Prepare a loading cocktail by combining 0.5-1.0 µl ILS with 9.5-9.0 µl formamide for a total volume of 10 µl per sample.

2.8.15.1.5.1 The volume of ILS used in the loading cocktail may be adjusted between 0.5 to 1.0 µl per sample to optimize size standard peaks. The amount of formamide shall be adjusted so that the total amount of loading cocktail for each well is 10 µl.

2.8.15.1.6 Vortex to mix.

2.8.15.1.7 Pipette 10 µl of the formamide/ILS mixture into each well containing sample, allelic ladder, or control. Add formamide or formamide/ILS mixture into empty wells to complete an injection set. Liquid must be present in every well in which an injection will occur.

2.8.15.1.8 Add 1 µl of amplified sample or allelic ladder to the appropriate wells. It is recommended that a minimum of one allelic ladder is present with every 24 samples injected (i.e., every three injections on the 8-capillary 3500 and within every injection on the 24-capillary 3500xL).

2.8.15.1.8.1   If amplified sample is in a 96-well plate, seal the amplification plate with a new aluminum foil seal for storage.

2.8.15.1.9   Cover the wells with the plate septa and briefly centrifuge to remove air bubbles.

2.8.15.1.10   Denature the samples at 95ºC for 3 minutes, then immediately chill on crushed ice or frozen plate block for ~3 minutes. Denature the samples just prior to loading the instrument. Avoid denaturing the samples for longer than 3 minutes as extended heat denaturing can lead to the appearance of artifacts.

2.8.15.2   Create a Plate Record

2.8.15.2.1   Open the 3500 Data Collection Software. The Dashboard screen will launch. Ensure that the **Consumable Information** and **Maintenance Notifications** are acceptable. The oven temperature should be set to 60ºC.

2.8.15.2.2   Select **Start Pre-Heat**. This should be done at least 30 minutes prior to the first injection.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

Dashboard:

2.8.15.2.3   Select **Create New Plate** or **Create Plate from Template**.

    2.8.15.2.3.1   Assign the plate name with the laboratory case number of the first case on the plate, the date, and the injection number of the plate (ex. 00A1234_01Jan16_01).

    2.8.15.2.3.1.1   If the plate is re-injected the "01" number shall increase sequentially (00A1234_01Jan16_02).

    2.8.15.2.3.1.2   If the plate is re-injected on a different day, the original date should still be used from when the plate was prepared.

    2.8.15.2.3.1.3   If a second preparation of a plate is made on the same date as the first, it shall be designated with a "-2" after the date (00A1234_01Jan16-2_01).

2.8.15.2.4   Select "96" for Number of Wells.

2.8.15.2.5   Select "HID" for Plate Type.

2.8.15.2.6   Select "36"cm for Capillary Length.

2.8.15.2.7   Select "POP4" for Polymer.

Create A Plate:
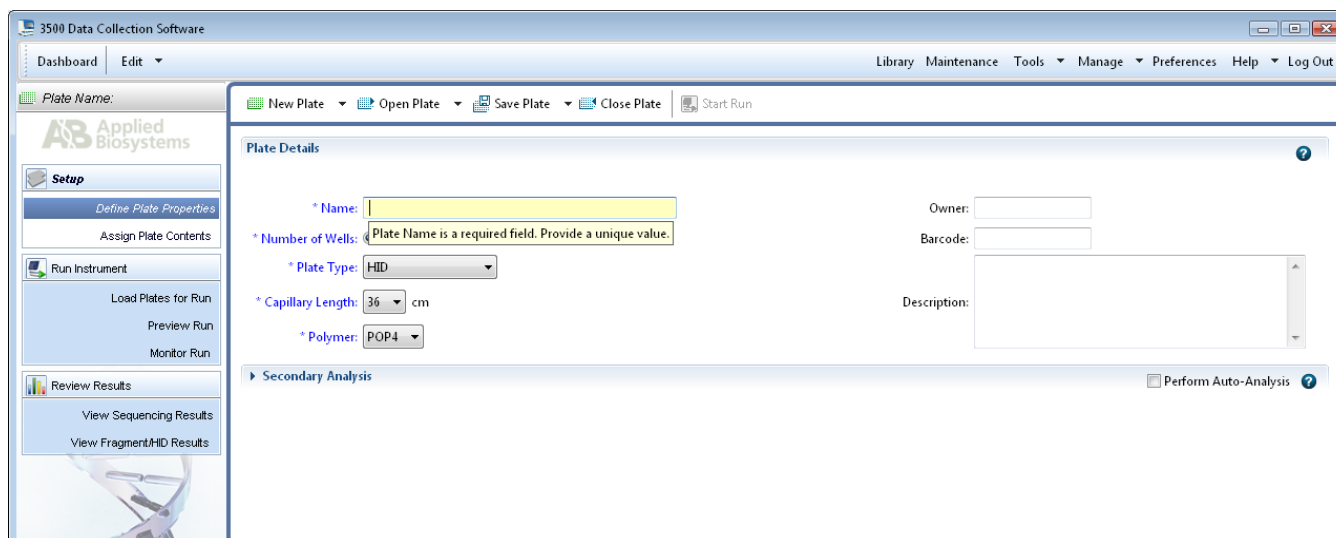
2.8.15.2.8   Click "Assign Plate Contents".

2.8.15.2.9   Enter sample information or import a plate record.

2.8.15.2.9.1   The sample name shall include the sample item/sub-item as well as the laboratory case number (ex. 1A1_00A1234) if more than one case is included on the plate. If only one case is present on the plate, then only the sub-item number is required.

2.8.15.2.9.2   In order to aid in GeneMapper™ ID-X sample analysis, it is recommended to place a "z" in front of the sample name for known standards (ex. z1A1_00A1234") here or in GeneMapper™ ID-X.

2.8.15.2.10   Under **Assays**, click "Add from Library" and select the appropriate Fusion6C assay. Double-click or click "Add to Plate" then "Close".

2.8.15.2.11   Under **File Name Convention**, click "Add from Library" and select "Fusion6C_FNC". Double-click or click "Add to Plate" then "Close".

2.8.15.2.12   Under **Results Groups**, click "Add from Library" and select "Fusion6C_RG". Double-click or click "Add to Plate" then "Close".

Assign Plate Contents:

2.8.15.2.13   On the bottom-right side of the screen expand the **Customize Sample Info** window so it appears as shown above.

    2.8.15.2.13.1   Use the drop-down box to select a sample type for each sample (ex. Ladder, Sample, Positive Control and Negative Control). For imported plates, this may be done automatically.

2.8.15.2.14   Highlight the sample wells and then select the appropriate boxes under **Assays**, **File Name Conventions**, and **Results Groups**.

2.8.15.2.15   Place the plate on the instrument in position A. Select "Link Plate for Run". Click "OK". The instrument automatically senses the plate and assigns the information to the Plate A field. Click "OK".

2.8.15.2.16   To add a second plate, follow steps 2.8.14.2.3 through 2.8.14.2.14. Place the plate in position B. Select the "Link Plate for Run" and click "OK". Assign the information to the Plate B field. Click "OK".

2.8.15.3   **Start a Plate Run**

2.8.15.3.1   A unique run name is automatically generated by the instrument for each plate.

2.8.15.3.2   To re-organize injections, click "Create Injection List" and use the arrows to arrange in the desired order.

2.8.15.3.3   Click "Start Run".

2.8.15.3.4   After the run is complete, click "Unlink Plate" and remove it from the instrument. The plate may be discarded or labeled with the same title as the plate record and

stored at -10°C to -30°C. The plate shall be destroyed after the case has been administratively reviewed.

2.8.15.3.4.1   If the CE plate requires re-injection after frozen storage, thaw, and centrifuge to remove any air bubbles. Denature the plate at 95ºC for 3 minutes, then immediately chill on crushed ice or frozen plate block for ~3 minutes prior to loading on the 3500 Genetic Analyzer.

2.8.15.3.5   The CE Plate Record Worksheet shall be maintained with each case record.

2.8.16 Setting Up GeneMapper^{TM} ID-X Version 1.6 Software User Accounts and Security System See [Appendix 4.](#)

2.8.17 GeneMapper^{TM} ID-X Version 1.6 Software - PowerPlex® Fusion 6C Data Analysis

2.8.17.1   Processing Sample Data

2.8.17.1.1   Import the sample files to be interpreted from a single run folder by selecting "Add Samples to Project".

2.8.17.1.2   Navigate to the run folder that contains the sample files. If the entire run folder is to be imported, click on the folder to highlight it, then click the "Add to List" button at the bottom of the window. If only a portion of samples need to be selected, expand the folder to view the samples. Highlight the appropriate samples, ensuring that the allelic ladder and all desired samples are selected. Once all the samples are selected click the "Add to List" button at the bottom of the window.

2.8.17.1.2.1   A run folder shall not be created manually by manipulating sample files.

2.8.17.1.3   Ensure that the necessary files are now located in the "Samples to Add" window by double-clicking on the folder in the right pane, then click "Add".

2.8.17.1.4   After the samples have been added to the project, first briefly scan the raw data to ensure that a bad injection did not occur.

2.8.17.1.4.1   To check the raw data, first expand the project folder in the left navigation pane, click on a sample file, then click on the "Raw Data" tab in the right GeneMapper^{TM} ID-X window. To return to the "Samples" window, click on the project folder at the top of the left navigation pane.

2.8.17.1.5   The GeneMapper^{TM} ID-X project shall contain at least one allelic ladder from each run folder included in the project for proper genotyping. Multiple allelic ladders within a run folder will be averaged by the software to calculate the allelic bins. If a ladder injection is of low quality, delete the ladder or change the sample type from "Allelic Ladder" to "Sample" to remove it from consideration in calculating the bins.

2.8.17.1.6   Set the **Table Setting** at the top of the screen to "STRmix_Fusion6C".

2.8.17.1.7   In the **Sample Type** column, verify that the correct sample types are displayed (i.e., Allelic Ladder, Sample, Positive Control, and Negative Control). Should a sample type need altered, use the drop-down menu to select the appropriate type.

2.8.17.1.8   In the **Analysis Method** column,

2.8.17.1.8.1   For unknown profiles: select "STRmix_Fusion6C_v3" from the drop-down menu in row 1. Click the column header cell to highlight the entire column and press Ctrl+D or select Edit → Fill Down.

2.8.17.1.8.2   For reference profiles: select "Fusion 6C_v3" from the drop-down menu in row 1. Click the column header cell to highlight the entire column and press Ctrl+D or select Edit → Fill Down.

     2.8.17.1.8.2.1   If a reference profile is degraded and/or exhibits allelic dropout, either analysis method may be used. Stutter will need to be manually deleted. Notification of the Technical Leader documented in the case record is required if the STRmix analysis method is utilized.

2.8.17.1.8.3   Positive controls can be analyzed with either analysis method; however, stutter will need to be manually deleted from positive control profiles.

2.8.17.1.8.4   Negative controls shall be analyzed with the lowest or lower analytical threshold of any associated samples.

2.8.17.1.8.5   A project may contain samples with different analysis methods. The project shall contain a separate ladder for each analysis method used.

2.8.17.1.8.6   A project does not need to be created for standards if no items of evidence in that case are amplified or demonstrate conclusive results.

2.8.17.1.9   In the **Panel** column,

2.8.17.1.9.1   For unknown profiles: select "STRmix_v3_Fusion_6C_ISP" from the drop-down menu in row 1. Click the column header cell to highlight the entire column and press Ctrl+D or select Edit → Fill Down.

2.8.17.1.9.2   For reference profiles: select "Fusion6C_v3_ISP" from the drop-down menu in row 1. Click the column header cell to highlight the entire column and press Ctrl+D or select Edit → Fill Down.

2.8.17.1.9.3   Ladders, positive and negative controls shall utilize the panel that corresponds to the analysis method used.

2.8.17.1.10   In the **Size Standard** column, select "WEN ILS 500" from the drop-down menu in row 1. Click the column header cell to highlight the entire column and press Ctrl+D or select Edit → Fill Down.

2.8.17.1.11   The Analysis Method, Size Standard, and Panel can be set as defaults when a GeneMapper™ ID-X project is opened. Select **File → Project Options**. Under the **Add Samples** tab select appropriate settings as the default in the drop-down menus for Analysis Method, Size Standard, and Panel. Click "OK".

2.8.17.1.12   Select the green **Analyze** arrow button to start data analysis. A pop-up window will ask for a project name to save. The project name shall contain the date the 3500 plate was prepared so that it matches the run folder name. See the DNA Server Instructions Appendix 9 for additional clarification. The case number is recommended but not required. Select the "ISP Casework Security Group". Click "OK".

2.8.17.2   Evaluating Sample Data

2.8.17.2.1   The **Sizing Quality** for unknown samples and allelic ladders shall be at least 0.75 for a sample to pass. The **Sizing Quality** for reference samples, negative controls and positive controls shall be at least 0.25 for a sample to pass. All appropriate ILS peaks shall be present and labeled correctly.

2.8.17.2.2 **Allelic Ladders** and **Samples**

2.8.17.2.2.1 Highlight the rows containing ladders or samples and click "Display Plots". In the Samples Plot screen, change the "Plot Setting" drop-down box to "Fusion6C". Magnify the area from about 50 to 500 bp ensuring that all ILS peaks are visible in the retained electropherogram.

2.8.17.2.2.2 Verify that the correct allele calls are made for each ladder peak and evaluate all sample calls as described in 2.8.21.3.

2.8.17.2.2.3 Retain the electropherograms.

2.8.17.2.3 **Negative Controls**

2.8.17.2.3.1 Highlight all sample rows containing negative controls and click "Display Plots". Verify the Plot Setting is set to "Fusion6C".

2.8.17.2.3.2 Ensure that no peaks above analytical threshold are present.

2.8.17.2.3.3 Retain the electropherograms ensuring that the primer peak is visible.

2.8.17.2.4 After all analysis is complete, select the **Genotypes** tab. Click "File", then select "Export Table". Export the genotypes table as a .txt file to be utilized for STRmix™ analysis and the generation of allele summary tables in the STRlite Excel program. The .txt file name shall be the same as the project name with the automatic extension "Genotypes Table" added during export.

2.8.17.2.5 The 3500 Data Collection run folder containing the sample files, associated GeneMapper™ ID-X projects, and exported genotypes table .txt file shall be saved under each associated laboratory case number and request folder located in the analyst's folders on the server. Projects should be deleted monthly from the "GeneMapper Manager" to maintain database space.

2.8.17.2.6 When exporting the GeneMapper™ ID-X project, ensure that the "Export with analysis settings" box is checked.

2.8.18 General Rules for PowerPlex® Fusion 6C Analysis On The Applied Biosystems® 3500 Genetic Analyzer

2.8.18.1 At least one allelic ladder shall be present within each run folder. It is recommended that an allelic ladder be run within each set of 3 columns. This will help account for possible migration shifts due to external environmental factors. If a single injection contains both unknown and reference samples, it is recommended that two allelic ladders are run for GeneMapper™ ID-X analysis.

2.8.18.2 Samples in the 96-well plate should be oriented to allow for the injection of unknown samples prior to the injection of any reference samples for that case.

2.8.18.3 A signal range of 5,000 to 20,000 RFU will produce optimal results.

2.8.18.4 If multiple amplifications are analyzed for a sample, the analyst shall use the amplification which he or she determines sufficiently represents the sample based on peak heights, artifacts, and noise levels. Analysts shall interpret data that most clearly represents the sample and try to achieve better resolution if a sample has peak heights that are too high (resulting in artifacts, increased instrument noise, and pull-up) or too low (resulting in allelic drop out and loss of data).

2.8.18.5 Only the data used for interpretation needs to be published in the case record. However, other runs shall be noted in the case record and all data shall be saved under each

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

associated laboratory case number and request folder located in the analyst's folder on the server.

2.8.19 Archiving Applied Biosystems® 3500 and GeneMapper™ ID-X Projects

2.8.19.1   The 3500 run folders containing all sample files for a case as well as the GeneMapper™ ID-X project and export files shall be saved under each associated laboratory case number and request folder located in the analyst's folder on the server and deleted from the hard drive of the instrument and/or analysis computer. The data stored on the server shall be routinely backed up to ensure security of data.

2.8.19.2   All electropherograms used in interpretation as well as the plate record shall be placed in the case record.

2.8.20 STRmix™ Installation and Set-up: See Appendix 5

2.8.21 Interpretation Guidelines for PowerPlex® Fusion 6C and STRmix™ Deconvolutions

2.8.21.1   Scope

2.8.21.1.1   The following interpretation guidelines shall aid in peak/true allele determinations and profile interpretations.

2.8.21.1.2   It shall be at the analyst's discretion, based on experience and training, as to which peaks are suitable for interpretation and input into STRmix™.

2.8.21.1.3   If an analyst has determined that a peak that has been labeled by the GeneMapper™ ID-X software is not a true allele peak, the analyst shall rename or delete the allele call label in GeneMapper™ ID-X.

2.8.21.1.4   Final allele calls shall be documented on the STRlite pages retained in the case record.

2.8.21.1.4.1   For unknown profiles, the STRlite Excel program utilizes a filter to display peaks in the allele table that fall below the mean + 3SD stutter value from the PowerPlex® Fusion 6C validation for backward (n-3/n-4/n-5) and forward (n+3/n+4/n+5) stutter and for n-2 stutter at SE33. This is only to aid in the analyst's ability to determine what are possibly true alleles for the determination of the number of contributors in the sample. STRmix™ analysis will make the official interpretative evaluations of these peaks.

2.8.21.1.5   STRmix™ is a probabilistic genotyping software program for the interpretation of autosomal STR DNA profiling results. STRmix™ can deconvolute (break down into individual contributors) a DNA mixture and attach a statistical weighting to comparisons of evidence sample profiles to reference profiles.

2.8.21.1.5.1   The ISP Laboratory has validated STRmix™ v2.7.0 for casework.

2.8.21.2   DNA Profile Interpretation

2.8.21.2.1   Assumptions used in the interpretation of mixtures and interpretation decisions shall be documented in the case record.

2.8.21.2.2   Analysts shall consider the additive effects of allele sharing during interpretation.

2.8.21.2.3   The flow of interpretation generally follows the steps listed below (Clayton, et al., 1998):

Identify the presence of a mixture.

Designate the allele peaks.

Identify the number of contributors.

Estimate the relative ratio of the individual contributors to the mixture.

Consider all genotype combinations.

Compare reference samples.

Statistical analysis (if necessary).

2.8.21.2.4 STRmix™ is utilized to perform the interpretation steps following the identification of the number of contributors. The general approach to profile interpretation with STRmix™ is outlined here:

A DNA profile or mixture previously analyzed in GeneMapper™ ID-X is assessed for suitability for STRmix™ analysis.

If suitable, the profile/mixture is interpreted/deconvoluted within STRmix™.

Reference samples are compared to the completed interpretation/deconvolution, and statistical calculations are performed reflecting that comparison (if necessary).

STRmix™ outputs are reviewed for quality and all appropriate results are reported.

2.8.21.2.5   Identify If the Profile Is A Mixture

2.8.21.2.5.1 Single Contributor DNA Profile Determination

2.8.21.2.5.1.1 Generally, a single source profile should contain no more than two alleles at each autosomal locus and no more than one allele at each Y-STR locus. However, three-peak allele patterns have been reported at autosomal loci for single-source stains and two-peak patterns have been reported at Y-STR loci, but these instances are rare. A profile where only one autosomal locus demonstrates three alleles or one Y-STR locus demonstrates two alleles could be an indication of:

2.8.21.2.5.1.1.1   A tri-allelic pattern for an autosomal locus. If a tri-allelic pattern is indicated, it should be confirmed by concordance to at least one additional sample contained within the case and/or re-amplification.

2.8.21.2.5.1.1.1.1   Any locus with a confirmed tri-allelic pattern shall not be used for statistical evaluation.

2.8.21.2.5.1.1.2   A duplication event for a Y-STR locus. If a duplication is indicated, the profile should be confirmed by concordance to at least one additional sample contained within the case and/or re-amplification and documented notification to the Technical Leader. Alternatively, the sample may be amplified with PowerPlex® Y23 to confirm the profile with documented approval from a Biology Unit supervisor and notification to the Technical Leader.

2.8.21.2.5.1.2 For single source profiles, the peak height ratio (the smallest peak divided by the largest peak) of heterozygous individuals at a locus should be within 0.65.

2.8.21.2.5.1.2.1   Samples may display peak height ratios less than expected when the sample is of poor quality or the amplification target is below the ideal input of template DNA.

2.8.21.2.5.1.3 Conclusions shall be reported for all results in which the assumption of a single contributor is made.

2.8.21.2.5.2 **Mixed DNA Profile Determination**

2.8.21.2.5.2.1 A profile is defined as a mixture of two or more individuals when a locus demonstrates an additional allele, with the exception of a tri-allelic pattern at an autosomal locus or duplication event at a Y-STR locus.

2.8.21.2.5.2.2 A peak height ratio less than 0.65 at a locus may indicate a mixed profile and that allele sharing may be occurring.

2.8.21.2.5.2.2.1 Samples may display peak height ratios less than expected when the sample is of poor quality or the amplification target is below the ideal input of template DNA.

2.8.21.2.5.2.3 Peaks in stutter position that exceed the mean +3 SD stutter percentage may indicate the presence of a mixture.

2.8.21.2.5.2.4 All loci shall be considered when determining the presence of a mixture.

2.8.21.2.5.3 **Single Contributor Profiles in Which A Single Additional Allele is Detected (not tri-allelic)**

2.8.21.2.5.3.1 If the additional allele is present at Amelogenin or a Y-STR locus:

2.8.21.2.5.3.1.1 If it is possible drop-in (≤200 RFU), the analyst shall document that they interpreted the peak as drop-in and shall utilize the single source report wording.

2.8.21.2.5.3.1.2 If it is >200RFU, this shall be considered an indication of an additional contributor. The profile shall be deconvoluted in STRmix™ with the assumed number of contributors increased to two contributors.

2.8.21.2.5.3.1.2.1 If the peak is in stutter position, the expected stutter RFU (mean) does not count toward the 200 RFU cut-off to manually allow for the additive effect of stutter and drop-in.

2.8.21.2.5.3.2 If the additional allele is present at an autosomal locus:

2.8.21.2.5.3.2.1 If it is possible drop-in (≤200 RFU), the assumed number of contributors should not be increased to two contributors and STRmix™ shall be allowed to model the peak.

2.8.21.2.5.3.2.1.1 If the sample is not being modeled in STRmix™ due to it being consistent with an assumed standard, the analyst shall document that they interpreted the peak as drop-in and shall utilize the single source report wording. If the peak is in stutter position, the expected stutter RFU (mean) does not count toward the 200 RFU cut-off to manually allow for the additive effect of stutter and drop-in.

2.8.21.2.5.3.2.2 If it is >200 RFU, this shall be considered an indication of an additional contributor. The profile shall be deconvoluted in

STRmix™ with the assumed number of contributors increased to two contributors.

2.8.21.3   Preliminary Evaluation of Allele Peaks

2.8.21.3.1   An analyst is required to visually confirm that all allelic ladders used for allele designation performed correctly.

2.8.21.3.2   The internally validated analytical threshold for Fusion 6C is 100 RFU for the Blue, Green, and Yellow dye channels, 120 RFU for the Red dye channel, and 60 RFU for the Purple and Orange dye channels. These values shall be used for interpretation all unknown samples.

2.8.21.3.2.1   Peaks below analytical threshold shall not be interpreted, but may be considered when deciding if the possible number of contributors can be reasonably assumed.

2.8.21.3.2.2   For reference standards, 150 RFU shall be used as the analytical threshold for all Fusion 6C dye channels.

2.8.21.3.3   There is no static stochastic threshold used for STRmix™ interpretation. However, a stochastic threshold value of 950 RFU will be utilized to evaluate reference samples for completeness when a single allele is detected at a locus.

2.8.21.3.3.1   If a reference sample has a locus with a single peak present $\leq$950 RFU, the locus shall be ignored in the STRmix™ deconvolution (if the reference sample is being utilized for conditioning) or likelihood ratio calculation (if the reference sample is being utilized for comparison). Technical Leader approval is not necessary for ignoring more than one locus due to possible allelic drop-out of reference sample.

2.8.21.3.4   All interpretable autosomal peaks in unknown samples shall not exceed 28,000 RFU. Exceptions may be allowed with Technical Leader approval documented in the case record.

2.8.21.3.4.1   Unknown profiles with autosomal peaks over 28,000 RFU should be interpreted with caution, as modeling parameters utilized in STRmix™ may not model profiles with saturated peaks optimally.

2.8.21.3.4.2   Reference samples and positive control profiles may have up to two autosomal loci with peaks over 28,000 RFU. These profiles should be interpreted with caution.

2.8.21.3.5   An initial interpretation of the data shall be made to distinguish non-allelic artifact peaks (e.g., pull-up, spikes, dye blobs, etc.) from true allelic data.

2.8.21.3.5.1   Artifacts have been observed and documented utilizing the PowerPlex® Fusion 6C amplification kit. Any artifact peaks called as alleles by the GeneMapper™ ID-X software shall be deleted or renamed in the software as an artifact. The case record electropherogram should indicate the reason for deletion, either manually or labeled in the software. Examples of documented artifacts are listed below. A complete list of artifacts, including nonhuman cross-reactivity, can be found in the PowerPlex® Fusion 6C Technical Manual. Some examples of artifacts that may be observed are:

Amelogenin, D1S1656, DYS391, FGA – n-1
D13S317, D18S51, vWA, D7S820, D5S818, D19S433, FGA – n-2

Other DNA-independent artifacts have been documented in the following dyes:

> FL-6C (Blue) - ~65-75 bp, ~113 – 120 bp, ~137 – 145 bp
> JOE-6C (Green) - ~60 – 66 bp
> TMR-5C (Yellow) - ~57-62 bp

2.8.21.3.5.2   Pull-up or bleed through peaks can occur if signal intensity of sample or ILS peaks are too high or if a new spectral calibration needs to be run. Any pull-up peaks called as alleles by the GeneMapper™ ID-X software shall be deleted or renamed in the software as pull-up or artifact. The case record electropherogram should indicate the reason for deletion, either manually or labeled in the software. The sample should be re-run if a pull-up peak interferes with the analyst's ability to evaluate the profile based on their experience and training.

2.8.21.3.5.3   Spikes are peaks that generally appear in all colors and are sharper than regular peaks; however, they can occur predominantly in one color. Spikes are a natural consequence of capillary electrophoresis and can be caused by dust present in the system as well as urea crystals in the system. It is essential that the instrumentation be maintained and cleaned regularly to minimize the appearance of spikes. All spikes called as alleles by the GeneMapper™ ID-X software shall be deleted or renamed in the software as spikes or artifacts. The case record electropherogram should indicate the reason for deletion, either manually or labeled in the software. A sample should be re-injected when a spike interferes with the analyst's ability to evaluate the profile based on their experience and training.

2.8.21.3.6   Stutter peaks are artifacts of the amplification process and display less intensity than the true parent peak. Stutter peaks will typically be observed one repeat unit shorter than the parent peak (ex. n-4 at a tetranucleotide STR), but may also occur two repeats less (ex. n-8 at a tetranucleotide STR), or one repeat more (n+4 at a tetranucleotide STR). Additionally, n-2 artifact peaks have been reported at several loci amplified with PowerPlex® Fusion 6C. Mean and mean + 3SD backward, forward, and double-backward stutter values can be found in [Appendix 6](#).

2.8.21.3.6.1   Stutter peaks in the backward (n-3/n-4/n-5) and forward (n+3/n+4/n+5) positions at the autosomal loci, as well as in the n-2 position for locus SE33, are modeled by STRmix™ and **shall not be removed** from GeneMapper™ ID-X data of unknown profiles destined for STRmix™ analysis.

2.8.21.3.6.2   The stutter peaks listed below are not modeled by STRmix™ and **shall be removed** from GeneMapper™ ID-X data of unknown profiles destined for STRmix™ analysis. GMID-X stutter filters can aid in the removal of these stutter peaks below the mean + 3SD values. Peaks above the stutter filter, but determined to be stutter shall be deleted or renamed in the software as stutter or artifact. The case record electropherogram should indicate the reason for deletion, either manually or labeled in the software. Examples include:

2.8.21.3.6.2.1   Stutter peaks in the double-backward position (n-6/n-8/n-10).

2.8.21.3.6.2.2   Partial repeat (n-2/n+2) stutter peaks not included above (most common for D1S1656, D19S433, SE33 n+2 only and FGA).

2.8.21.3.6.2.3   All stutter peaks at the Y-STR loci.

2.8.21.3.6.2.4   In a mixture, GMID-X stutter filters may remove a double-back stutter off a major allele that is of a similar height to minor peaks. In this instance, the locus shall be ignored in the decon.

2.8.21.3.6.3   For reference profiles, all stutter peaks shall be deleted.

2.8.21.3.7   Rare variants (microvariants) have been described in the literature. These peaks will have a similar intensity to the other allelic peaks for that locus but will not line up with the allelic ladder and will fall outside of established bins.

2.8.21.3.7.1   Any allele peak that is not present in the allelic ladder and does not have an associated "bin" in the GeneMapper™ ID-X analysis software is labeled "OL" (Off-Ladder) and shall be renamed by the analyst in the software.

2.8.21.3.7.1.1   Alleles which are located between two alleles on the ladder shall be described as the short repeat followed by the number of base pairs it is larger (e.g., 0.1, 0.2, 0.3, or 0.4). Therefore, if a peak is 1 base pair larger than the 5 allele it shall be designated as 5.1. A microvariant 3 base pairs larger for than an allele on the ladder for a tri-nucleotide, 4 base pairs larger for a tetranucleotide, or 5 base pairs for a pentanucleotide may be designated with the full repeat number (e.g., A peak 4 base pairs larger than the 5 allele could be designated a 6; 5 base pairs larger a 6.1).

2.8.21.3.7.1.2   Alleles which are located outside the range of the ladder or bin set but within the designated marker range of the locus shall be evaluated to determine the appropriate repeat number that should be assigned to the allele.

2.8.21.3.7.1.2.1   Any allele recognized by the CODIS software to fall outside of the allelic ladder shall be renamed during CODIS entry as "<" the smallest allele or ">" the largest allele for the locus.

2.8.21.3.7.1.3   Alleles which are labeled "OMR" (Outside Marker Range) by the GeneMapper™ ID-X analysis software shall be evaluated to determine which locus the allele likely belongs to. If the analyst can reasonably determine which locus the allele belongs to, the allele can be assigned to that locus in GeneMapper™ ID-X and the allele call can be made. If the analyst cannot reasonably determine which locus the allele belongs to, both loci surrounding the allele shall be ignored from the STRmix™ deconvolution.

2.8.21.3.7.1.4   In instances where non-numeric alleles (e.g., OL and/or OMR) are marked and retained in the GeneMapper™ ID-X data, the resulting STRmix™ run shall be analyzed with that locus ignored from analysis.

2.8.21.3.7.2   Unresolved peaks may be observed with alleles and/or stutter peaks that are one bp apart. Most often this is observed at loci that have partial repeat alleles (e.g., D1, D12) and it occurs if the allele peak is very large and the allele or stutter peak one bp away is small. Sometimes in this situation, the GeneMapper™ ID-X software does not recognize and call the smaller peak.

It can typically be observed as a shoulder on the larger peak. Unresolved peaks shall be closely evaluated using the following guidelines:

- If the unresolved peak is estimated to be below the analytical threshold, no action is required, proceed with analysis.

- If the unresolved peak is above the analytical threshold, determine if the peak is potentially allelic or likely stutter. If the peak is potentially allelic, the locus shall be ignored in deconvolution.

- If the unresolved peak is likely stutter in a single source sample, the unlabeled stutter shall be documented in the notes and analysis can proceed as normal. Careful evaluation of the STRmix™ deconvolution shall be done as the stutter variance will be affected.

- If the unresolved peak is in a stutter position in a mixture, the estimated peak height shall be evaluated. If the estimated peak height is roughly equal or greater than the RFU height of minor alleles, the locus shall be omitted in the STRmix™ deconvolution. The Technical Leader shall be notified.

2.8.21.4   Evaluation of Controls

2.8.21.4.1   Failed controls require documented notification to the Technical Leader with appropriate documentation in the case record.

2.8.21.4.2   The appearance of pull-up or known artifact peaks does not render the following controls inconclusive.

2.8.21.4.3   Reagent Blank:

2.8.21.4.3.1   The purpose of the reagent blank is to determine if the reagents used to extract the associated samples were contaminated by human DNA. Therefore, no signal should be detected in this sample well other than the internal lane standard. If a signal is detected in the reagent blank, all results of samples associated with that reagent blank shall be considered inconclusive.

2.8.21.4.3.2   A reagent blank with peaks below the analytical threshold shall not prevent associated samples from being interpreted.

2.8.21.4.3.3   A reagent blank with peaks at or above the analytical threshold shall be considered a failed negative control.

2.8.21.4.4   Positive Control:

2.8.21.4.4.1   The 2800M DNA is used as a positive control to demonstrate that the kit is performing properly. A positive control is considered a failed positive control if expected alleles are not detected, a single autosomal locus peak is $\leq$ 950 RFU, or if extra alleles are detected.

Positive Control 2800M (PowerPlex® Fusion 6C):

X, Y	17, 18	12, 13	10, 14	13, 15	9, 11	7, 14
Amelogenin	D3S1358	D1S1656	D2S441	D10S1248	D13S317	Penta E

9, 13	16, 18	22, 25	12	12, 13
D16S539	D18S51	D2S1338	CSF1PO	Penta D

6, 9.3	16, 19	29, 31.2	8, 11	12	11
TH01	vWA	D21S11	D7S820	D5S818	TPOX

14, 15	18, 23	13, 14	15, 16	16
D8S1179	D12S391	D19S433	SE33	D22S1045

10	20, 23	18	17
DYS391	FGA	DYS576	DYS570

2.8.21.4.5 Amplification Blank:

2.8.21.4.5.1 The purpose of the amplification blank is to determine if human DNA contaminated the samples at the amplification step. Because no template DNA was placed in the reaction, the sample should be blank except for the internal lane standard peaks.

2.8.21.4.5.2 An amplification blank with peaks below the analytical threshold shall not prevent associated samples from being interpreted.

2.8.21.4.5.3 An amplification blank with peaks at or above the analytical threshold shall be considered a failed negative control.

2.8.21.4.6 Interpreting, Comparing and Reporting DNA results associated with Failed Controls and Contamination Events

2.8.21.4.6.1 The Technical Leader shall be notified if a positive or negative control fails and/or if sample contamination occurs. Sample contamination is defined as additional peaks present in an unknown or reference sample that can be conclusively identified to not have originated in the sample (e.g., case to case contamination or a mixture in a reference sample).

2.8.21.4.6.2 Assessment of the integrity of the associated DNA results shall be done in consultation with the Technical Leader. This shall include the possible cause and effect of the failed control or contamination. Additionally, an assessment of the risk associated with moving forward with interpretation shall be documented, as well as the risks associated with retesting (e.g., unnecessary consumption of evidence).

2.8.21.4.6.2.1 If retesting is performed, the reasons for doing so shall be documented.

2.8.21.4.6.2.2 If results are determined to be suitable for interpretation without retesting, the appropriate interpretations, comparisons, and statistical results and a description of the failed control or contamination event shall be reported per the Report Writing Guidelines.

2.8.21.4.6.2.3 If results are determined to not be suitable for interpretation and retesting cannot be performed, the results shall be reported as not suitable for comparison per the Report Writing Guidelines.

2.8.21.4.6.3 Documentation in the case record shall include at a minimum: the forensic sample, reference, or control test result that failed or was contaminated, the likely or known cause of the failed control or contamination, the likely or known source of the contamination, the impact on the integrity of the DNA

results, and the determination if the test result is suitable or unsuitable for interpretation.

2.8.21.5   Determination of the Assumed Number of Contributors

2.8.21.5.1   The assignment of the number of contributors to a profile should be made by the analyst prior to the comparison of the profile to any reference samples. Reference sample profiles from individuals who are assumed to be present in a sample and will be used to condition on in the STRmix™ deconvolution may be used to aid in the determination of number of contributors for that sample.

2.8.21.5.1.1   The profile from the corresponding fraction of a differential extraction may also be used to aid in the determination of the number of contributors.

2.8.21.5.2   Determination of the number of contributors shall be done at the analyst's discretion, based on experience and training, and should take into account peak height ratios and possible genotype pairings, as well as sub-analytical threshold and high stutter peaks.

2.8.21.5.3   Depending on sample data, an analyst may assign the number of contributors to a sample as one, two, three, four, or at least five.

2.8.21.5.4   In circumstances when the number of contributors cannot be determined, the profile is reported as too complex to interpret (inconclusive). Such profiles include:

- 5 person or greater mixtures

- Sample results with data at less than two autosomal loci

- Sample results with poor quality data in which peak number, peaks below analytical threshold, peak height ratios and other qualitative factors prevent the reasonable determination of the number of contributors

2.8.21.5.5   The following steps should be followed in assigning the number of contributors to a profile:

2.8.21.5.5.1   Review the profile as a whole, assessing the level of degradation, presence of low level peaks, noisy or clean baseline and the general quality of the profile.

2.8.21.5.5.2   If replicate amplifications have been undertaken, all qualifying replicate profiles should be reviewed when determining the number of contributors.

2.8.21.5.5.3   Identify possible stutter peaks (both forward and backward) by reference to stutter table (see Appendix 6).

2.8.21.5.5.4   Find the locus with the highest number of unambiguous allelic peaks to determine the minimum number of contributors. Peak height imbalance between alleles may mean the presence of an additional contributor(s) above that indicated by allele count alone.

2.8.21.5.5.5   If one or more contributors at this locus is a clear major, check that this pattern is represented at other loci.

2.8.21.5.5.6   Apply the general pattern of contributors (e.g., number of alleles, peak height ratios) to other loci in the profile.

2.8.21.5.5.7   Discriminating loci such as FGA and SE33 are likely to be the most informative when determining the number of likely contributors.

2.8.21.5.5.8   The presence of a single minor peak or elevated stutter may indicate the presence of drop-in and not an additional contributor.

2.8.21.5.5.8.1   If a single additional allele at a Y-STR locus that is $\leq$200 RFU is the only indication of an additional contributor, the peak shall be deleted and documented on the electropherogram as drop-in. The number of contributors shall not be increased.

2.8.21.5.5.8.2   If the peak is in stutter position the expected stutter RFU (mean) does not count toward the 200 RFU cut-off to account for the potential for drop-in and stutter being additive.

2.8.21.5.5.9   After initial assignment of the number of contributors and subsequent STRmix™ analysis, and prior to comparison with the person of interest reference profiles, the analyst may determine that the deconvolution did not conform to scientific expectation and may be re-run under a different number of contributors or with increased MCMC iteration accepts. In this case, the course of action shall be detailed in the case notes and the electronic file of the first deconvolution shall be maintained in the case record.

2.8.21.5.5.10   The Variable Number of Contributors (varNOC) function in STRmix™ may be utilized if the analyst is unable to determine a single number of contributors estimate, but feels the data quality is suitable for STRmix™ analysis.

2.8.21.5.5.10.1   It is recommended that the analyst utilize other troubleshooting methods to resolve the number of contributors (such a reamplification) prior to utilization of varNOC.

2.8.21.5.5.10.2   Technical Leader permission is required if varNOC is utilized. DBLR software shall not be used with varNOC.

2.8.21.5.5.10.3   The range of contributors evaluated in varNOC shall not exceed a difference of one contributor.

2.8.21.6   **General Interpretation Considerations for STRmix™**

2.8.21.6.1   STRmix™ should be used to deconvolute all qualifying samples, where a qualifying sample is defined as:

2.8.21.6.1.1   All evidence DNA samples where the number of contributors can be assumed (up to four contributors) unless the profile is completely accounted for by conditioning.

2.8.21.6.1.1.1.1   A sample demonstrating a partial profile may be interpreted if the number of contributors can be reasonably assumed. STRmix™ requires a minimum of two autosomal loci for interpretation.

2.8.21.6.2   Evidence samples with identical profiles shall be analyzed independently with STRmix™.

2.8.21.6.3   Y-STR loci present in PowerPlex® Fusion 6C shall only be used for interpretation purposes and not for statistical evaluations. The Y-STR loci are not evaluated within the STRmix™ software.

2.8.21.6.4 The basis of STRmix™ is that, given a set of mass parameters (e.g., DNA amount, degradation, and amplification efficiency), the software can determine the expected height of a peak of a certain size at a given locus. If there are reasons for the expected peak height to be incorrect or for issues with allele designation, STRmix™ is unable to handle loci with these effects. Examples include:

      2.8.21.6.4.1   Tri-allelic loci

      2.8.21.6.4.2   Somatic and primer binding site mutations

      2.8.21.6.4.3   Technical issues (saturated or poorly resolved GeneMapper™ ID-X data)

2.8.21.6.5 If these effects are discovered after the original deconvolution (e.g. upon inclusion of a tri-allelic standard), a second deconvolution should be performed ignoring the affected locus.

      2.8.21.6.5.1   If the effect causes an analyst to change their original number of contributors decision, Technical Leader approval is required.

2.8.21.6.6 Non-numeric values such as OL, <, or > are not permitted within the STRmix™ input files.

2.8.21.6.7 The Ignore Locus function within STRmix™ may be used to ignore an affected locus within an interpretation. A locus with this option selected will be removed from the subsequent deconvolution or LR from Previous calculation.

   2.8.21.6.7.1   Analyst discretion may be used to ignore a single locus within the STRmix™ deconvolution or LR from Previous calculation with reasoning documented in the case record. Ignoring more than one locus requires Technical Leader approval with documentation in the case record, except in the situation where ignoring multiple loci is necessary due to possible allele drop-out in a reference sample.

   2.8.21.6.7.2   If a locus has been ignored due to the inability of STRmix™ to handle a biological effect (e.g., a tri-allelic locus), an analyst may document their manual interpretation for CODIS entry purposes only.

2.8.21.6.8 A replicate is defined as a repeat amplification of the same extract and may be analyzed together in STRmix™ to aid sample interpretation, including determination of the number of contributors.

   2.8.21.6.8.1   Replicates may only be analyzed together through STRmix™ with Technical Leader approval documented in the case record.

2.8.21.6.9 If it has been determined that a sample is unsuitable for interpretation, an explanation as to why the sample is un-interpretable should be given. Examples include:

   2.8.21.6.9.1   No results: No peaks were detected in the electropherogram.

   2.8.21.6.9.2   Inconclusive: Peaks were observed at one or more loci; however, no conclusive results could be drawn due to an inability to reasonably determine the number of contributors.

2.8.21.6.10 Test results from an unknown sample that are suitable for comparison to a reference sample may have the following conclusions reported:

2.8.21.6.10.1 Included: The STRmix™ calculated HPD (Highest Posterior Density) Likelihood Ratio favors inclusion.

2.8.21.6.10.2 Excluded: The profile obtained from the unknown stain had discrepant genotypes as compared to the profile of the reference sample (i.e., was not the same) and can be visually excluded or the STRmix™ calculated HPD Likelihood Ratio favors exclusion.

2.8.21.6.10.3 Uninformative: The profile obtained from the unknown stain has sufficient ambiguity when compared to the profile of the reference sample and the HPD Likelihood Ratio falls in the uninformative range.

2.8.21.6.11 If conclusive results are obtained from a sample, appropriate CODIS eligible profiles which do not match the victim shall be entered into CODIS for searching. The analyst and technical reviewer shall evaluate CODIS eligibility before submitting profiles for entry.

2.8.21.7 **Setting Up Propositions for Calculating the Likelihood Ratio (LR)**

2.8.21.7.1 The Likelihood Ratio (LR) assesses the probability of the evidence given two alternate propositions or hypotheses; one that aligns with the prosecution (H_1 or H_p) and one that aligns with the defense (H_2 or H_d). H_1 is typically inclusionary of the person(s) of interest. The defense proposition aligns with exclusion of the person(s) of interest.

2.8.21.7.2 Analysts will need to review all case information in order to determine which likelihood ratio calculations should be calculated and reported. Multiple propositions may need to be considered.

2.8.21.7.2.1 Examples:

2.8.21.7.2.1.1 Two-person mixture from intimate swab collected from victim. The victim and the Person of Interest (POI) cannot be excluded as possible contributors to the sample.

H_1: DNA originates from victim and POI

H_2: DNA originates from victim and unknown contributor

2.8.21.7.2.1.2 Three-person mixture from firearm located at scene. The POI cannot be excluded as a possible contributor to the sample.

H_1: DNA originates from POI and two unknown contributors

H_2: DNA originates from three unknown contributors

2.8.21.7.2.1.3 Three-person mixture from firearm located at scene. POI1 and POI2 both cannot be excluded as possible contributors to the sample. Three separate LRs may be calculated:

H_{1-1}: DNA originates from POI1 and two unknown contributors

-Or-

H_{1-2}: DNA originates from POI2 and two unknown contributors

-Or-

H_{1-3}: DNA originates from POI1, POI2 and one unknown contributor

H_2: DNA originates from three unknown contributors

2.8.21.7.3 Conditioning is defined as a DNA profile that is present in both propositions (H_1 and H_2) because it is reasonable to concede the presence of the individual's DNA. Conditioning where appropriate provides a more accurate likelihood ratio for the person of interest's possible contribution to that profile.

2.8.21.7.3.1 At this time, only DNA reference samples may be used for conditioning, except as indicated for CODIS purposes only.

2.8.21.7.3.2 The reference profiles shall have at least 20 complete autosomal loci (loci with no possible allelic drop-out). In cases where degradation of the reference sample results in less than 20 complete autosomal loci and conditioning may be beneficial to the sample, consultation with the Technical Leader is required to determine the best course of action.

2.8.21.7.3.3 If a reference profile being used for conditioning is a partial profile or has loci with possible allelic drop-out (a single allele ≤ 950 RFU), the loci with incomplete allelic information shall be omitted from the STRmix™ deconvolution.

2.8.21.7.3.4 The determination for the use of a reference profile for conditioning should be based on the analyst's visual determination if the individual is included in the evidence profile. A likelihood ratio should not be performed to determine if a reference is included or excluded for this purpose.

2.8.21.7.3.5 Intimate samples should be conditioned on the owner, unless a single source foreign profile is obtained (e.g., sperm fractions or blood of another on one's person).

2.8.21.7.3.5.1 Intimate samples should also be conditioned on an elimination standard from a consensual partner.

2.8.21.7.3.6 Non-intimate samples may be conditioned on elimination standards, depending on the profile and case information (e.g., steering wheel profile conditioned on owner or clothing profile conditioned on wearer).

2.8.21.8 Initial STRmix™ Analysis for Evidentiary Sample Data (Deconvolution)

2.8.21.8.1 To start a new STRmix™ deconvolution, from the STRmix™ main menu select **Interpretation.**

2.8.21.8.1.1 Alternately, **Batch Mode** for deconvolutions may be utilized for multiple samples, which is accessed from the main menu and functions as below.

2.8.21.8.2 In the **Interpretation** window, enter the case number in the "Case Number" box. The deconvolution number should be entered after the item number in the "Sample ID" box. For subsequent deconvolutions of the same item number, increase "Decon#" sequentially (e.g., 001A_Decon1, 001A_Decon2). If additional characters are added to the "Sample ID" designation, those characters shall be added after the specified naming convention.

2.8.21.8.3 Enter the proposed number of contributors for the sample (1, 2, 3, or 4), and select **Confirm.**

2.8.21.8.4 Ensure the **Contributor Range** box is not checked if varNOC is not being utilized.

    2.8.21.8.4.1 If varNOC is being utilized, check the **Contributor Range** box. Enter the minimum and maximum number of contributors. The range of contributor number shall be +/- 1. Select **NIST_2017_Caucasian** as the population for the range.

2.8.21.8.5 Select **Run Settings** and ensure that the number of burn-in accepts per chain is set to **10,000**, and the number of post burn-in accepts per chain is set to **50,000**, and click **Apply,** then **Next**.

    2.8.21.8.5.1 For troubleshooting purposes, an analyst may increase the number of burn-in accepts per chain to **100,000**, and the number of post burn-in accepts per chain to **500,000** with notification to the Technical Leader documented in the case record.

    2.8.21.8.5.2 Aside from number of accepts, no other changes should be made in **Run Settings** without Technical Leader approval.

2.8.21.8.6 Verify that **ISP_Fusion6C_v3** is the selected DNA kit used.

2.8.21.8.7 Add a new profile to the box marked **Evidence Profile Data**. This may be done by selecting **+** and navigating the file tree, or by dragging and dropping the appropriate GeneMapper™ ID-X export file into the box. If multiple samples are present in the file, select the desired sample for analysis.

2.8.21.8.8 If conditioning on a reference sample, add a new profile to the box marked **Reference Profile Data**. This may be done by selecting **+** and navigating the file tree, or by dragging and dropping the appropriate GeneMapper™ ID-X export file or previous STRmix Evidence file into the box. Multiple reference profiles may be added if you are conditioning on multiple individuals.

2.8.21.8.9 After the reference profile is added, check the **Contributor to: Hd** box to reflect that it is a conditioned sample.

2.8.21.8.10 Ensure that the **Perform Database Search** box is checked.

2.8.21.8.11 To ignore loci from deconvolution, select **Kit Settings**, then select **Loci**, then select **Ignore?** for appropriate loci, then select **Apply**.

2.8.21.8.12 Select **Start**. This will begin a deconvolution analysis of the chosen sample and search the results against the ISP QA DNA Database.

2.8.21.8.13 When the deconvolution is complete, STRmix™ will generate several data files and save them to the STRmix™ Results folder on the C: drive.

2.8.21.8.14 Save each deconvolution folder to the DNA server for the appropriate case.

2.8.21.9 **Review of Run Diagnostics**

    2.8.21.9.1 Upon completion, the Deconvolution Report shall be reviewed for quality by assessing the deconvolution diagnostics. Skewed diagnostics may indicate that the sample is in need to further scrutiny (e.g., inclusion of inappropriate artifacts in import file or incorrect contributor number), that the deconvolution has not reached the appropriate sample space and additional accepts may be needed, or that the sample is of poor quality.

2.8.21.9.1.1   The weights of the genotype combinations generated by STRmix™ should be assessed at each locus for their intuitiveness when compared visually to the accompanying sample electropherogram. These may be found under the **Weights** section of the Deconvolution report (example below).

2.8.21.9.1.1.1 Rarely, due to limitations in stutter modeling for loci with complex repeat structures, STRmix™ may not model stutter peaks as expected (ex. 13 stutter peak from 14 allele at vWA). If an analyst finds that a peak has not modeled as expected, they may refer to the STRbase website (NIST STRBase) to investigate if the allele is one that may contain variations in the Longest Uninterrupted Sequence (LUS) value. This would provide weight to the decision to ignore the locus in the STRmix™ deconvolution.

WEIGHTS

LOCUS	CONTRIBUTORS		WEIGHT
	1 (52%)	2 (48%)	(HIGHLIGHT ≥ 0.99)
D3S1358	15, 18	16, 16	2.7918E-1
	15, 16	16, 18	2.7166E-1
	16, 18	15, 16	2.3937E-1
	16, 16	15, 18	2.0979E-1
D1S1656	15.3, 17	15.3, 16	2.9155E-1
	15.3, 16	15.3, 17	2.5167E-1
	15.3, 15.3	16, 17	2.3808E-1
	16, 17	15.3, 15.3	2.1871E-1
D2S441	14, 14	11.3, 13	4.6170E-1
	13, 14	11.3, 14	2.9570E-1
	11.3, 13	14, 14	1.2257E-1
	11.3, 14	13, 14	1.1996E-1
	13, 14	11.3, 11.3	5.0760E-5
	11.3, 11.3	13, 14	1.6656E-5

2.8.21.9.1.2   Template (RFU) and Mixture Proportions

2.8.21.9.1.2.1 Mixture proportions are calculated from the template mass parameter per contributor. The template amount in RFU should approximate the lowest molecular weight alleles/loci for a heterozygous peak (the value would need to be doubled for a homozygous genotype). The template and mixture proportions should be intuitive to the analyst's interpretation of the profile. Significant deviations from the expected template and/or mixture proportions may require further scrutiny and additional rework.

2.8.21.9.1.3   Degradation

2.8.21.9.1.3.1 The degradation mass parameter per contributor is displayed in the STRmix™ report as two values. The first value is the Linear approximation (RFU/bp) which demonstrates the average decrease in RFU per base pair. The second value is the Degradation exponential

curve which is the actual mass parameter utilized by STRmix^TM for the calculation of Total Allelic Product. This value has a maximum threshold of 0.01 in the standard kit settings. For either value a larger value indicates a steeper curve and more degradation; however, a sample with significant degradation cannot exceed the 0.01 exponential curve threshold and may result in additional skewed diagnostics and/or unintuitive genotype weightings. If an elevated degradation value is observed (likely greater than 0.009) and potentially hindering the interpretation of the sample, consultation with the Technical Leader is recommended to consider increasing the threshold up to 0.02.

2.8.21.9.1.4   Total Iterations and Acceptance Rate

2.8.21.9.1.4.1 The Total Iterations value displayed in the **Post Burn-In Summary** indicates the total number of post burn-in MCMC iterations. This value, along with the number of accepts chosen for the analysis can inform the user as to how often a new proposed set of parameters was accepted. This is referred to as the acceptance rate.

2.8.21.9.1.4.2 A very low acceptance rate (e.g., 1 in thousands to millions) may, in combination with the other diagnostics, indicate that the analysis may benefit from a run with additional iterations. On its own (and without any other indication of sub-optimal results) a low acceptance rate is not an indication that rework is required.

POST BURN-IN SUMMARY

Total iterations	532,424	Acceptance rate	1 in 1.33
Effective sample size	5,141.61	log(likelihood)	49.66
Gelman-Rubin convergence diagnostic	1.07		
Allele variance (mode = 8.769)	10.884	Back Stutter variance (mode = 5.992)	10.385
Forward Stutter variance (mode = 3.186)	4.165	SE33 -2 Stutter variance (mode = 0.694)	1.732

2.8.21.9.1.5   The Effective Sample Size (ESS) value displayed in the **Post Burn-In Summary** is the number of independent samples the MCMC has taken from the posterior distribution of all parameters. A low ESS in relation to the total number of iterations suggests that the MCMC has not moved very far with each step or has had a low acceptance rate. A low absolute value of ESS (e.g., 10s or 100s) will mean that there is potential for a large difference in weights if the analysis was run again. On its own (and without any other indication of sub-optimal results) a small ESS is not an indication that rework is required.

2.8.21.9.1.6   The log(likelihood) value displayed in the **Post Burn-In Summary** is the log of the average likelihood (or probability) value created at each of the post burn-in MCMC iterations. The larger this value the better STRmix™ has been able to describe the observed data. A negative value suggests that STRmix™ has not been able to describe the data very well given the information it has been provided. Reasons why this value may be low or negative are:

- The profile is simply very low level and there is very little data making up the likelihood.

- The proposed number of contributors does not model well (and may be incorrect), forcing an interpretation of large stochastic events (e.g., large heterozygote peak imbalances or variation in mixture proportions across the profile) in the STRmix™ run.

- Data has been removed that was real, (e.g., stutter peaks), forcing an interpretation of dropout by STRmix™.

- Artifacts have been left labelled (not removed) in the imported data, forcing an interpretation of drop-in by STRmix™.

2.8.21.9.1.6.1 A low or negative value for the average log(likelihood) may indicate to users that the analysis requires additional scrutiny.

2.8.21.9.1.6.2 Good quality mixed DNA profiles are likely to give higher average log(likelihood) values than good quality single source profiles.

2.8.21.9.1.6.3 Low average log(likelihood) values alone are not necessarily an indicator of an issue, especially if the profile is single source.

2.8.21.9.1.7 The Gelman-Rubin Convergence Diagnostic (GR) value displayed in the **Post Burn-In Summary** informs the user whether the MCMC analysis has likely converged. STRmix™ uses multiple MCMC chains to carry out the deconvolution, and ideally each chain will be sampling in the same space after burn-in. Too few accepts may result in a failure to converge. If the chains spend their time in different spaces, then it is likely that the analysis has not run for sufficient iterations. Whether or not the chains have spent time in the same space can be gauged by the within-chain and between-chain variances. These two variances are used to calculate the variance of what is called the 'stationary distribution'. If all chains have sampled the same space, then the within chain variance and the variance of the stationary distribution will be approximately equal. If chains have spent time in different spaces, then the variation between the chains is likely to be larger than the variation within the chains. The GR is the ratio of the stationary distribution and within-chain variances. For a perfectly converged analysis, GR = 1.

2.8.21.9.1.7.1 If the GR is above 1.2 then there exists the possibility that the analysis hasn't converged. If the GR value is above 1.2, STRmix™ will automatically run an additional 50,000 post burn-in accepts. This may decrease the GR to below 1.2, yielding a clearer deconvolution.

2.8.21.9.1.7.2 If the GR continues to remain above 1.2 after the additional post burn-in accepts, this may be an indicator that the sample requires rework.

2.8.21.9.1.8 The allele, stutter, and Locus Specific Amplification Efficiency (LSAE) variance data depicted in the **Variance Charts** (see below for allele and back stutter variance only) can be used as a guide as to the level of stochastic variation in peak heights that is present in the profile. The values listed in the **Post Burn-In Summary** of the report include the average value for allele, stutter, and LSAE variance constants across the entire post burn-in MCMC analysis.

VARIANCE CHARTS

ALLELE VARIANCE — $\alpha = 7.54$, $\beta = 1.34$, mode $= 8.77$, $C^2 = 10.88$

BACK STUTTER VARIANCE — $\alpha = 1.70$, $\beta = 8.62$, mode $= 5.99$, $K^2 = 10.39$
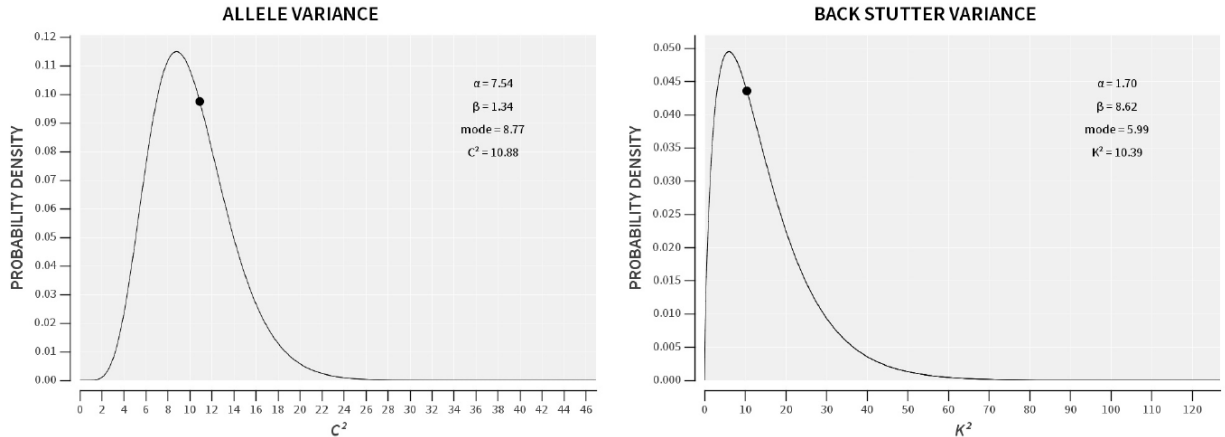
2.8.21.9.1.8.1 If the variance constant has increased markedly from the mode of the prior distribution, then this may indicate that the DNA profile is sub-optimal or that the number of contributors is incorrect.

2.8.21.9.1.8.2 Used in conjunction with the average log(likelihood), a large allele variance or stutter variance constant can indicate poor PCR.

2.8.21.9.1.8.2.1 A low-level sample may result in a low average log(likelihood) and an average variance constant.

2.8.21.9.1.8.2.2 Data with missing information, extra information (e.g., artifact peaks), or misinterpreted data may result in a low average log(likelihood) and high variances.

2.8.21.10 Indiana State Police Laboratory Division Quality Assurance DNA Database Search Review

2.8.21.10.1 In the Database Search Report, verify that no positive associations with LR $\geq$ 10,000 have occurred.

2.8.21.10.2 Any database matches with a LR $\geq$ 10,000 should be evaluated for the likelihood of potential contamination. The Technical Leader shall be consulted for evaluation of the match. If the match is determined to be a suspected contamination event a root cause analysis shall be performed to determine the possible cause. Appropriate report wording shall be utilized if contamination is suspected.

2.8.21.10.2.1 Database matches (LR $\geq$ 10,000) shall be interpreted with caution and acknowledged in the report wording.

2.8.21.10.2.1.1 The database profile shall be utilized and conditioned upon in an additional STRmix™ deconvolution.

2.8.21.10.2.1.2 The database Excel tool shall be used to convert the database profile into the correct STRmix™ reference profile format.

2.8.21.10.2.1.3　　Any LRs calculated for the samples shall be performed on the deconvolution conditioned on the database profile.

2.8.21.11　　Comparing Sample Deconvolutions to Reference Standards (LR from Previous)

2.8.21.11.1　　A reference standard may be visually excluded without STRmix™ analysis.

2.8.21.11.2　　To compare reference STR profiles to a previously run STRmix™ deconvolution, from the STRmix™ main menu, select **Investigation,** then **LR from Previous.**

2.8.21.11.2.1　　Alternately, a **Batch Mode** for LR from Previous may be utilized for multiple samples, which is accessed from the main menu. In the **Add to Batch** drop down menu, select **add LR from Previous** and then follow the instructions below.

2.8.21.11.3　　In the **Previous Interpretation** box, select **Browse** and navigate to the config.xml file for the appropriate STRmix™ deconvolution or drag and drop the deconvolution folder into the window.

2.8.21.11.4　　Leave the Case Number as it is carried over. In the "Sample ID" box the appropriate LR number should be added behind LRPrev (e.g., 001A_Decon1_LRPrev1). Each LR calculated from a deconvolution shall have a unique identifier. If additional characters are added to the "Sample ID" designation at this step, those characters shall be added after the specified naming convention. Select **Next**.

2.8.21.11.4.1　　The number of contributors, number of burn-in accepts per chain, and number of post burn-in accepts per chain will be grayed out and cannot be changed. No changes should be made to **Run Settings.**

2.8.21.11.5　　Add a new profile to the box marked **Reference Profile Data**. This may be done by selecting **+** and navigating the file tree, or by dragging and dropping the appropriate GeneMapper® ID-X export file or previous STRmix™ evidence file into the box. Multiple reference profiles may be added, one at a time or highlight and add multiple profiles at one time.

2.8.21.11.6　　To ignore loci from deconvolution, select **Kit Settings**, then select **Loci**, then select **Ignore?** for appropriate loci, then select **Apply**.

2.8.21.11.6.1　　If a locus in the reference profile has potential allelic drop-out (a single allele ≤ 950 RFU), the locus shall be omitted from the LR from Previous calculation.

2.8.21.11.7　　If a varNOC deconvolution was performed, select **LR Settings**. Under **Contributor Range**, select **MLE** for the priors method, then select **Apply**.

2.8.21.11.7.1　　Upon request by the submitting agency, Prosecuting or Defense attorney or with Technical Leader permission documented in the casefile, a User Defined LR may be performed.

2.8.21.11.7.1.1　　Select **LR Settings**. Under **Contributor Range**, select **User Defined** for the priors method, enter the selected number of contributors for Hp and Hd, then select **Apply.**

2.8.21.11.8　　Select **Start**. This will begin the LR calculation of the chosen sample.

2.8.21.11.9　　When the analysis is complete, STRmix™ will generate several data files and save them to the Results folder on the C: drive.

2.8.21.11.10 Save the results folder for the LR calculation containing all appropriate STRmix™ files to the corresponding deconvolution master folder located on the DNA server for the appropriate case.

2.8.21.12   LR Calculations

2.8.21.12.1 Each analyzed proposition (and therefore each LR From Previous report) will contain a generated set of calculations including a Likelihood Ratio (LR) Total (sub-sub-source), Factor of N! (sub-source) LR, a 99% 1-Sided Lower HPD Interval, and a Stratified LR.

2.8.21.12.2 The (sub-sub-source) LR Total is a ratio between the probability of the evidence given two competing propositions or hypotheses and forms the basis for subsequent statistical calculations.

2.8.21.12.2.1   Calculations for (sub-sub-source) LR Total utilize the National Research Council recommendation 4.2, equations 4.10, more commonly referred to as the Balding & Nichols formulae.

2.8.21.12.2.1.1   Homozygous $\dfrac{[2\theta + (1-\theta)p][3\theta + (1-\theta)p]}{[1+\theta][1+2\theta]}$

2.8.21.12.2.1.2   Heterozygous $\dfrac{2[\theta + (1-\theta)p][\theta + (1-\theta)q]}{[1+\theta][1+2\theta]}$

2.8.21.12.2.1.3   Each locus LR shall be multiplied together to form the multi-locus LR for the profile.

2.8.21.12.3 The Factor of N! (sub-source) LR is a calculation that uses the (sub-sub-source) LR Total with additional consideration for the number of ways to combine the different contributor probabilities in a mixture.

2.8.21.12.4 The HPD calculation uses the Factor of N! (sub-source) LR with additional consideration for the uncertainty in the allele frequencies and deconvolution process. HPD is the most conservative of the three calculations and will be used for reporting the significance of a comparison.

2.8.21.12.5 The Stratified LR utilizes the HPD calculations and population settings to produce a single LR that samples across all the calculated populations.

2.8.21.12.6 Additionally, a Unified LR may be calculated under propositions that consider only a single person of interest. The unified LR takes into account that the unknown contributor(s) are made up of both unrelated people and relatives of the person of interest. These calculations may be most useful when the alternate proposition is that the true donor of the evidence sample is a relative of the person tested.

2.8.21.12.7 The varNOC Maximum Likelihood Estimate LR (MLE) assigns an LR using the most probable number of contributors under H_1 and H_2. This means the number of contributors in the numerator and denominator may be the same or they may be different. The number of contributors will depend on which number of contributor analysis has the highest likelihood given the proposition for each.

2.8.21.13   Troubleshooting STRmix™ Results

2.8.21.13.1 After a STRmix™ analysis has completed, the results are interpreted by examining the weightings of various genotypes and the DNA results observed.

There are instances when the results obtained do not intuitively seem correct. Examples of this are:

- Large LRs (greater than 1) are obtained for each locus, except one where the LR is 0.

- The mixture proportions do not reflect what is observed.

- The interpreted contributor genotypes are not intuitively correct.

2.8.21.13.2   Possible causes for these issues include:

- The MCMC has not run for enough iterations.

- The number of contributors has not been correctly chosen.

- An artifact was left in the GeneMapper™ ID-X interpretation.

2.8.21.13.3   Should the weights and/or the diagnostics highlight to the user that further scrutiny is necessary then a number of rework options are available, if required. For example, a review of the proposed number of contributors could be considered. Further analytical work such as a re-electrophoresis, a re-amplification, or a clean up to strengthen the number of contributors assumption or assist with allele designation/sub optimal PCR performance. Extended total iterations (x10) may be considered, if the acceptance rate is low, the ESS is low and/or the GR value is significantly above 1.2. If extended iterations are manually utilized, the Technical Leader shall be notified with documentation included in the case record.

2.8.21.13.3.1   Additional troubleshooting options may be available after consultation with the Technical Leader (documented in the case record). This may include utilizing informed priors to designate the mixture proportions utilized in the deconvolution.

2.8.21.13.4   If after comparison to a reference sample from a person of interest, an analyst suspects an error was made during the interpretation of the sample (e.g., determining the wrong number of contributors or an artifact was not deleted), independent analysis and interpretation of the profile by the Technical Leader or their designee shall be performed. A referee analysis is not required if the error determined after comparison to a reference profile of an individual who may be assumed present in the sample. If the error is caught during technical review of the case, and the reviewer has not examined the profiles of the person(s) of interest, the referee analysis is not required.

2.8.21.13.4.1   The analyst shall only provide the .hid files for the unknown sample of concern and the allelic ladder to the Technical Leader. If a reference standard may be assumed, the .hid file for that reference may also be included. If the analysis method and panels and bins in GMID-X are not current methods, the analyst shall notify the Technical Leader of the appropriate analysis methods and settings to utilize. No additional case information or information regarding the interpretation issue shall be given. The Technical Leader may request limited additional information only if it is needed for analysis.

2.8.21.13.5   In a single source sample, if an error is discovered that did not affect the STRmix deconvolution of the sample (ex. a pull-up peak was not deleted, but it was modeled as drop-in), a second deconvolution does not need to be performed.

The error shall be documented in the case record with an explanation why it did not affect the interpretation.

2.8.21.13.5.1 An error of this type requires a new deconvolution in a mixed sample.

2.8.21.14   CODIS Eligibility and Entry

2.8.21.14.1   Casework profiles shall be carefully evaluated for CODIS eligibility in accordance with National DNA Index System (NDIS) Operational Procedures "DNA Records Accepted at NDIS" and the flowchart "A Guide to Determining What is Allowable in the Forensic Index at NDIS," both available on the CODISPortal. Refer to the CODIS Test Method for details.

2.8.21.14.2   Each year, every qualified analyst shall complete the 'Annual Review of DNA Data Accepted as NDIS'. The timing of this review is specified by NDIS and completion is documented within the CODIS learning management system. Appropriate extensions, if needed, will be maintained by the CODIS Administrator.

2.8.21.14.3   If a casework profile is determined to be ineligible after CODIS entry and upload, that profile shall be deleted by a CODIS administrator. The case analyst shall issue a supplemental report stating that the profile was removed.

2.8.21.15   Use of STRmix™ to aid in deconvolution of mixtures for CODIS entry

2.8.21.15.1   Additional STRmix™ deconvolutions utilizing conditioning may be performed to aid in the deconvolution of a mixture for improved CODIS entry.

2.8.21.15.1.1   If a mixture includes support for inclusion of a suspect reference profile (an LR has been calculated in support for H_1), the mixture may be deconvoluted conditioning on that reference profile to provide improved genotype weightings of an additional contributor to be entered into the database.

2.8.21.15.1.1.1   The case documentation of this deconvolution shall be clearly marked as for CODIS purposes only.

2.8.21.15.1.1.2   The analyst shall ensure all alleles to be entered in CODIS are present in the original mixture.

2.8.21.15.1.1.3   The original deconvolution should generally be utilized for comparison if an evidentiary reference standard is submitted at a later date.

2.8.21.15.1.2   If a mixture indicates that an additional single source unknown profile in the case cannot be excluded as a contributor, that unknown profile may be utilized to improve genotype weightings of an additional contributor for CODIS entry.

2.8.21.15.1.2.1   The single source unknown profile may be reanalyzed in GeneMapper™ ID-X utilizing reference profile settings.

2.8.21.15.1.2.2   The mixture may be deconvoluted conditioning on the reanalyzed single source unknown profile.

2.8.21.15.1.2.3   The analyst shall ensure all alleles to be entered in CODIS are present in the original mixture.

2.8.21.15.1.2.4   The case documentation of the reanalysis and the deconvolution shall be clearly marked as for CODIS purposes only.

2.8.21.15.1.2.5   The original deconvolution with no conditioning on the unknown profile shall be utilized for all LR calculations.

2.8.21.16   **Use of the STRlite Excel program**

2.8.21.16.1   The ISP STRlite Excel program shall be utilized to provide a summary of GeneMapper™ ID-X allele calls for reference and unknown samples; as well as information collated from the STRmix™ deconvolution, database search and LR from Previous reports for sample analysis utilized in the interpretation of the profile.

2.8.21.16.1.1   STRlite v2.0 (or most current version as approved by the Technical Leader) shall be utilized.

2.8.21.16.1.2   Worksheets, as appropriate, from the STRlite program shall be maintained in the case record. These worksheets may include Pre-STRmix, Deconvolution, Likelihood Ratios, Standards, CODIS, 1P Combo, and 2P Combo. An electronic copy of STRlite containing results shall not be maintained on the DNA server to avoid duplication of items between LIMS and the server.

2.8.21.16.1.2.1   The STRlite Deconvolution page or a 1P/2P Combo worksheet shall be maintained for all deconvolutions that represent a valid interpretation of the profile.

2.8.21.16.1.2.1.1   If an analyst error is made during a deconvolution run (e.g., An unintended number of contributors was entered, a pull-up peak was not deleted etc.), a deconvolution worksheet does not need to be generated for that run, but the STRmix™ deconvolution files shall be maintained electronically and the case record shall document why the run was not used.

2.8.21.16.1.2.2   On STRlite Deconvolution pages, Combo worksheets, and CODIS pages, the use of "{ }" at a locus with one or two alleles clarifies that the 99% cumulative weighting contains more than one possible genotype (mixture or partial) and would be searched with moderate stringency in CODIS.

2.8.21.16.1.2.3   For single source profiles, the analyst shall manually add results for Amelogenin to the CODIS worksheet.

2.8.21.16.1.2.4   For mixed profiles, the analyst may manually add results for Amelogenin to the CODIS worksheet if they feel the data supports the determination.

2.8.21.16.1.3   The electronic STRmix™ reports saved to the case folder on the DNA server shall be referenced for further troubleshooting as needed.

2.9   Records:

2.9.1   The appropriate worksheets as contained in the Worksheet Manual, STRlite, or the equivalent workbooks shall be used to record all procedures.

2.9.2   All data sheets, notes, photographs, and other information generated from the laboratory examination shall be kept in the case record.

2.9.3   The technical review of the case record shall be recorded on the technical review worksheet.

2.9.4   Electronic records shall be retained as indicated in Appendix 9.

2.10 **Interpretations of Results:**

2.10.1 Interpretation guidelines are located within the **Procedures Section 2.8.**

2.11 **References:**

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2.11.5   Promega Corporation. PowerPlex® Fusion 6C System Technical Manual. Part No. TMD045. Most current issue.

2.11.6   Promega Corporation. PowerPlex® Matrix Standards, 3100/3130 Technical Bulletin. Part No. TBD022. 1/06 or most current issue.

2.11.7   Promega Corporation. Maxwell® 16 Instrument Operation Manual. Part No. TM295. 2008 or most current issue

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2.11.19 Gill, P. et al. Considerations from the European DNA profiling group (EDNAP) concerning STR nomenclature. *Forensic Science International* (1997) 87, 185-192.

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2.11.22 Institute of Environmental Science and Research Limited. STRmix™ V2.7 Operation Manual. 11 October 2019 or most current version.

2.11.23 Institute of Environmental Science and Research Limited. STRmix™ V2.7 Installation Manual. 4 September 2018 or most current version.

2.11.24 Institute of Environmental Science and Research Limited. STRmix™ V2.7 User's Manual. 8 September 2019 or most current version.

2.11.25 Hill, C.R. et al. U.S. population data for 29 autosomal STR loci. *Forensic Sci. Int. Genet*. (2013) 7, e82-e83.

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INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

3. **Forensic Relationship Comparison Methods:**

3.1. Scope:

3.1.1. Forensic relationship comparisons may be requested as part of some criminal investigation cases. Types of relationship comparisons that can be performed include paternity, maternity, reverse paternity, sibling relationship (sibship), etc. These comparisons may establish potential relationships among individuals and/or aid in the identification of human remains and missing persons. Samples for forensic relationship testing shall be processed in accordance with the test methods outlined in the DNA methods section of this document.

3.2. Precautions/Limitations:

3.2.1. Cases submitted for relationship comparisons should be evaluated by a member of the relationship comparison team before any testing is performed. It may be necessary to outsource some types of cases to a vendor laboratory for analysis. Cases shall be refused when they do not meet the requirements of forensic relationship comparison analysis.

    3.2.1.1. Cousin comparisons should not be performed unless it is part of a pedigree.

3.3. Related Information:

3.3.1. See Relationship Comparison Statistical Reference Sheet (Appendix 10)

3.4. Instruments:

3.4.1. See DNA Test Methods Section 2.4

3.5. Reagents/Materials:

3.5.1. See DNA Test Methods Section 2.5

3.6. Hazards/Safety:

3.6.1. See DNA Test Methods Section 2.6

3.7. Reference Materials/Controls/Calibration Checks:

3.7.1. See DNA Test Methods Section 2.7

3.8. Procedures/Instructions:

3.8.1. See DNA Test Methods Section 2.8

3.9. Interpretation of Relationship Testing Results:

3.9.1. Possible Outcomes of Relationship Comparisons

    3.9.1.1. The genetic profiles from standards and samples are compared to evaluate relationship. The following conclusions may be reported:

        3.9.1.2. For relationship cases using DBLR™:

- [Limited/moderate/strong/very strong] support for the alleged relationship: CRI > 1.

- Uninformative: CRI = 1

- Limited support for unrelatedness: $2 \leq 1/CRI < 100$

- Unrelated: $1/CRI \geq 100$ or visual exclusion for parentage cases

3.9.2. Inconsistent Profiles in Parentage Relationship Cases

3.9.2.1. If the alleged father's profile is inconsistent with the child's obligate paternal alleles at more than three loci with PowerPlex® Fusion 6C, then no relationship/exclusion shall be concluded.

3.9.2.2. If the alleged father's profile is inconsistent with the child's obligate paternal alleles at fewer than four loci with PowerPlex® Fusion 6C, then other alternatives may be evaluated for these inconsistencies. Other alternatives may include possible mutation, null allele, first degree relative, or true exclusion. If it is determined that a possible mutation or a null/silent allele could account for the inconsistencies, then the mutation or null allele shall be included into the statistical calculations.

3.9.2.2.1 A mutation is typically observed to be a 1-step (repeat unit) smaller or larger from the parental allele (family study estimates ~90%). 2-step (~9%) or 3-step (~1%) mutations are possible, but very uncommon.

3.9.2.2.2 A null/silent allele may be observed when both the child and either parent appear homozygous at a locus without a concordant allele and the overall peak height of the homozygous allele is approximately half expectation based on the peak heights of other loci in the same dye channel. Additional indicators may include a possible peak below AT that would be concordant between the parent and child and/or requiring more than a 1-step mutation to explain the discordant locus. The Technical Leader shall be consulted for incorporation of a null allele into the statistical calculation.

3.9.3. Mixtures

3.9.3.1. With product of conception/fetal samples, it may be necessary to have a standard from the mother. Using the biological mother's standard, a deconvolution may be performed to determine the genotype of the child.

3.9.3.2. Secondary standards, including personal effects believed to belong to a missing person, may be suitable for comparison. Additional standards may be requested to aid in interpretation or to be conditioned upon, if appropriate.

3.10. Guidelines for Additional Testing

3.10.1. Y-STR testing for kinship comparisons should only be done in the following circumstances:

3.10.1.1. Kinship analysis involving male individuals proposed to be paternally related that demonstrate limited support for H1 or H2 or an uninformative result shall have additional Y-STR analysis performed to provide further information regarding a potential relationship.

3.10.1.2. Kinship analysis involving male individuals proposed to be paternally related that demonstrate moderate support for H1 may have additional Y-STR analysis performed to provide further information regarding a potential relationship.

3.10.1.3. If a mutation is suspected at one or two Y-STR loci with PowerPlex® Fusion 6C, additional genetic testing should be performed to evaluate additional Y-STR loci.

3.10.2. If there are additional Y-STR discordances that do not support an alleged relationship (such as full siblings), the analyst shall attempt to confirm with the contributing agency if there is potential for a different relationship (such as half-siblings). If the agency is unable to confirm a different relationship is possible, the results shall be reported as no support for the originally designated relationship and additional family standards should be requested. If confirmation of a potential relationship in which Y-STR profiles are not expected to match is obtained, the new relationship statistic should be performed along with a report statement indicating that the Y-STR results do not demonstrate support for the original relationship.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

3.11. Rules for Relationship Statistical Calculations using DBLR™:

3.11.1. Calculations shall be performed using DBLR™ version 1.2.24 or currently approved version.

3.11.2. Statistical calculations shall be performed and reported in forensic relationship comparison cases where Results/Opinions/Interpretations are being given in regards to relationship.

3.11.3. A minimum of eight autosomal loci with no indication of allelic drop-out are required for comparison and statistical calculations. For STRmix deconvolutions, the 99% contributor designation shall be utilized for this purpose.

3.11.4. Statistical calculations shall not be performed in parentage cases with more than three genetic inconsistencies.

3.11.5. Linkage shall be enabled for all calculations, regardless of the type of relationship under evaluation.

3.11.6. The addition of Y-STR loci (DYS391, DYS576, and DYS570) into the PowerPlex® Fusion 6C system is intended to assist with interpretation of the number of male contributors in a sample and with samples demonstrating a possible null Y allele at Amelogenin. Therefore, statistical evaluation of the Y-STR loci shall not be performed or incorporated in the Combined Relationship Index unless a mutation is suspected.

3.11.7. See Relationship Comparison Statistical Reference Sheet (Appendix 10) for a list of formulas for calculations.

3.11.8. If more than one standard/sample is available for comparison, the most representative standard/sample or the standard/sample where the most obligate paternal alleles can be determined shall be used for statistical calculations.

3.11.9. Relationship statistical calculations utilize the race of the alleged individual. The calculations shall be performed with the appropriate NIST 2017 population data using the racial information provided by the contributing agency. If racial information is unknown or reported as multi-racial, calculations shall be performed using the Combined population data.

3.11.10. Manual calculations performed by an analyst shall be verified by a qualified technical reviewer and/or a qualified unit supervisor.

3.11.11. Mutations shall only be enabled in DBLR™ when necessary.

    3.11.11.1. The appropriate Maternal or Paternal mutation rate file shall be selected in DBLR™ based on the suspected mutation source.

    3.11.11.2. In the event that a mutation is suspected at one Y-STR locus other than DYS570, DYS576, DYS612, and DYF387S1, a mutation factor of 0.001 shall be used as a haplotype Relationship Index and incorporated through manual calculation into the Combined Relationship Index derived from DBLR™. DYS570, DYS576, DYS612, and DYF387S1 are generally regarded as rapidly mutating Y-STR loci. The mutation factor for a single discordant Y-STR locus defined as rapidly mutating will be incorporated as a haplotype Relationship Index and incorporated manually into the CRI by multiplying by 0.01. TL consultation is required for two suspected Y-STR mutations.

3.11.12. Mutation rates for historical loci (PowerPlex® 16 HS and SE33) have been reported in the AABB Annual Report Summary. For any locus without a published mutation rate, the rate

shall be calculated by averaging the available tetranucleotide STR mutation rates (excluding SE33).

3.11.13. For parentage calculations utilizing a mother's standard and an alleged father's standard in which a null allele from the alleged father is suspected, the Paternity Index (PI) for a locus demonstrating a possible null allele should be manually calculated using the formula described in Appendix 10. The frequency of a silent allele(s) is 0.001 based on the typical observation of a null allele in modern STR typing kit loci is less than 1 in 1,000. The Technical Leader shall be consulted if a null allele is suspected.

3.11.14. The F_{ST} Coancestry Coefficient shall be enabled for all calculations. An F_{ST} of 0.01 shall be utilized for all populations.

3.12. Relationship Calculations using DBLR™

3.12.1. To start a new DBLR™ relationship calculation, from the DBLR™ main menu select **Kinship**.

3.12.2. In the H1 screen, construct the pedigree.

3.12.2.1. The symbols used should reflect the sex of the corresponding sample, with squares for males and circles for females.

3.12.2.2. A template may be chosen from the drop-down menu.

3.12.2.3. A custom pedigree may be constructed by right clicking on the symbol for Individual A and adding parents or offspring as required.

3.12.2.4. The default sample names may be edited to reflect case information by highlighting the assigned letter and typing the desired label.

3.12.3. Add H2 by clicking the "**+**" symbol next to H1.

3.12.3.1. Add the unknown, unrelated individual by choosing the male or female symbol from the toolbar, as appropriate.

3.12.4. Verify that all symbols and labels are visible and adjust spacing as needed.

3.12.4.1. The pedigrees can be rearranged by clicking and dragging symbols.

3.12.5. Choose **Numeric Kinship** in the bottom, right corner.

3.12.6. Add the profiles to the box marked **Load Samples**. This may be done by dragging and dropping the appropriate files, or by selecting **Browse** and navigating the file tree.

3.12.6.1. If a Genotypes Table file is used, ensure that all samples in the project have unique names, including controls such as Ladders.

3.12.6.2. DBLR™ does not have an option to omit a locus; therefore, a STRmix™ deconvolution shall be performed if it is necessary to omit loci.

3.12.7. Under **Configuration**, add the appropriate Analysis Name. At a minimum, the name shall include the case number, followed by an underscore and the DBLR run number, increasing sequentially (e.g., 23L1234_DBLR1, 23L1234_DBLR2). If additional characters are added to the "Analysis Name" designation, those characters shall be added after the specified naming convention.

3.12.8. Under **Parameters**, choose the appropriate Allele Frequencies file. Verify that "Model mutations" is unchecked unless a mutation is suspected. Verify that "Model Fst" is checked and set to 0.01. Verify that "Model linkage" is checked and the appropriate Linkage map is selected.

3.12.9. Select **Confirm Inputs** once all profiles are loaded and settings are verified.

3.12.10. Link all loaded profiles to the appropriate positions in H1 and H2. This may be accomplished by dragging the sample name to the appropriate position in the pedigree, or by clicking **Add Relationship** and selecting the person in the pedigree and its corresponding sample from the drop-down menus.

    3.12.10.1. Verify that the correct Contributor Position is selected if a STRmix deconvolution is used as a sample input.

    3.12.10.2. When F_{ST} is enabled, a warning message will show that there are unexplained contributor positions until all loaded samples are linked to the pedigree. This must be resolved before a likelihood ratio can be calculated.

3.12.11. Click **Compute Likelihood Ratios** and review the data.

3.12.12. Save a copy of the Kinship Report in the casefile and the run folder on the DNA server (See Appendix 9).

    3.12.12.1. The report will list linked loci together whenever Linkage is enabled. However, the model is only applied when needed based on the pedigree and sample profiles.

3.13. Report Writing for Relationship Comparisons Using DBLR™ General Guidelines:

3.13.1. The Combined Paternity Index shall be reported in the Results/Opinions/Interpretations area of the Certificate of Analysis when applicable as detailed in the report wording section (Appendix 7).

    3.13.1.1. No statistical calculations shall be reported for exclusions determined from greater than three inconsistencies in parentage comparisons.

3.13.2. All reports associated with a Paternity/Relationship Comparison Request shall include an Appendix (Appendix 7). Each profile development statement in the report shall contain the citation for the appropriate kit utilized statement in the Appendix.

3.13.3. The items used for comparison will be reported in the format listed in the report wording section (Appendix 7).

3.13.4. Alternate report wording may be used depending on the type of relationship and calculation performed.

3.13.5. Terminology of Parentage, Paternity, Maternity, Relationship, Kinship, and Sibling Relationship (Sibship) may be used interchangeably where applicable.

3.13.6. The Combined Paternity Index shall be reported to two significant figures.

    3.13.6.1. Combined Relationship/Paternity Index values supporting the relationship (H1) shall be rounded down.

    3.13.6.2. Combined Relationship/Paternity Index values supporting exclusion (H2) shall be inverted (1/CRI) and rounded up.

    3.13.6.3. Combined Relationship/Paternity Index values less than 2 shall be rounded down to 1.0 (uninformative).

3.13.7. Wording of Relationship Comparisons for Results/Opinions/Interpretations are located in Appendix 7.

3.14. Support for Investigative Genetic Genealogy

3.14.1. Y-STR testing may be utilized to aid in paternal lineage identification.

3.14.2. At a minimum, 12 Y-STR loci are required to draw a conclusion on paternal lineage.

3.14.3. Interpretations will be based on the number of loci evaluated and mismatches observed.

   3.14.3.1. When at least 20 Y-STR loci are available for comparison the following interpretation guidelines shall apply:

   - 0-1 mismatches: support the donors of the Y-STR profiles are related

   - 2 mismatches: support the donors of the Y-STR profiles are probably related

   - 3 mismatches: support the donors of the Y-STR profiles are possibly related

   - 4+ mismatches: support the donors of the Y-STR profiles are not related within four generational steps

   3.14.3.2. When 12-19 Y-STR loci are available for comparison the following interpretation guidelines shall apply:

   - 0-1 mismatches: support the donors of the Y-STR profiles are probably related

   - 2 mismatches: support the donors of the Y-STR profiles are possibly related

   - 3+ mismatches: support the donors of the Y-STR profiles are not related within four generational steps

3.14.4. Interpretations of Y-STR testing for paternal lineage identification shall be issued to the interested parties via a memo as shown in Appendix 7.

3.15. Definitions:

3.15.1. **Random Man Not Excluded (RMNE)** – The frequency of selecting a random man from the population that could not be excluded as the biological father**.**

3.15.2. **Probability of Exclusion/Power of Exclusion (PE)** – The probability of excluding a random man from the population as being the biological father; is dependent on the genotypes of the child and mother and the race of the alleged father.

3.15.3. **Parentage Index (PI)/Relationship Index (RI)** – A likelihood ratio based on two different conditional probabilities; PI/RI is calculated for each locus in a system.

3.15.4. **Combined Parentage Index (CPI)/Combined Relationship Index (CRI)** – CPI/CRI is the product of all PIs/RIs for the loci in a system; a measure of the strength of the genetic evidence.

3.15.5. **Probability of Parentage (W)** – Also known as the probability of relatedness. It is the measure of the strength of the genetic evidence and the non-genetic evidence that an individual is a biological relative as compared to an unrelated, random individual in the same population.

3.15.6. **Prior Probability (Pr) –** The strength of the non-genetic evidence that the alleged individual is a biological relative.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

4. Y-STR Test Methods:

4.1. **Scope**: Y-STR analysis may be performed in situations where male DNA needs to be differentiated from female DNA. DNA analysis performed prior to Y-STR analysis shall be in accordance with the test methods outlined in the DNA Methods section of this document.

4.2. **Precautions/Limitations:** Samples that may be suitable for Y-STR analysis should be evaluated by a member of the Y-STR team to determine the most appropriate sample(s) to analyze in a case. The following should be considered:

4.2.1. Cases from sexual assault/misconduct should be considered for analysis.

4.2.1.1. Due to the limitations of the technology, property crimes generally will not be considered for analysis.

4.2.2. Cases that have not provided meaningful autosomal results should be considered for analysis.

4.2.2.1. Y-STR analysis should be considered if a sample from a more intimate/informative area qualifies for Y-STRs if autosomal results are only from a non-intimate/uninformative area.

4.2.2.2. Due to the high probability of extraneous transfer and non-probative DNA results, clothing and hand swabs generally will not be considered for analysis if the victim and suspect live in the same household. Exceptions require supervisor notification.

4.3. **Related Information:**

4.3.1. Worksheet Manual

4.3.2. [Appendix 13](#) PowerPlex® Y23 Stutter Table

4.4. **Instruments:**

4.4.1. See DNA Test Methods Section 2.4

4.5. **Reagents/Materials:**

4.5.1. See DNA Test Methods Section 2.5

4.6. **Hazards/Safety:**

4.6.1. See DNA Test Methods Section 2.6

4.7. **Reference Materials/Controls/Calibration Checks:**

4.7.1. See DNA Test Methods Section 2.7

4.8. **Procedures/Instructions:**

4.8.1. **See DNA Test Methods Section 2.8 for extraction procedures**

4.8.1.1. Performing Y-STR analysis before or after autosomal STR analysis will be evaluated on a case by case basis.

4.8.1.2. Y-STR analysis may be performed on extracts previously used for autosomal STR testing or may require re-extraction of a sample.

4.8.1.3. The male quantification value using Quantifiler® Trio shall be used for amplification of the sample.

4.8.2. **PowerPlex® Y23 (Applied Biosystems® 3500 Genetic Analyzer)**

4.8.2.1. **Introduction**

4.8.2.1.1. Short tandem repeat (STR) markers on the Y chromosome (Y-STR) have qualities that are distinct from autosomal markers and are useful for human male identification. The Promega PowerPlex® Y23 System allows co-amplification of twenty-three loci (DYS576, DYS389 I, DYS448, DYS389 II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385 a/b, DYS456, and YGATAH4) in a single reaction. Separation and detection occur using capillary electrophoresis with overlapping loci visualized simultaneously by using PCR primers labeled with four different fluorescent tags (Fluorescein, JOE, TMR-ET, and CRX-ET).

4.8.2.1.2. The Applied Biosystems® 3500 Genetic Analyzer utilizes electrokinetic injection of DNA molecules into polymer-filled capillaries which separates the DNA fragments by size. The fluorescent tag labeled primers incorporated into the PowerPlex® Y23 amplification products are responsive to the frequency of the 20 mW solid state laser. Upon excitation, the fluorophores are raised to a higher energy level. When the fluorophores return to their normal energy level, a fluorescent signal is emitted. This signal is then detected by a camera within the 3500 capillary electrophoresis instrument which converts the signal to a computer image where it is visualized in an electropherogram as a peak.

4.8.2.1.3. The data produced by the Applied Biosystems® 3500 Genetic Analyzer is analyzed with GeneMapper™ ID-X Software which results in peaks labeled with their allele designation. The Internal Lane Standard (WEN ILS 500) is injected with each sample and it contains 21 fragments of 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500 bases in length. Each fragment is labeled with WEN dye and can be detected separately (as a fifth color) in the presence of PowerPlex® Y23 amplified material. The Internal Lane Standard determines the base pair size of the fragments in the sample and the software compares the sizes to an allelic ladder to determine the allele designation.

4.8.2.1.4. The PowerPlex® Y23 locus-specific and allelic ladder information is available at *www.promega.com/protocols/* in the PowerPlex® Y23 System Technical Manual TMD035.

4.8.2.1.5. All PowerPlex® Y23 components should be stored at -10°C to -30°C upon receipt in the appropriate laboratory locations. The PowerPlex® Y23 Allelic Ladder is packaged separately for shipping and should be stored in the post-amplification kit. Once thawed, the pre-amplification kit may be stored at 2°C to 10°C for up to one year or refrozen at -10°C to 30°C. The post-amplification kit, including the WEN Internal Lane Standard (WEN ILS) 500 Y23, should be stored at 2°C to 10°C after thawing. Do not refreeze.

4.8.2.1.6. The PowerPlex® Y23 Primer Pair Mix, PowerPlex® Y23 Allelic Ladder Mix, and the WEN ILS 500 Y23 shall be protected from excessive exposure to light.

4.8.2.1.7. The preparation of the amplification reactions shall be performed in the PCR amplification set-up area.

4.8.2.2. **PowerPlex® Y23 Amplification Set-Up**

4.8.2.2.1. Thaw the Amplification Grade Water, PowerPlex® Y23 5X Master Mix, and PowerPlex® Y23 10X Primer Pair Mix. Centrifuge briefly to bring contents to the bottom, then vortex to mix. Do not centrifuge after vortexing as this may cause the primers/dNTPs to be concentrated at the bottom of the tube. Once thawed, the PowerPlex® Y23 kit can be stored at 2-10ºC for up to one year or refrozen.

4.8.2.2.2. Determine the number of samples to be amplified, including controls. Add 2 to 4 reactions to this number to compensate for the loss that occurs during reagent transfers.

4.8.2.2.3. Place one 0.2 ml PCR reaction tube for each sample into a rack and label appropriately. A 96-well plate may be used.

4.8.2.2.3.1. If utilizing a 96-well plate for amplification, well locations where allelic ladder will be utilized for capillary electrophoresis shall be left empty during amplification.

4.8.2.2.4. 0.1 ng to 0.5 ng of template DNA is recommended. Amplification of greater than 0.5 ng of template DNA should not be used due to off-scale peak heights and may result in increased artifacts and interfere with interpretation. Targeting less than 0.5 ng of template DNA should be used only with samples whose concentration falls between 0.006 ng/µl and 0.029 ng/µl. Such samples shall be interpreted with caution. Samples with concentrations greater than 0.029 ng/µl should target 0.5 ng but shall not exceed 1.0 ng for amplification.

4.8.2.2.5. For samples that will amplify 5 µl of template DNA, use the **PowerPlex Y23® Amplification Worksheet – 5 µl Input** to calculate the required amount of each component of the PCR master mix. Multiply the volume per reaction by the total number of reactions to obtain the final volume.

PCR Master Mix Component	Volume per Reaction (µl)
Amplification Grade or Nuclease Free H_2O	12.5
PowerPlex® Y23 5X Master Mix	5
PowerPlex® Y23 10X Primer Pair Mix	2.5
Total Volume	20

4.8.2.2.6. Add the calculated volume of each component to a 1.5 ml tube. Mix gently.

4.8.2.2.7. Add 20 µl of PCR master mix to each sample tube or well.

4.8.2.2.8. Pipette 5 µl of sample, reagent blank, positive amplification control, or negative amplification control into the respective tube or well.

4.8.2.2.8.1. For the positive amplification control, dilute the 2800M or other approved positive DNA standard supplied with the PowerPlex® Y23 kit to 0.02 ng/µl to 0.1 ng/µl and record the final concentration on the amplification worksheet. A positive control shall be included in each thermal cycler. The diluted positive control should be stored at 2-10°C.

4.8.2.2.8.2. Amplification grade H_2O or NF H_2O shall be used as a negative amplification control included with each thermal cycler as the last sample in the batch.

4.8.2.2.9. For samples requiring more than 5 µl of sample volume, use the **PPY23® Amplification Worksheet – Variable Input** to calculate the required amount of water and template DNA for individual samples. Prepare the PCR Master Mix without water. Multiply the volume per reaction by the total number of reactions to obtain the final volume.

PCR Master Mix Component	Volume per Reaction (µl)
PowerPlex® Y23 5X Master Mix	5
PowerPlex® Y23 10X Primer Pair Mix	2.5
Total Volume	7.5

4.8.2.2.10. Add the calculated volume of each component to a 1.5 ml tube. Mix gently.

4.8.2.2.11. Add 7.5 µl of PCR master mix to each sample tube or well.

4.8.2.2.12. Add the calculated volume of Amplification Grade H_2O or NF H_2O to each sample tube or well.

4.8.2.2.13. Pipette the appropriate amount of each sample, reagent blank, positive amplification control into the respective tube or well containing master mix.

4.8.2.2.14. For samples amplified in a 96-well plate, seal the plate with adhesive foil. Press the foil well using a roller tool.

4.8.2.3. **PowerPlex® Y23 Amplification**

4.8.2.3.1. Place the tubes or 96-well plate in a Veriti™ thermal cycler.

4.8.2.3.2. Select and run the thermal cycling protocol below:

4.8.2.3.3. **Y23 Veriti™ Thermal Cycler Protocol**
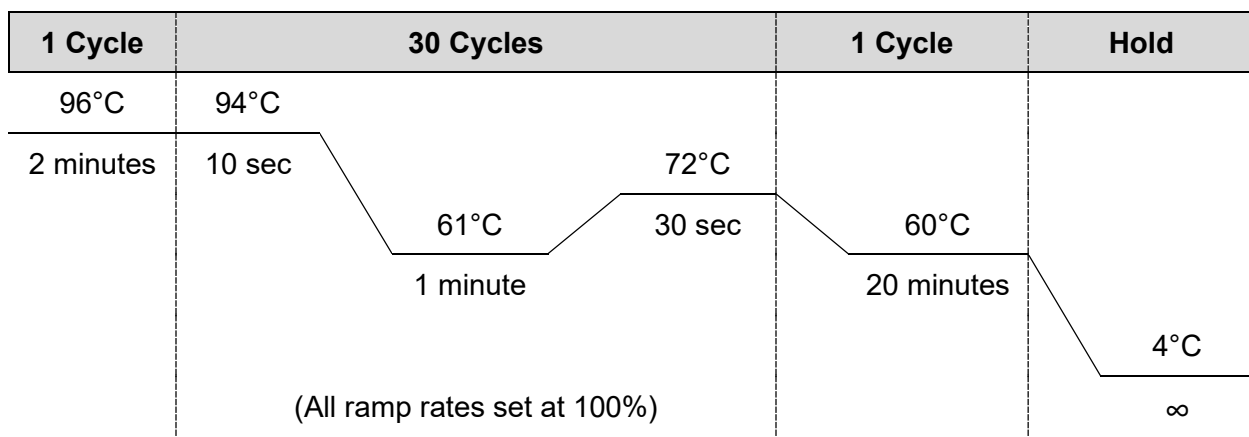
1 Cycle	30 Cycles			1 Cycle	Hold
96°C	94°C		72°C		
2 minutes	10 sec	61°C	30 sec	60°C	
		1 minute		20 minutes	4°C
	(All ramp rates set at 100%)				∞

4.8.2.3.3.1. Select 25 µl for the volume in the amp tubes.

4.8.2.3.4.　　Remove samples after the amplification process is completed and proceed with capillary electrophoresis or store the amplified samples at -10°C to -30°C. Alternatively, amplified samples may be stored at 2-10°C if they are to be used within 2 days.

    4.8.2.3.4.1. Any remaining amplified products shall be discarded after the case is administratively reviewed.

4.8.2.4. **PowerPlex® Y23 Direct Amplification Sampling for Standards**

4.8.2.4.1. (Oral swabs only) Place the head of one swab in a deep well plate or a 1.5ml microcentrifuge tube.

4.8.2.4.2. Using a fresh pipette tip every time, place 1ml SwabSolution™ into each well or tube containing a swab head.

4.8.2.4.3. For the reagent blank, place solution only into an empty well or tube.

4.8.2.4.4. Incubate at 70° C for approximately 1 hour.

4.8.2.4.5. Store the samples in the refrigerator at 2-10°C. Samples may be stored for up to three weeks prior to direct amplification.

4.8.2.5. **PowerPlex® Y23 Direct Amplification Set-Up for Standards**

4.8.2.5.1. Thaw the Amplification Grade Water, PowerPlex® Y23 5X Master Mix, and PowerPlex® Y23 10X Primer Pair Mix. Centrifuge briefly to bring contents to the bottom, then vortex to mix. Do not centrifuge after vortexing as this may cause the primers/dNTPs to be concentrated at the bottom of the tube. Once thawed, the PowerPlex® Y23 kit can be stored at 2-10°C for up to one year or refrozen.

4.8.2.5.2. Determine the number of samples to be amplified, including controls. Add 2 to 4 reactions to this number to compensate for the loss that occurs during reagent transfers.

4.8.2.5.3. Place one 0.2 ml PCR reaction tube for each sample into a rack and label appropriately. A 96-well plate may be used.

    4.8.2.5.3.1. If utilizing a 96-well plate for amplification, well locations where allelic ladder will be utilized for capillary electrophoresis shall be left empty during amplification.

4.8.2.5.4. For samples and controls, use the **PowerPlex® Y23 Direct Amplification Worksheet** to calculate the required amount of each component of the PCR master mix. Multiply the volume per reaction by the total number of reactions to obtain the final volume .

PCR Master Mix Component	Volume per Reaction (µl)
Amplification Grade or Nuclease Free H_2O	15.5
PowerPlex® Y23 5X Master Mix	5
PowerPlex® Y23 10X Primer Pair Mix	2.5
Total Volume	23

4.8.2.5.5. Add the calculated volume of each component to a 1.5 ml tube. Mix gently.

4.8.2.5.6. Add 23 µl of PCR master mix to each sample tube/well.

4.8.2.5.7. Pipette 2 µl of sample, reagent blank, positive amplification control, or negative amplification control into the respective tube or well containing Master Mix.

4.8.2.5.7.1. For the positive amplification control, dilute the 2800M or other approved positive DNA standard supplied with the PowerPlex® Y23 kit to 1 ng/µl to 1.5 ng/µl and record the final concentration on the amplification worksheet. A positive control shall be included in each thermal cycler. The diluted positive control should be stored at 2-10°C.

4.8.2.5.7.2. Amplification grade H_2O or NF H_2O shall be used as a negative amplification control included with each thermal cycler as the last sample in the batch.

4.8.2.5.8. For samples amplified in a 96-well plate, seal the plate with adhesive foil. Press the foil well using a roller tool.

4.8.2.6. **PowerPlex® Y23 Direct Amplification for Standards**

4.8.2.6.1. Place the tubes or 96-well plate in a Veriti™ thermal cycler.

4.8.2.6.2. Select and run the thermal cycling protocol below:

4.8.2.6.3. **Y23direct Veriti™ Thermal Cycler Protocol**

1 Cycle	25 Cycles		1 Cycle	Hold
96°C	94°C			
2 minutes	10 sec	72°C	60°C	
	61°C	30 sec	20 minutes	
	1 minute			4°C
	(All ramp rates set at 100%)			∞
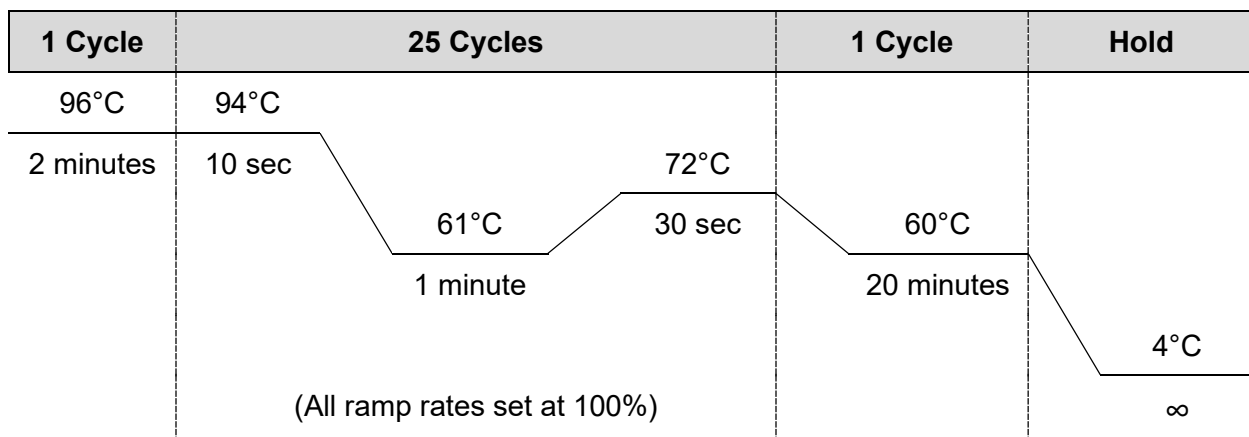
4.8.2.6.3.1. Select 25 µl for the volume in the amp tubes.

4.8.2.6.4. Remove samples after the amplification process is completed and proceed with capillary electrophoresis or store the amplified samples at -10°C to -30°C. Alternatively, amplified samples may be stored at 2-10°C if they are to be used within 2 days.

4.8.2.6.4.1. Any remaining amplified products shall be discarded after the case is administratively reviewed.

4.8.3. **Applied Biosystems® 3500 Genetic Analyzer - Data Collection Software version 2.0 – Instrument Set-up. See Appendix 11**

4.8.4. **Applied Biosystems® 3500 Genetic Analyzer - Data Collection Software version 2.0 - PowerPlex® Y23 Electrophoresis**

4.8.4.1. **Sample Preparation**

4.8.4.1.1. **Note**: The quality of formamide is critical for the successful detection of a DNA profile. Deionized formamide shall be used that has a conductivity of less than

100µS/cm, such as Hi-Di™ Formamide. The formamide shall be frozen in aliquots at -20°C and the remainder of each aliquot shall be discarded after it is thawed. Multiple freeze-thaw cycles or long-term storage at 4°C may cause breakdown of the formamide which can create ions that compete with DNA during injection. This will cause lower peak heights and decreased sensitivity.

4.8.4.1.2. **Caution:** Formamide is an irritant and teratogen; therefore, universal precautions and a fume hood shall be utilized when manually working with formamide to avoid inhalation and contact with the skin.

4.8.4.1.3. Thaw the ILS (WEN ILS 500 Y23), the allelic ladder, and an aliquot of Hi-Di™ Formamide. Once thawed initially, the ILS shall not be refrozen and should be stored at 2-10°C. When all components are thawed, centrifuge briefly then vortex to mix. Do not centrifuge the ILS after vortexing.

4.8.4.1.4. Determine the number of samples to be injected, including controls and allelic ladders. Add 2 to 4 reactions to this number to compensate for the loss that occurs during reagent transfers.

4.8.4.1.5. Prepare a loading cocktail by combining 0.5-1.0 µl ILS with 9.5-9.0 µl Hi-Di™ Formamide for a total volume of 10 µl.

4.8.4.1.5.1. The volume of ILS used in the loading cocktail may be adjusted between 0.5 to 1.0 µl per sample to optimize size standard peaks. The amount of formamide shall be adjusted so that the total amount of loading cocktail for each well is 10 µl.

4.8.4.1.6. Vortex to mix.

4.8.4.1.7. Pipette 10 µl of the formamide/ILS mixture into each well. Add formamide or formamide/ILS mixture into empty wells to complete an injection set of eight. Every well in which an injection is occurring must contain liquid.

4.8.4.1.8. Add 1 µl of amplified sample or 1 µl of the allelic ladder to each well. It is recommended that one allelic ladder is injected within each set of 3 injections on the 8 capillary Applied Biosystems® 3500 instruments to ensure that a usable ladder injection occurs. At least one allelic ladder is required within each run folder.

4.8.4.1.8.1. If amplified sample is in a 96-well pate, seal the amplification plate with a new aluminum foil seal for storage.

4.8.4.1.9. Cover the wells with the plate septa and briefly spin down to remove air bubbles.

4.8.4.1.10. Denature the samples at 95°C for ~3 minutes, then immediately chill on crushed ice or a frozen plate block for ~3 minutes. Denature the samples just prior to loading the instrument. Avoid denaturing the samples for longer than 3 minutes as extended heat denaturing can lead to the appearance of artifacts.

4.8.4.2. **Creating a Plate Record**

4.8.4.2.1. Open the 3500 Data Collection Software. The Dashboard screen will launch. Ensure that the **Consumable Information** and **Maintenance Notifications** are acceptable. The oven temperature should be set to 60ºC.

4.8.4.2.2. Select **Start Pre-Heat**. This should be done at least 30 minutes prior to the first injection.

Dashboard:
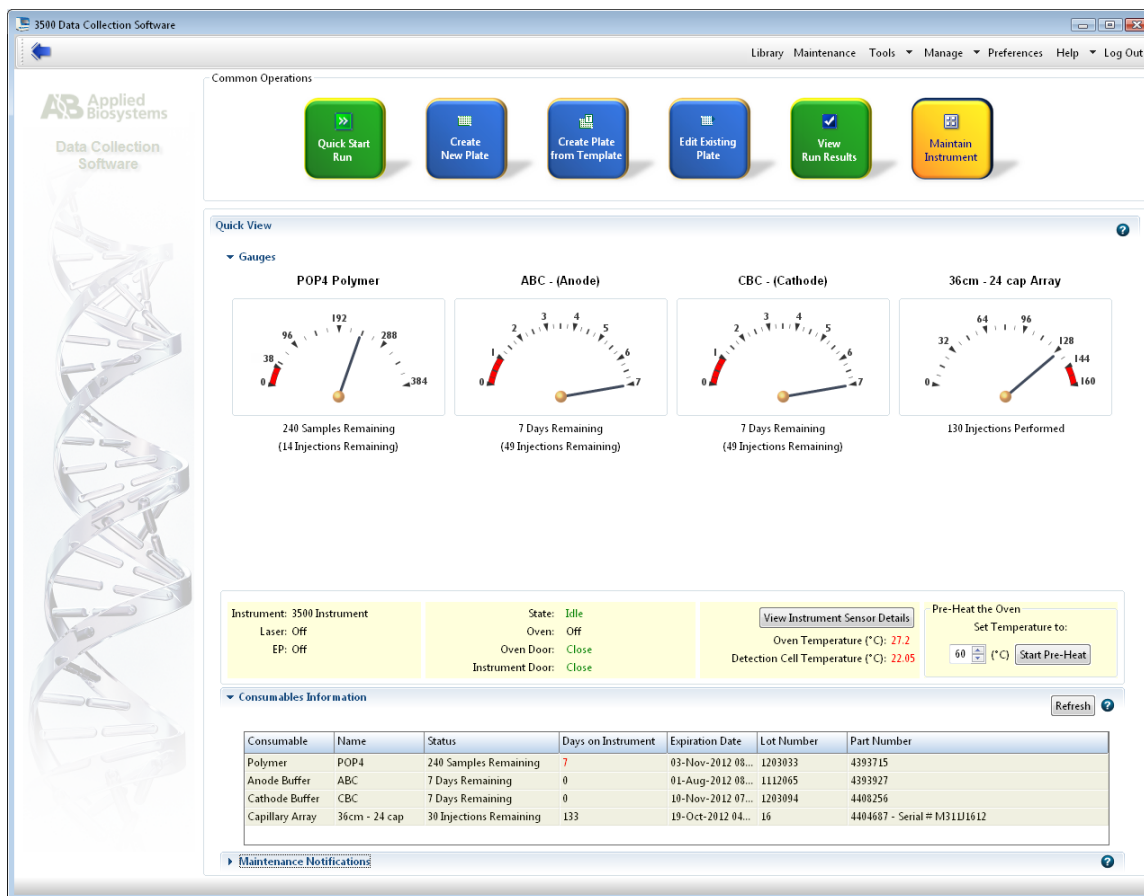
4.8.4.2.3. Select **Create New Plate**. Alternatively, **Create Plate from Template** may be used.

4.8.4.2.3.1. Assign the plate name with the laboratory case number, the date and the injection number of the plate (ex. 00A1234_01Jan13_01).

4.8.4.2.3.1.1. If the plate is re-injected the "01" number shall increase sequentially (00A1234_01Jan13_02).

4.8.4.2.3.1.2. If the plate is re-injected on a different day, the original date should still be used from when the plate was prepared.

4.8.4.2.3.1.3. If a second preparation of a plate is made on the same date as the first, it shall be designated with a "-2" after the date (00A1234_01Jan13-2_01).

4.8.4.2.4. Select "96" for Number of Wells.

4.8.4.2.5. Select "HID" for Plate Type.

4.8.4.2.6. Select "36" for Capillary Length.

4.8.4.2.7. Select "POP4" for Polymer.

Create A Plate:
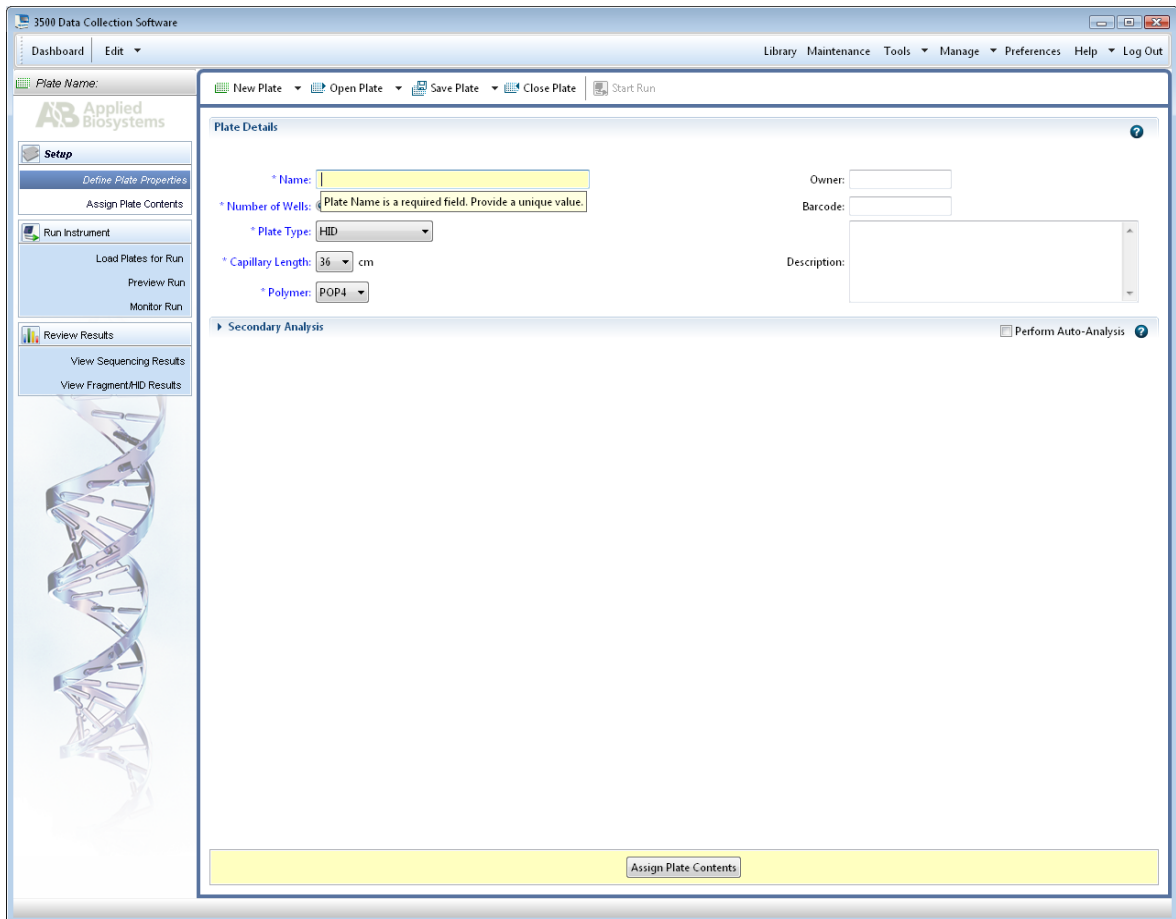
4.8.4.2.8. Click on "Assign Plate Contents".

4.8.4.2.9. Enter sample information or import a plate record.

4.8.4.2.9.1. The sample name shall include the sample item/subitem as well as the laboratory case number (ex. 1A1_00A1234) if more than one case is included on the plate. If only one case is present on the plate, then only the item/subitem number is required.

4.8.4.2.9.2. In order to aid in GeneMapper™ ID-X sample analysis, it is recommended to place a "z" in front of the sample name for reference standards (ex. z1A1_00A1234") here or in GeneMapper™ ID-X under sample name.

4.8.4.2.10. Under **Assays** click "Add from Library" and select "PPY23_1.2kV15s". Click "Add to Plate" then "Close".

4.8.4.2.11. Under **File Name Convention**, click "Add from Library" and select "PPY23_FNC". Click "Add to Plate" then "Close".

4.8.4.2.12. Under **Results Groups**, click "Add from Library" and select "PPY_ResultsGroup". Click "Add to Plate" then "Close".

Assign Plate Contents:
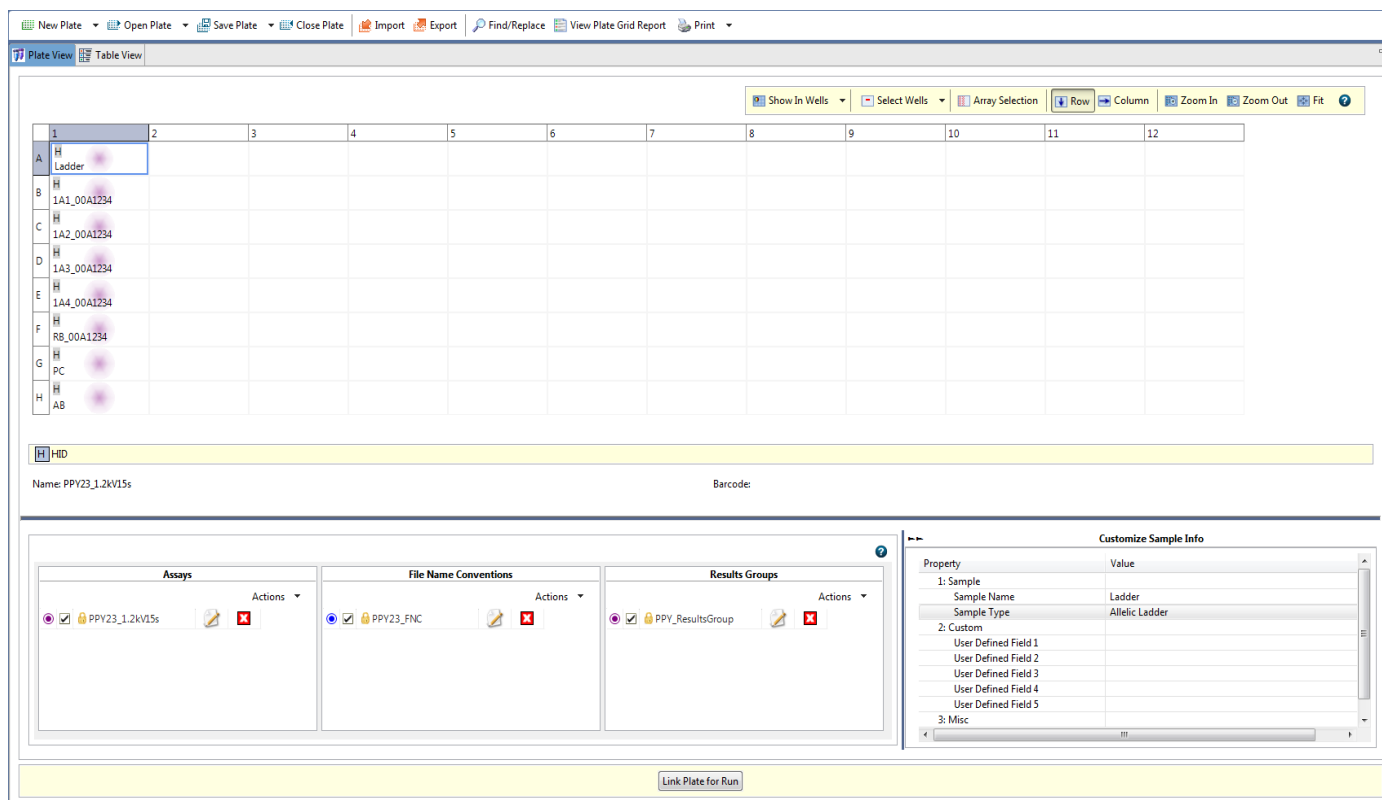
4.8.4.2.13. On the bottom-right side of the screen expand the **Customize Sample Info** window so it appears as shown above.

4.8.4.2.13.1. Use the drop-down box to select a sample type for each sample (e.g., Ladder, Sample, Positive Control and Negative Control). For imported plates, this will be done automatically.

4.8.4.2.13.2. Minimize the "Customize Sample Info" window.

4.8.4.2.14. Highlight the sample wells and then select the boxes in the **Assays, File Name Conventions,** and **Results Groups** that pertain to those samples.

4.8.4.2.15. Place plate on instrument in position A. Select "Link Plate for Run". Click "OK". The instrument automatically senses the plate and puts the information in the Plate A field. Click "OK".

4.8.4.2.16. To add a second plate, follow steps 4.8.4.2.3 through 4.8.4.2.15. Place the plate in position B. Select the "Link Plate for Run" and click "OK". Assign the information to the Plate B field. Click "OK".

4.8.4.3. **Start a Plate Run**

4.8.4.3.1. A unique run name is automatically generated by the instrument for each plate.

4.8.4.3.2. To re-organize injections, click "Create Injection List" and use the arrows to re-order the injections.

4.8.4.3.3. Click "Start Run".

4.8.4.3.4. After the run is complete, click "Unlink Plate" and remove it from the instrument. The plate may be discarded or labeled with the same title as the plate record and stored at -10°C to -30°C. The plate shall be destroyed after the case has been administratively reviewed.

4.8.4.3.4.1. If the CE plate requires re-injection after frozen storage, thaw and centrifuge to remove any air bubbles. Denature the plate at 95°C for 3 minutes, then immediately chill on crushed ice or frozen plate block for ~3 minutes prior to loading on the 3500 Genetic Analyzer.

4.8.4.3.5. The CE Plate Record Worksheet shall be maintained with each case record.

4.8.5. **See Casework Test Methods Appendix 4 for GeneMapper™ ID-X Version 1.6 Software User Accounts and Security Setting Procedures. GeneMapper™ ID-X Version 1.6 Software - PowerPlex® Y23 Software Settings. See Appendix 12**

4.8.7. **GeneMapper™ ID-X Version 1.6 Software - PowerPlex® Y23 Data Analysis**

4.8.7.1. **Processing Sample Data**

4.8.7.1.1. Import the sample files from a single run folder by "Edit", then selecting "Add Samples to Project".

4.8.7.1.2. In the "Add Samples to Project" screen, navigate to the run folder that contains the sample files. If the entire run folder is to be imported, click on the folder to highlight it; then click the "Add to List" button at the bottom of the window. If the run folder was shared between multiple cases, expand the folder to view the samples. Highlight the appropriate samples, ensuring that the allelic ladder and all the desired samples are selected. Once all the samples are selected click the "Add to List" button at the bottom of the window.

4.8.7.1.2.1. A run folder shall not be created manually by manipulating sample files.

4.8.7.1.3. Ensure that the necessary files are now located in the "Samples to Add" window by double-clicking on the folder in the right pane, then click "Add".

4.8.7.1.4. After the samples have been added to the project, first briefly scan the raw data to ensure that a bad injection did not occur. To check the raw data, first expand the project folder in the left navigation pane, then click on a sample file, then click on the "Raw Data" tab in the right GeneMapper™ ID-X window. To return to the "Samples" window, click on the project folder at the top of the left navigation pane.

4.8.7.1.5. The GeneMapper™ ID-X project shall contain at least one allelic ladder from each run folder included in the project for proper genotyping. Multiple allelic ladders within a run folder will be averaged by the software to calculate the allelic bins. If a ladder injection is of low quality, delete the ladder or change the sample type from "Allelic Ladder" to "Sample" to remove it from consideration in calculating the bins.

4.8.7.1.6. Ensure that the **Table Setting** at the top of the screen is set to "PPY23".

4.8.7.1.7. In the **Sample Type** column, verify that the correct sample types are displayed (e.g., Allelic Ladder, Sample, Positive Control, and Negative Control). Should a sample type need altered, use the drop-down menu to select the appropriate type.

4.8.7.1.8. In the **Analysis Method** column, select the appropriate method from the drop-down menu: "PowerPlex Y23 WEN". Click the column header cell to highlight the entire column, then select "Edit", then "Fill Down" (or the shortcut Ctrl + D).

4.8.7.1.9. In the **Panel** column, select the appropriate panel file: Open the "PowerPlexY23_Panels_IDX_v2.0" folder then select "PowerPlexY23_IDX_v2.0". Click the column header cell to highlight the entire column, then select "Edit", then "Fill Down" (or the shortcut Ctrl + D).

4.8.7.1.10. In the **Size Standard** column, select the appropriate ILS from the drop-down menu: "WEN ILS 500". Click the column header cell to highlight the entire column, then select "Edit", then "Fill Down" (or the shortcut Ctrl + D).

4.8.7.1.11. The Analysis Method, Size Standard, and Panel can be set as defaults when a GeneMapper™ ID-X project is opened. Select **File → Project Options**. Under the "Add Samples" tab select the above settings as the default in the drop-down menus for Analysis Method, Size Standard and Panel. Click "OK".

4.8.7.1.12. Select the green **Analyze** arrow button to start the data analysis. At the Project name prompt, save the project. The project name shall contain the date the 3500 plate was prepared so that it matches the run folder name. See the DNA Server Instructions Appendix 9 for additional clarification. The case number is recommended but not required. Select the "ISP Casework Security Group".

4.8.7.2. **Evaluating Sample Data**

4.8.7.2.1. The **Sizing Quality** for unknown samples and allelic ladders shall be at least 0.75 for a sample to pass. The **Sizing Quality** for reference samples, negative controls, and positive controls shall be at least 0.25 for a sample to pass. All appropriate ILS peaks shall be present and labeled correctly.

4.8.7.2.2. **Allelic Ladders** and **Samples**

4.8.7.2.2.1. Highlight all sample rows containing ladders or samples and click "View", then "Display Plots". In the Samples Plot screen, change the "Plot Setting" drop-down box to "PPY23". Magnify the area from approximately 50 bp to 500 bp ensuring that all ILS peaks are visible in the retained electropherogram.

4.8.7.2.2.2. Verify the correct allele calls are made for each ladder peak and evaluate all allele calls as described in 4.8.10.

4.8.7.2.2.3. Retain the electropherograms.

4.8.7.2.3. **Negative Controls** (e.g., amplification blanks and reagent blanks).

4.8.7.2.3.1. Highlight all sample rows containing negative controls and click "View", then "Display Plots". In the Samples Plot screen, change the "Plot Setting" drop-down box to "PPY23".

4.8.7.2.3.2. Ensure that no peaks above analytical threshold are present.

4.8.7.2.3.3. Retain the electropherograms ensuring that the primer peak is visible.

4.8.7.2.4. After all analysis is complete, save the 3500 Data Collection Run Folder and associated GeneMapper™ ID-X projects under each associated laboratory case number and request folder located in the analysts' folders on the server. Projects should be deleted monthly from the "GeneMapper Manager" to maintain database space.

4.8.7.2.4.1. When exporting the GeneMapper™ ID-X project, ensure that the "Export with analysis settings" box is checked.

4.8.8. General Rules for PowerPlex® Y23 Analysis On The Applied Biosystems® 3500 Genetic Analyzer

4.8.8.1. It is required that at least one allelic ladder is present within a run folder. However, it is recommended that an allelic ladder is run within each set of 3 injections on the 8 capillary 3500 (one ladder per 24 capillaries). External environmental factors during a plate run can cause a shift in the migration of DNA fragments within the capillary which may cause a small difference in the base pair length determined for an allele. If an allelic ladder sample was not run within a reasonable time as the sample, this may cause an allele to be called off ladder.

4.8.8.2. The placement of unknown samples in the 96-well plate should be done so that the orientation allows for the injection of unknown samples prior to the injection of any reference samples for that case.

4.8.8.3. Peak heights of analyzed samples should not exceed 28,000 RFU. This level of peak height is approaching saturation and will lead to the appearance of artifacts. Use of data with more than two peaks >28,000 RFU may be allowed with the approval of a supervisor and notification to the Technical Leader documented in the case record.

4.8.8.4. If multiple amplifications are analyzed for a sample, the analyst shall use the amplification which he or she determines sufficiently represents the sample based on peak heights, artifacts, and noise levels. Analysts shall interpret data that most clearly represents the sample and try to achieve better resolution if a sample has peak heights that are too high (resulting in artifacts, increased instrument noise, and pull-up) or too low (resulting in allelic drop out and loss of data).

4.8.8.5. Only the data used for interpretation needs to be published in the case record. However, other runs shall be noted in the case record and all data shall be saved under each associated laboratory case number and request folder located in the analysts' folders on the server.

4.8.9. Archiving Applied Biosystems® 3500 and GeneMapper™ ID-X Projects

4.8.9.1. The 3500 run folders containing all sample files for a case as well as the GeneMapper™ ID-X project file shall be saved under each associated laboratory case number and request folder located in the analysts' folders on the server and deleted from the hard drive of the instrument and/or analysis computer. The data stored on the server shall be routinely backed up to ensure security of data.

4.8.9.2. All electropherograms used in interpretation as well as the plate record shall be placed in the case record.

4.8.10. Interpretation Guidelines for PowerPlex® Y23

4.8.10.1.1. An analyst is required to visually confirm all allelic ladders used for allele designation.

4.8.10.1.2.   The internally validated analytical threshold for data interpretation is 100 RFU for all dye channels.

4.8.10.1.2.1.   Peaks below 100 RFU shall not be interpreted or marked on the STR summary sheet but may be considered when determining suitability for interpretation and/or number of contributors for a sample.

4.8.10.1.3.   The stochastic threshold for data interpretation of the DYS385 a/b locus is 225 RFU. There is no stochastic threshold for the remaining loci.

4.8.10.1.3.1.   Peaks between 100 and 225 RFU at the DYS385 a/b locus shall be reported with a bracket (e.g., [13]) on the Y-STR summary sheet.

4.8.10.1.3.2.   Interpretation of peaks with RFU from 100 to 225 RFU at the DYS385 a/b locus is a qualitative assessment. If the DYS385 a/b locus demonstrates a single allele that is above 100 RFU and below 225 RFU, true homozygosity cannot be determined due to possible stochastic effects.

4.8.10.1.4.   An initial interpretation of the data shall be made to distinguish non-allelic artifact peaks (e.g., pull-up, spikes, dye blobs, etc.) from true allelic data.

4.8.10.1.4.1.   Artifacts have been observed and documented utilizing the PowerPlex® Y23 amplification kit. Any artifact peaks called as alleles by the GeneMapper™ ID-X software shall be deleted or renamed on the electropherogram as an artifact. Many DNA-independent and DNA-dependent artifacts have been observed. A complete list of artifacts can be found in the PowerPlex® Y23 Technical Manual.

4.8.10.1.4.2.   Pull-up or bleed through peaks can occur if signal intensity of sample or ILS peaks is too high or if a new spectral calibration needs to be run. Any pull-up peaks called as alleles by the GeneMapper™ ID-X software shall be labeled on the electropherogram as pull-up or artifact. The sample should be re-run if a pull-up peak interferes with the analyst's ability to evaluate the profile based on their experience and training.

4.8.10.1.4.3.   Spikes are peaks that generally appear in all colors and are sharper than regular peaks; however, they can occur predominantly in one color. Spikes are a natural consequence of capillary electrophoresis and can be caused by dust present in the system as well as urea crystals in the system. It is essential that the instrumentation be maintained and cleaned regularly to minimize the appearance of spikes. All spikes called as alleles by the GeneMapper™ ID-X software shall be clearly labeled as spikes or artifacts on the electropherogram. A sample should be re-injected when a spike interferes with the analyst's ability to evaluate the profile based on their experience and training.

4.8.10.1.5. Stutter peaks are artifacts of the amplification process. The peak heights of stutter peaks will be less intense than that of the major peak. Stutter peaks will typically be observed one repeat unit shorter than the parent peak (e.g., n-4 position at a tetranucleotide repeat locus) but may also occur two repeat units less (e.g., n-8 at a tetranucleotide repeat locus) or one repeat unit more (e.g., n+4 at a tetranucleotide repeat locus). Additionally, n-2 and n+2 artifact peaks have been observed. Any peaks determined to be stutter that were not filtered out by the GeneMapper™ ID-X software shall be labeled at stutter or artifact on the

electropherogram. Mean and mean + 3SD stutter values can be found in [Appendix 13](#).

4.8.10.1.6. Rare variants have been described in the literature. The causes of these rare variants are microvariants or chromosomal mutations (duplication or deletion).

4.8.10.1.6.1.   Microvariants represent a partial repeat for a given allele. These peaks have a similar intensity to other allelic peaks but will not line up with the allelic ladder and will fall outside established bins.

4.8.10.1.6.1.1. Any allele peak that is not present in the allelic ladder and does not have an associated "bin" in the GeneMapper™ ID-X analysis software is labeled "OL" (Off-Ladder) and shall be renamed by the analyst on the electropherogram.

4.8.10.1.6.1.2. Alleles which are located between two alleles on the ladder shall be described as the short repeat followed by the number of base pairs it is larger (e.g., 0.1, 0.2, 0.3, 0.4, or 0.5). Therefore, if a peak is 1 base pair larger than the 5 allele it shall be designated as 5.1. A microvariant 3 base pairs larger than an allele on the ladder for a trinucleotide, 4 base pairs larger for a tetranucleotide, 5 base pairs larger for a pentanucleotide, or 6 base pairs larger for a hexanucleotide may be designated with the full repeat number (A peak 4 base pairs larger than the 5 allele could be designated a 6; 5 base pairs larger a 6.1).

4.8.10.1.6.1.3. Alleles which are located outside the range of the ladder or bin set but within the designated marker range of the locus shall be evaluated to determine the appropriate repeat number that should be assigned to the allele. The allele may also be described as "<" or ">" the largest or smallest allele for that locus with a set of ( ) placed around the off ladder allele. For example, if a peak is located above the largest allele for the DYS19 locus, it would be designated as "(>19)".

4.8.10.1.6.1.4. Alleles which are labeled "OMR" (Outside Marker Range) by the GeneMapper™ ID-X analysis software shall be evaluated to determine which locus the allele likely belongs to. If the analyst can reasonably determine which locus the allele belongs to, the allele can be assigned to that locus in GeneMapper™ ID-X and the allele call can be made. If the analyst cannot reasonably determine which locus the allele belongs to, both loci surrounding the loci shall be marked inconclusive.

4.8.10.1.6.2.   A duplication occurs a when a DNA sequence on the Y-chromosome is duplicated and results in more than one allele at a locus. Duplications have been documented with the PowerPlex® Y23 amplification kit. Instances of multiple duplications within a sample have been documented as well. Most duplications have a one repeat difference; however, two, three, and four repeat unit differences has been observed.

4.8.10.1.6.2.1. If a locus other than DYS385 a/b demonstrates more than one allele, evaluate the sample to distinguish between a mixture and a duplication.

4.8.10.1.6.2.1.1. Determine the number of loci containing more than one peak and consider the position on the Y-chromosome if multiple loci have two peaks.

4.8.10.1.6.2.1.2. Determine if the repeat spread is >1 repeat unit.

4.8.10.1.6.2.1.3. Evaluate the peak height ratio of the two peaks present at the locus.

4.8.10.1.6.2.1.4. Examine DYS385 a/b for the presence of >2 peaks.

4.8.10.1.6.2.2. If a two-peak pattern is observed for a single-source unknown sample and is believed to be a duplication, the profile should be confirmed by concordance to at least one additional sample contained within the case and/or re-amplification and documented notification to the Technical Leader.

4.8.10.1.6.3. Null alleles can occur due to a deletion of a portion of the Y chromosome or a mutation to the primer binding site for a particular locus. The result is a lack of a peak at a single locus or multiple loci.

4.8.10.1.6.3.1. The lack of an allele at a locus that is believed to be a null allele may be distinguished from an undetected allele due to allelic drop out by evaluating the overall quality of the sample, including peak heights and degradation. The location of the missing allele may also be considered. Documented notification of the null allele to the Technical Leader is required.

4.8.10.1.6.3.1.1. A deletion has been reported at the DYS448 locus. Samples with this deletion will show two peaks (duplication) in DYS576 and a null allele in DYS448.

4.8.10.2. **Evaluation of Controls**

4.8.10.2.1. Failed controls require documented notification to the Technical Leader with appropriate documentation in the case record.

4.8.10.2.2. The appearance of pull-up or known artifact peaks does not render the following controls inconclusive.

4.8.10.2.3. **Reagent Blank**:

4.8.10.2.3.1. The purpose of the reagent blank is to determine if the reagents used to extract the associated samples were contaminated by male DNA. Therefore, no signal should be detected in this sample well other than the internal lane standard. If a signal is detected in the reagent blank, all results of samples associated with that reagent blank shall be considered inconclusive.

4.8.10.2.3.2. A reagent blank with peaks below analytical threshold shall not prevent associated samples from being interpreted.

4.8.10.2.3.3. A reagent blank with peaks at or above the analytical threshold shall be considered a failed negative control.

4.8.10.2.4. **Positive Control**:

4.8.10.2.4.1. The 2800M or other appropriate male DNA standard supplied with the PowerPlex® Y23 kit is used as a positive control to demonstrate that the kit is performing properly. A positive control is considered a failed positive control if expected alleles are not detected or if extra alleles are detected.

Y-STR Locus	2800M
DYS576	18

DYS389 I	14
DYS448	19
DYS389 II	31
DYS19	14
DYS391	10
DYS481	22
DYS549	13
DYS533	12
DYS438	9
DYS437	14
DYS570	17
DYS635	21
DYS390	24
DYS439	12
DYS392	13
DYS643	10
DYS393	13
DYS458	17
DYS385 a/b	13,16
DYS456	17
Y-GATA-H4	11

4.8.10.2.5. **Amplification Blank**:

4.8.10.2.5.1. The purpose of the amplification blank is to determine if male DNA contaminated the samples at the amplification step. Because no template DNA was placed in the reaction, the sample well should be blank except for the internal lane standard peaks.

4.8.10.2.5.2. An amplification blank with peaks below analytical threshold shall not prevent associated samples from being interpreted.

4.8.10.2.5.3. An amplification blank with at or above analytical threshold shall be considered a failed negative control.

4.8.10.2.6. **Interpreting, Comparing and Reporting DNA Results Associated with Failed Controls and Contamination Events**

4.8.10.2.6.1. The Technical Leader shall be notified if a positive or negative control fails and/or if sample contamination occurs. Sample contamination is defined as additional peaks present in an unknown or reference sample that can be conclusively identified to not have originated in the sample (e.g., case to case contamination or a mixture in a reference sample).

4.8.10.2.6.2. Assessment of the integrity of the associated DNA results shall be done in consultation with the Technical Leader. This shall include the possible cause and effect of the failed control or contamination. Additionally, an assessment of the risk associated with moving forward with interpretation shall be documented, as well as the risks associated with retesting (e.g., unnecessary consumption of evidence).

4.8.10.2.6.2.1.   If retesting is performed, the reasons for doing so shall be documented.

4.8.10.2.6.2.2.   If results are determined to be suitable for interpretation without retesting, the appropriate interpretations, comparisons, and statistical results and a description of the failed control or contamination event shall be reported per the Report Writing Guidelines.

4.8.10.2.6.2.3.   If results are determined to be not suitable for interpretation and retesting cannot be performed, the results shall be reported as not suitable for comparison per the Report Writing Guidelines.

4.8.10.2.6.3.   Documentation in the case record shall include at a minimum: the forensic sample, reference or control test results that failed or was contaminated, the likely or known cause of the failed control or contamination, the likely or known source of the contamination, the impact on the integrity of the DNA results, and the determination if the test result is suitable or unsuitable for interpretation.

4.8.10.3.   **Y-STR DNA Profile Interpretation**

4.8.10.3.1.   All interpretation decisions shall be documented prior to comparison of reference profiles. If an interpretation error is discovered after comparison to a reference profile, the Technical Leader shall be consulted to determine the appropriate action. The error and Technical Leader consultation shall be documented in the case record.

4.8.10.3.2.   If available, a reference profile from a male victim who is not paternally related to the person of interest may be assumed for single source and major Y-STR profiles and no statistical analysis is required.

4.8.10.3.3.   During interpretation, the analyst and technical reviewer shall each compare all single source and major Y-STR profiles to available staff profiles to ensure that samples have not been contaminated. All instances of profiles consistent with a staff member shall be reported to the Technical Leader. If the match is determined to be a suspected contamination event, a root cause analysis shall be performed to determine the possible cause.

4.8.10.3.4.   There are four possible outcomes of Y-STR analysis:

4.8.10.3.4.1. No results: No peaks were detected on the electropherogram.

4.8.10.3.4.2. Inconclusive: Peaks were observed at one or more loci; however no conclusive results can be drawn from them.

4.8.10.3.4.3. Inclusion: The profile obtained from the unknown sample had no discrepant alleles as compared to the profile of the reference standard. Allelic drop-out may occur in low concentrations or mixtures.

4.8.10.3.4.4. Exclusion: The profile obtained from the unknown sample was not the same as the reference sample.

4.8.10.3.5.   **Single Source Profile Interpretation**

4.8.10.3.5.1. Generally, a single source profile should contain no more than one allele at each locus except for DYS385 a/b, which may demonstrate up to two

alleles. A profile where only one locus demonstrates two alleles could be an indication of:

4.8.10.3.5.2. A mixture where only one allele from a minor contributor is detected above analytical threshold.

4.8.10.3.5.2.1. Extraneous DNA.

4.8.10.3.5.2.2. A duplication.

4.8.10.3.5.2.3. In situations 1 and 2, the sample shall be reported with the additional allele wording outlined in the Report Wording Guidelines (Appendix 7). In situation 3, no mention is required in the Certificate of Analysis.

4.8.10.3.5.3. At least twelve loci eligible for statistical calculations are required for interpretation of a single source profile. Profiles demonstrating fewer than twelve loci shall be reported as inconclusive.

4.8.10.3.5.4. If DYS385 a/b demonstrates two alleles, the two peaks should have a peak height ratio of 65%. If the peak height ratio is less than 65%, evaluate the sample quality to determine if the locus should be used in statistical analysis.

4.8.10.3.5.5. If DYS385 a/b demonstrates two alleles in which one or both alleles are above 100 RFU and below 225 RFU, both alleles are interpretable and may be used for statistical analysis.

4.8.10.3.5.6. If only one allele is present at DYS385 a/b and is below 225 RFU, the locus shall not be used for statistical analysis.

4.8.10.3.6. **Mixture Interpretation**

4.8.10.3.6.1. A profile is defined as a mixture between two or more males when two or more loci demonstrate two or more alleles, with the exception of two alleles at DYS385 a/b or a duplication event.

4.8.10.3.6.2. If the profile is determined to be a mixture, each locus should be evaluated and classified as below:

4.8.10.3.6.2.1. Indistinguishable mixture between two or more males

4.8.10.3.6.2.2. Mixture demonstrating a major contributor.

4.8.10.3.6.2.3. Demonstrates the potential for allelic drop-out.

4.8.10.3.6.3. For mixtures of two male individuals, a major single source profile shall be calculated based on the following criteria:

4.8.10.3.6.3.1. If the peak height ratio between the peaks is less than 20%, then the highest peak can be called a major at that locus.

4.8.10.3.6.3.2. If there is only one peak at a particular locus (and a major can be determined at one or more loci with more than one peak), this peak can be called a major. All loci must be considered in making this determination.

4.8.10.3.6.3.3. A mixture threshold of 500 RFU shall be used to interpret single peaks and major peaks at a locus. Peaks below 500 RFU shall not be used for interpretation of the mixture.

4.8.10.3.6.3.4. When evaluating DYS385 a/b, a major consisting of two peaks should have a peak height ratio of 65% and the tallest of the minor peak shall have a peak height ratio of less than 20% of the shortest major peak.

4.8.10.3.6.3.5. If the sample does not meet these criteria at 12 or more loci, a major profile cannot be determined for the sample.

4.8.10.3.6.4. Mixtures of three males which meet the above criteria should be interpreted with caution.

4.8.10.3.6.5. Mixtures of four or more males shall be evaluated based on the following criteria. These samples should be interpreted with caution and only with approval of the Technical Leader.

4.8.10.3.6.5.1. If the peak height ratio between the highest peak and tallest minor peak is less than 15%, the highest peak can be called a major at that locus.

4.8.10.3.6.5.2. A mixture threshold of 1000 RFU shall be used to interpret single peaks and major peaks at a locus.

4.8.10.3.6.5.3. When evaluating DYS385 a/b, a major consisting of two peaks should have a peak height ratio of 65% and the tallest of the minor peaks shall have a peak height ratio of less than 15% of the shortest major peak.

4.8.10.3.6.5.4. If the sample does not meet these criteria at 12 or more loci, a major profile cannot be determined for the sample.

4.8.10.3.6.6. If a major profile cannot be determined in a mixture of two or more males, the mixture is considered indistinguishable. In this case, since no statistical interpretations can be made, the sample shall be reported as inconclusive.

4.8.10.4. Y-STR Statistical Analysis and Database Searching

4.8.10.4.1. A Y-STR profile is treated as a single locus because it is a haplotype.

4.8.10.4.2. Once a comparison between a reference sample(s) and an unknown sample is completed and an inclusion for a single source or major Y-STR profile is reported, the significance of that inclusion shall be estimated. It shall be reported that all paternally related males will share the same Y-STR profile.

4.8.10.4.3. The unknown haplotype developed for the evidence profile shall be searched against the Y-Chromosome Haplotype Reference Database (YHRD), found online at http://yhrd.org.

4.8.10.4.4. The unknown haplotype shall be searched by selecting the PowerPlex® Y23 Kit and the Y17 Dataset. Loci from the PowerPlex® Y23 kit include DYS576, DYS389 I, DYS448, DYS389 II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385 a/b, DYS456, and Y-GATA-H4.

4.8.10.4.4.1. If the Y-STR profile contains a locus with a duplication, the duplication can be entered in the format of "X, Y".

4.8.10.4.4.2. If the Y-STR profile contains a locus with a null allele, a "0" will be entered.

4.8.10.4.5. The counting method with the application of a 95% upper confidence interval using the Clopper and Pearson method along with a theta-corrected match probability are used for statistical calculations.

    4.8.10.4.5.1. The feature National Database (with Subpopulations, 2014 SWGDAM-compliant) shall be added to the statistical report after the Y-STR profile has been searched.

4.8.10.4.6. A statistic result sheet from YHRD shall be generated for every unique Y-STR haplotype/profile searched. The applicable sample ID(s) shall be clearly documented on the sheet.

4.8.10.4.7. A likelihood ratio that incorporates the Theta-corrected Match Probability (without Native American) shall be included in the Certificate of Analysis. The likelihood ratio is the inverse of the match probability. A verbal scale shall accompany the likelihood ratio result in an effort to provide additional explanation of the weight of the evidence.

Likelihood Ratio	Support for Inclusion
1	Uninformative
$2 \leq LR < 100$	Provides limited support
$100 \leq LR < 10,000$	Provides moderate support
$10,000 \leq LR < 1,000,000$	Provides strong support
$1,000,000 \leq LR$	Provides very strong support

4.8.10.4.8. The Certificate of Analysis shall acknowledge the date of the search and the release number of YHRD at the time of the search.

    4.8.10.4.8.1. Statistics from a previous database search will not be recalculated. Exceptions require permission from the Technical Leader.

4.9. Records:

4.9.1. The appropriate worksheets or the equivalent workbooks shall be used to record all procedures.

4.9.2. All data sheets, notes and other information generated from the laboratory examination shall be kept in the case record.

4.9.3. The technical review of the case record shall be recorded on the technical review worksheet.

4.9.4. Electronic records shall be retained as indicated in Appendix 9.

4.10. Interpretation of Results:

4.10.1. Interpretation guidelines are located with the **Procedures Section 4.8.**

4.11. Reporting of Y-STR Analysis:

4.11.1. See Appendix 7 for formatting and Y-STR reporting for specific examples

4.12. References:

4.12.1. Promega Corporation, (2014 or most current version). PowerPlex® Y23 System Technical Manual. Part No. TMD035. Madison, WI.

4.12.2. Butler, J.M. 2011. Y Chromosome DNA Testing. <u>Advanced Topics in Forensic DNA Typing: Methodology</u>. pp 371-403. Elsevier Academic Press. Burlington, MA.

4.12.3. Butler, J.M. 2010. Lineage Markers: Y Chromosome and mtDNA testing. Fundamentals <u>of Forensic DNA Typing.</u> pp 363-374. Academic Press. Burlington, MA.

4.12.4. Butler, J.M. 2005. Y Chromosome DNA Testing. <u>Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers</u>. Second Edition. pp 201-239. Elsevier Academic Press. Burlington, MA.

4.12.5. SWGDAM (2014). SWGDAM Interpretation Guidelines for Y-Chromosome STR Typing by Forensic DNA Testing Laboratories. Available at http://swgdam.org/SWGDAM_YSTR_Guidelines_APPROVED_01092014_v_02112014_FINAL.pdf

4.12.6. Ballantyne, J. and Fatolitis, L. The U.S. Y-STR database. Profiles in DNA. 2008. 11(1): 13 – 14.

4.12.7. Y-Chromosome Haplotype Reference Database (YHRD). http://yhrd.org

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

5. DNA Sample Processing using the Biomek NX^P and Biomek 3000 Automated Workstations:

5.1. Scope: This test method is designed for the direction of laboratory personnel who will operate the Biomek NX^P and Biomek 3000 automation workstations to process DNA samples from extraction/purification through CE (capillary electrophoresis) setup. The Biomek NX^P workstation is capable of extracting up to 87 samples and blanks in two hours, setting up quantification plates, normalizing samples and setting up amplification. The Biomek 3000 workstation then transfers amplified product to a CE plate to be run on a genetic analyzer. This test method may be expanded or altered as techniques and/or new genetic analyses are found applicable and validated.

5.2. Precautions/Limitations:

5.2.1. Due to the hands-off nature of automated processing, it is vital that submitted samples be prepared following procedures determined by the automation validation and that the ordered flow of data throughout the process be maintained.

5.2.2. Customization or modification of sample processing procedures will not be allowed without consultation with the automation team and documented approval of the Technical Leader.

5.3. Related Information:

5.3.1. N/A

5.4. Instruments:

5.4.1. Biomek NX^P Automated Laboratory Workstation— robotic DNA processing system.

5.4.2. Biomek 3000— robotic DNA processing system.

5.5. Reagents/Materials:

5.5.1. Digest/Wash Buffer
5.5.2. Isopropyl Alcohol
5.5.3. Nuclease Free Water (NFH$_2$O)
5.5.4. DTT 1M
5.5.5. Proteinase K (10 mg/ml)
5.5.6. Proteinase K (18 mg/ml)
5.5.7. Ethanol
5.5.8. Sarkosyl 20% w/v
5.5.9. Stain Extraction Buffer for Automation
5.5.10. TRIS/EDTA/NaCl Solution
5.5.11. DNA IQ™ Extraction Kit
    5.5.11.1. Lysis Buffer
    5.5.11.2. Elution Buffer
    5.5.11.3. Magnetic Resin
    5.5.11.4. Wash Buffer

5.6. Hazards/Safety:

5.6.1. All chemicals shall be handled in a safe method as referenced in the specific Safety Data Sheets (SDS).

5.6.2. Universal Precautions shall be observed whenever biological materials are being handled.

5.6.3. Biological waste shall be disposed of in the appropriate waste receptacle.

5.6.4. On the Biomek NX^P, a light curtain prevents interaction with the deck or the robotic arm while a procedure is running.

5.6.5. There is an emergency stop button on the front of the Biomek 3000.

5.7. **Reference Materials/Calibration Checks:**

5.7.1. DNA processing using the automated workstation does not require any additional controls to those used in manual DNA processing.

5.7.2. Annual calibration of the pipetting accuracy will be performed by Beckman Coulter Operational Qualification 3 (OQ3).

5.8. **Procedures/Instructions:**

5.8.1. **General Rules**

5.8.1.1. Batch ID numbers shall be named sequentially "year-run number" (ex. 2011-001).

5.8.1.2. Each case shall contain at least one reagent blank for unknowns when processed on the automated workstation.

5.8.1.3. Each group of reference samples (per analyst) processed on the automated workstation shall contain at least one reagent blank. On the pre-extraction worksheet (submission form), the case number for the grouped reagent blank shall be the first case number in the batch it is associated with.

5.8.1.4. A submitting analyst may request that an assumed reference standard (for any type of case) be included within the same extraction batch as the associated unknowns as long as:

5.8.1.4.1. All pre-robot processing steps were performed separately (separated by time and/or space); AND

5.8.1.4.2. There is no additional evidence within the batch where the reference cannot be assumed. (For example: If sexual assault kit samples are submitted to the robot along with a penile swab from the suspect, the assumed victim standard cannot be included on that robot batch plate.)

5.8.1.5. Reagent blanks shall contain "RB" in the name on the pre-extraction worksheet.

5.8.1.6. At the Indianapolis Lab: Within 24 hours of incubation, analysts shall deliver samples to be processed to the automation room. An e-mail shall be sent to the automation team at DAutomation@ISP.IN.gov with the completed pre-extraction worksheet attached. The worksheet filename shall include the analyst's initials, PE number, and the date the samples were submitted (ex. AB1234_01Jan11). The tube rack containing the samples

shall be labeled to include the same information. See Section 5.9 for more information regarding retention of records for chain of custody.

> 5.8.1.6.1.   Analysts from Evansville, Fort Wayne, and Lowell shall deliver samples to their designated transfer location within 72 hours of incubation. Tubes shall be placed in a rack with a lid, and the lid shall be secured with tape. The covered rack shall be placed in a convenience package and labeled with the worksheet filename. An email with the completed pre-extraction worksheet attached shall be sent on the day of incubation to DAutomation@ISP.IN.gov.

5.8.1.7. To maximize efficiency of each run, the automation team may process samples up to 14 days from their submission date.

5.8.1.8. In addition to the documentation of data as described in the Forensic Biology Section Test Method (DNA Test Methods, section 2), the associated automation documents to be retained in the case record are: emails documenting submission of samples and return of samples to the analyst, the pre-extraction worksheet, the extraction/quantification plate record, the Samples Not Amplified summary and/or the Normalization/Amplification summary sheet as appropriate, and the amplification/capillary electrophoresis plate record if case samples are amplified.

5.8.2. Sample Triage with Automated Sample Processing

5.8.2.1. General Triage Rules

> 5.8.2.1.1.   The Automation Workbook will triage samples based on their quantification data, their letter code(s) as assigned on the "Samples" tab (initially provided by the analyst on the Pre-Extraction Worksheet**)**, and their context within a case as defined by their case number.

> 5.8.2.1.2.   No samples containing less than 0.01 ng/uL small autosomal DNA will be amplified, regardless of sample code or "locked" status.

> 5.8.2.1.3.   Whenever comparisons between code M samples are made (Differential Triage or Priority System Triage), the "better" or "winning" sample is defined as the one with more actual male DNA input into the amplification reaction.

>> 5.8.2.1.3.1.   This calculation prioritizes maximum male DNA input rather than only the F:M ratio or only the Y quantification value.

>> 5.8.2.1.3.2.   The calculation takes into account the normalization of the small autosomal DNA amount (i.e., normalizing a high autosomal value requires dilution, which also lowers the amount of total male DNA input).

>> 5.8.2.1.3.3.   *Example: SampleA has 0.018 ng/uL of autosomal DNA and 0.008 ng/uL of male DNA, for an F:M ratio of 1.25 and 0.12 ng of total male input in a 15 uL reaction. SampleB has 0.072 ng/uL of autosomal DNA and 0.02 ng/uL of male DNA, for an F:M ratio of 2.6 and 0.3 ng of total male input in a 15 uL reaction. SampleB "wins" despite having a higher F:M ratio than sample A.*

5.8.2.1.4.   Case numbers, item numbers, and sample codes will be read as-is. Typos in any of these may result in extra samples being amplified beyond the triage process.

5.8.2.2.   Sample Codes

5.8.2.2.1.   Sample codes are specific letters or combinations of letters entered by the analyst in the "Code" column of the Pre-Extraction Worksheet. These codes determine certain properties of the sample and how it will be processed on the Automation platform. Code letters may be entered in any order and are case-insensitive.

5.8.2.2.2.   **Code T (Tube)**: Code T merely indicates that the extract will be returned to a tube after processing; it does not affect any other analysis. It can be combined with other codes.

5.8.2.2.3.   **Code S (Standard)**: Code S indicates that the sample is a standard, or a reagent blank associated with a standard. Standards are "freed" from their case numbers; they do not undergo case-level triage and are assigned to extraction sets differently. If a sample is marked S, an M or L code will not affect it.

5.8.2.2.3.1.   This allows one reagent blank to be associated with standards from multiple cases.

5.8.2.2.4.   **Code M (Male DNA)**: Code M is the equivalent of "stop if no male," used formerly. It indicates that the sample must be Priority 3 or greater to amplify. The sample will also undergo case-level triage, which includes the Priority system ratings, comparison between differential fractions (Differential Triage), and selection of the five best samples to amplify based on total male DNA input (Priority System Triage). The function of code M is not affected by code T, but code S will cancel the functionality of code M.

5.8.2.2.4.1.   Code M samples will not be amplified if the F:M ratio is greater than 50, regardless of whether they are locked.

5.8.2.2.5.   **Code L (Locked)**: Code L is combined with code M to "lock" a sample for amplification. It is a way of bypassing the case-level triage while still requiring sufficient male DNA for amplification (i.e., Priority 3 or greater, and sufficient autosomal DNA). Code L does not affect any other code except M.

5.8.2.2.5.1.   Locked samples will not affect the triage of other samples within the same case. In other words, locking samples that turn out to be poorer quality will *not* prevent better quality, "unlocked" samples from being amplified. Locked samples will be amplified in addition to better quality unlocked samples.

5.8.2.3.   Differential Triage

5.8.2.3.1.   This section only applies to code M samples designated as sperm or non-sperm fractions as described below.

5.8.2.3.1.1.   Non-sperm fraction samples are designated by the presence of "ns" in the item name. Note that this includes either "ns" or "nsf" (e.g., "1C1_nsf" or "1C1_ns").

5.8.2.3.1.2.   Sperm fraction samples are designated by the presence of "sf" or "sp" and not "ns" in the item name (e.g., "1C1-sf" or "1F2sf_2").

5.8.2.3.2.   A pair of samples with *identical* case numbers and item numbers, except for the "ns/sf" designations, will be detected as a differential set.

5.8.2.3.2.1.   *Example: "99F9999_1Cnsf" and "99F9999_1Csf" will be detected as a differential set.*

5.8.2.3.2.2.   *Example: "99F9999_1C-ns" and "99F9999_1Csf" will not be detected as a differential set because of the dash in the non-sperm sample name.*

5.8.2.3.3.   If a differential set is detected, the two fractions will be compared to each other and only the better fraction (see General Triage Rules, above) will proceed to Priority System Triage.

5.8.2.3.3.1.   The "losing" fraction will not be amplified unless it is locked.

5.8.2.4.   Priority System Triage

5.8.2.4.1.   This section only applies to code M samples.

5.8.2.4.2.   Non-differential samples and the "winning" fractions of differential sets (see Differential Triage section) are sorted by their F:M ratio into Priority 1, 2, or 3 categories (P1, P2, or P3).

5.8.2.4.3.   The best five samples within a case will be amplified.

5.8.2.4.3.1.   These samples are chosen by Priority level (P1>P2>P3) and then by total male DNA amplified (see General Triage Rules, above).

5.8.2.4.3.2.   *Example: A case contains seven samples marked code M. Of these, one is P1, five are P2, and one is P3. The P1 sample is amplified, and the P2 samples are compared to find the best four. The remaining P2 sample and the P3 sample are not amplified.*

5.8.2.4.4.   After the best five samples are chosen, any remaining locked (code L) samples will also be amplified if eligible.

5.8.3. Sample Preparation

5.8.3.1.   Preparation of Samples for Blood, Hair, Saliva, Epithelial Cell Samples And Blood Or Buccal Swab Standards

5.8.3.1.1.   For each sample, combine 350 µl Stain Extraction Buffer for Automation (SEBA) with 10 µl Pro K (18 mg/ml) and 40 µl DTT (1M). *Example: for 16 samples, combine 5,600 µl Incubation Buffer (or SEBA) with 160 µl Pro K and 640 µl DTT.*

5.8.3.1.2.   Place sample at the bottom of a Qiagen® Investigator® Lyse&Spin basket with 2 ml collection tube. Do not use "dolphin-style" or other microcentrifuge tubes. Each group of samples being extracted shall include a reagent blank as the last sample in the batch.

5.8.3.1.3.   To the sample add 400 µl Buffer/Pro K/DTT Master Mix. Vortex and spin briefly in a microcentrifuge or tap the tube to force the cutting into the extraction fluid.

5.8.3.1.3.1.   Do NOT add more than 400 µl of liquid per sample.

5.8.3.1.4.   Incubate the sample at 56°C for at least one hour. Alternatively, samples may be incubated overnight.

5.8.3.1.5.   Spin in a microcentrifuge at 10,000 – 14,000 rcf for at least 1 minute. Remove and discard the basket insert.

5.8.3.1.6.   Close the lid of the sample tube and cut the hinge.

5.8.3.1.7.   Save the extract until ready for automated DNA extraction. Do not refrigerate or freeze sample. Prior to submission to the automation team, processed samples may be left at room temperature (22-25°C) up to 24 hours, if necessary.

5.8.3.2. Preparation of Samples for Differential Extraction

5.8.3.2.1.   For each sample, combine 240 µl Tris/EDTA/NaCl, 6 µl 20% Sarkosyl, 54 µl NFH$_2$O and 3 µl Proteinase K (10 mg/ml). *Example: for 16 samples, combine 3840 µl Tris/EDTA/NaCl, 96 µl 20% Sarkosyl, 864 µl NFH$_2$O, and 48 µl Pro K.*

5.8.3.2.2.   Place the sample in 1.5 ml microcentrifuge tube or 2 ml Qiagen® Investigator® Lyse&Spin collection tube. Qiagen® Investigator® Lyse&Spin baskets shall not be used for differential extractions. Each group of samples being extracted shall include reagent blanks (sperm and non-sperm fraction) as the last samples in the batch.

5.8.3.2.2.1.   For extraction on the Biomek NX^P workstation, samples shall be loaded specifically into Qiagen® Investigator® Lyse&Spin 2 ml collection tubes. Do not use "dolphin-style" or other microcentrifuge tubes. If the differential steps are performed with an alternate 1.5 ml tube the sample extract shall be transferred to a Lyse&Spin 2 ml collection tube prior to submission.

5.8.3.2.3.   To the sample add 300 µl of the Master Mix. Vortex and spin briefly in a microcentrifuge or tap the tube to force the cutting into the extraction fluid.

5.8.3.2.4.   Incubate the sample at 37°C for 2 hours.

5.8.3.2.5.   Spin briefly in a microcentrifuge to force the condensate into the bottom of the tube.

5.8.3.2.6.   Using a wooden applicator stick, remove the cutting and spin in a microcentrifuge for about 5 minutes at approximately 14,000 rcf.

5.8.3.2.7.   Alternately transfer the cutting into a spin basket insert. Place the basket insert into the tube containing the stain extract. Spin in a microcentrifuge at approximately 14,000 rcf for about 5 minutes. Remove and discard the basket insert.

5.8.3.2.8.   While being very careful to not disturb any pelleted material, remove the supernatant to a clean, labeled Qiagen® Investigator® Lyse&Spin 2 ml collection tube. This is the non-sperm fraction. Analysis of the non-sperm fraction resumes at 5.8.2.2.11. The pellet remaining in the tube is the sperm fraction.

5.8.3.2.9.   Wash the sperm fraction by resuspending it in 500 µl digest/wash buffer, vortexing the suspension briefly, and spinning the tube in a microcentrifuge at approximately 14,000 rcf for about 5 minutes. Remove and discard the supernatant fluid, being careful not to disturb the cell pellet.

5.8.3.2.10.   Repeat step 5.8.3.2.9 two times for a total of three washes.

5.8.3.2.11.   Lysis buffer step:

    5.8.3.2.11.1.   For each sample pair (sperm and non-sperm fraction) combine 600 µl of Lysis Buffer with 6 µl of 1M DTT.

    5.8.3.2.11.2.   Add 400 µl of the Lysis Buffer master mix to each sperm fraction sample and 200 µl to each non-sperm fraction sample.

5.8.3.2.12.   Vortex sample and spin down briefly. Cut the hinge of the sample tube.

5.8.3.2.13.   Save until ready for automated DNA extraction. Do not refrigerate or freeze sample. Prior to submission to the automation team, processed samples may be left at room temperature (22−25°C) overnight, if necessary.

5.8.4. Biomek NX^P Preparation

5.8.4.1.   Wipe down the deck with 70% ethanol, 3-6% hydrogen peroxide (with 70% ethanol rinse) or 10% bleach.

5.8.4.2.   Ensure that the workstation trash is not full.

5.8.4.3.   Ensure that the waste carboy is not full.

5.8.4.4.   Ensure that there is enough distilled water in the intake carboy (the valve must be underwater).

5.8.4.5.   Ensure that the software is connected to the instrument and not in Simulate mode:

5.8.4.5.1.   "Instrument" menu → "Hardware Setup".

5.8.4.5.2.   Set Port option to "COM1".

5.8.4.5.3.   Click "Reconnect" and then "Accept".

5.8.4.6.   Run "Home All Axes" under the Instrument menu.

    5.8.4.6.1.   Let the tips flush until there are no bubbles created when the pistons move.

    5.8.4.6.2.   There may be a large bubble stationary at the tops of the syringes; this is normal.

5.8.4.7.   Ensure that ethanol and isopropyl alcohol have been added to the alcohol wash buffer in the DNA IQ™ kit (35 mL of each).

5.8.5. Initial Tube Transfer

5.8.5.1.   Place sample tubes in the tube racks (4x6 with white adapters), arranging the tubes according to the order shown in the Automation Workbook. Do not skip columns or spaces between samples.

5.8.5.2.   Ensure that the sample tube lid hinges have been cut.

5.8.5.3.   Spin down the tube racks (containing the sample tubes) in the centrifuge. Carefully remove the sample tube lids and discard.

    5.8.5.3.1.   CAUTION: Once the lids are removed, the Automation Workbook is the only record of the order and identity of each sample.

5.8.5.4.   Arrange the deck layout as shown in the figure below:

    5.8.5.4.1.   Place sample tube racks on positions P8, P9, P11, and P12, again paying attention to sample order according to the Automation Workbook.

    5.8.5.4.2.   Place a new Promega (Madison, WI) 2.2 ml Square-Well Deep Well Plate (Cat. no. V6781) on position P5. Orient the plate so that the clipped corner is on the bottom left.

    5.8.5.4.3.   Place a new box of Beckman Coulter (Fullerton, CA) Biomek P250 Span-8 Barrier Tips (Cat. no. 379503) on the Rapid-Shuck module (top left).
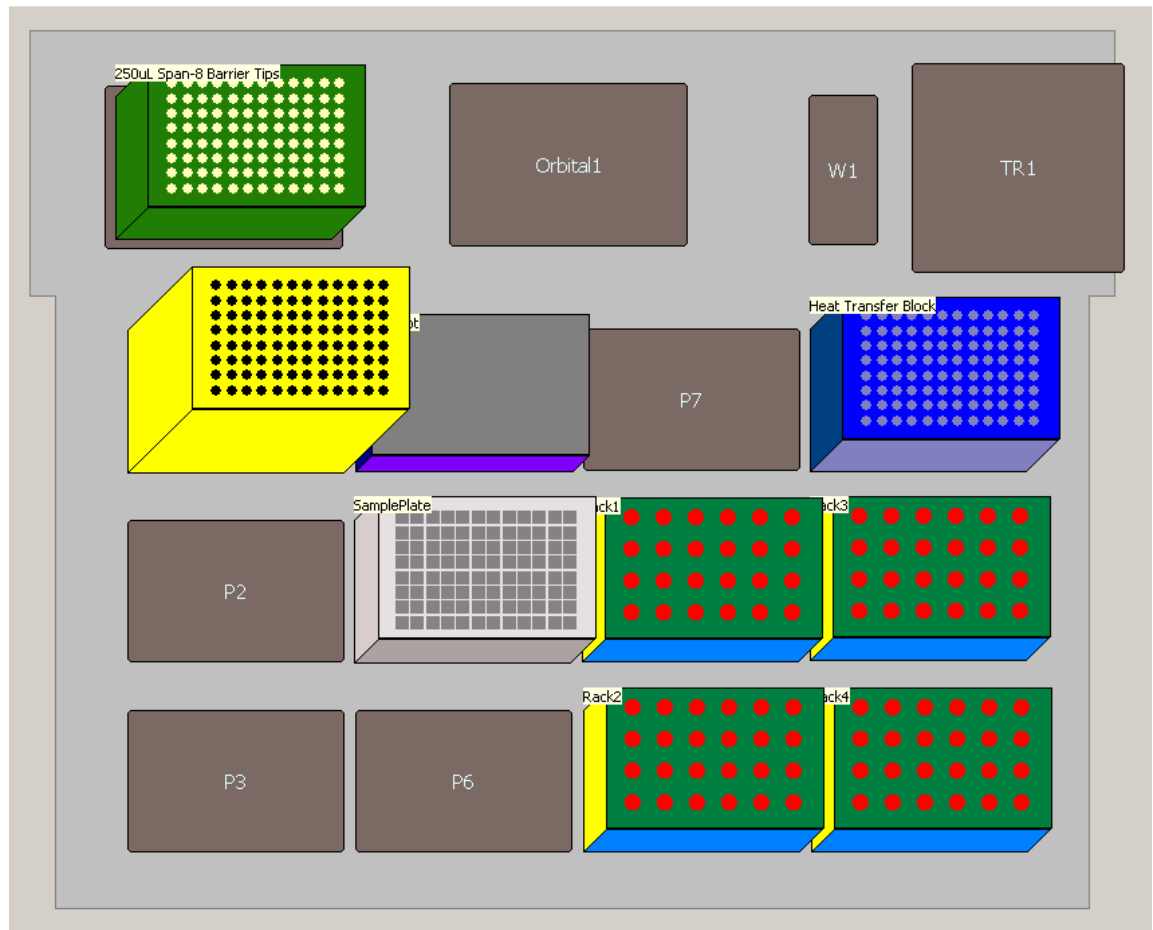
5.8.5.5.   Open the "Initial Tube Transfer" method contained in the Promega project.

5.8.5.6.   Click the green "Run" button at the top of the screen.

5.8.5.7.   The method will prompt for two values:

5.8.5.7.1.   SampleColumns = the number of full or partial columns of sample on the extraction plate.

5.8.5.7.1.1.   *Example: 20 samples/blanks will use two full columns and half of a third. Enter "SampleColumns" as 3.*

5.8.5.7.2.   SampleVolume = the approximate volume of liquid in the sample tubes, in microliters. Round to the next highest hundred microliters, to a maximum of 400 µl.

5.8.5.7.2.1.   *Example: Most of the sample tubes contain 300 µl, but one tube contains 340 µl. Enter "SampleVolume" as 400.*

5.8.5.8.   When the instrument has finished transferring sample from the tubes to the sample plate, remove the tube racks from the deck and discard the empty sample tubes.

5.8.5.9. Leave the used P250 Span-8 Barrier Tips on the Rapid-Shuck module for subsequent use in DNA IQ™ automated extraction. Each tip is now associated with a single sample and will only be used to pipet that sample.

5.8.6. DNA IQ™ Automated Extraction Procedure

5.8.6.1. Instrument Deck Setup

5.8.6.1.1. Turn on the heating block via the switch to the right of the Biomek NX^P and ensure that temperature is set to 85^{0}C.

5.8.6.1.2. Place the 2.2 ml Square-Well Deep Well Plate sample plate [containing sample transferred during Initial Tube Transfer (5.8.4)] on position P5.

5.8.6.1.3. The box of P250 Span-8 Barrier Tips used in the Initial Tube Transfer (5.8.4) should be unchanged on the Rapid-Shuck module. DO NOT change the orientation of this box.

5.8.6.1.4. Place the following labware on the deck as shown in the figure below:

5.8.6.1.4.1. Box of Beckman Coulter Biomek P1000 Span-8 Tips (Cat. no. 987925) on position P1. If there are fewer than five columns of tips available, the program will prompt for a full box of P1000 tips on position P3, otherwise, a partial box may be used.

5.8.6.1.4.2. Box of new P250 Span-8 Barrier Tips on position P11.

5.8.6.1.4.3. Promega 1.2 ml Round-Bottom Deep Well Plates (Cat. no. V6771) on positions P6 and P4 (aligned on top of the MagnaBot module in P4). Orient each plate so that the clipped corner is on the bottom left.

5.8.6.1.4.4. Promega 1.1 ml Square-Well V-Bottom Deep Well Plate (Cat. no. V6821) on position P12. Orient the plate so that the clipped corner is on the bottom left.
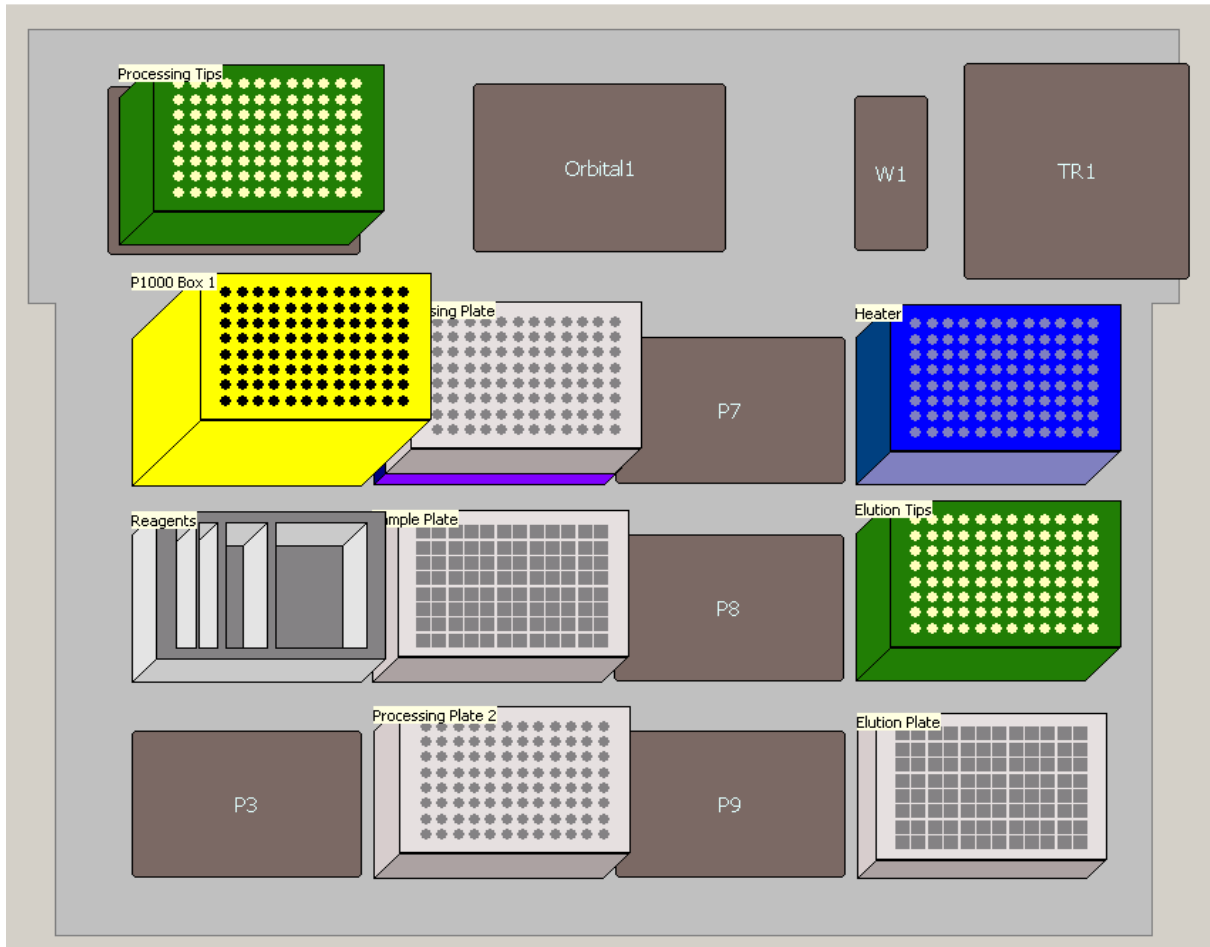
5.8.6.2. Reagent Setup

5.8.6.2.1. Open the Automation Workbook from the "Robot Team" network folder.

5.8.6.2.2. The "Reagents" tab has a description of the reagents and supplies needed to run automated DNA IQ™ extraction.

5.8.6.2.2.1. Enter the number of samples on the plate, including reagent blanks.

5.8.6.2.2.2. Enter the desired elution volume (between 50-100 µl per sample).

5.8.6.2.2.3. Do not count the No Template Control (NTC) or quantification standards.

5.8.6.2.2.4. Do not add extra samples for excess volume; it is already calculated into the program.

5.8.6.2.3. In a reservoir holder, place the following clean reservoirs in order from left to right: one Quarter Module reservoir (Beckman Coulter, Fullerton CA, cat. no. 372790) divided by length, one additional Quarter Module reservoir, and one Half Module reservoir (Beckman Coulter, cat. no 372786).

5.8.6.2.4. Load reagents in each reservoir as indicated in the "Reagents" tab, adding elution buffer last.

5.8.6.2.5. Place the reservoir holder on deck position P2.

5.8.6.3. Running the DNA IQ™ Method

5.8.6.3.1. Open the "Extraction_v2.1.0.ISP" method, contained in the Promega project.

5.8.6.3.2. Click the "Run" button at the top of the screen.

5.8.6.3.3. The method will prompt for three values:

5.8.6.3.3.1. UsedP1000TipCols = the number of **used** full or partial columns in the 1000 µl tip box on position P1. For a new box, enter 0.

5.8.6.3.3.1.1. *Example: If two and a half columns of P1000 tips have been used, enter "UsedP1000TipCols" as 3.*

5.8.6.3.3.2. UsedP200TipCols = the number of **used** full or partial columns in the 250 µl tip box on the Rapid-Shuck module. Assuming a new box was used during the Initial Tube Transfer (5.8.4), the value should be 0.

5.8.6.3.3.3. SampleCount = the number of total samples to be extracted, up to 87. Wells may not be skipped; this number corresponds to the last well of the plate containing a sample, counting down columns then across rows (e.g., well G11 = 87th well)

5.8.6.3.3.3.1. For a default (aqueous) extraction, simply enter the sample count number (e.g., "87"). This extraction type accommodates a 400 uL starting sample volume and 75 uL elution volume.

5.8.6.3.3.3.2. To re-extract/purify a previously extracted lysate plate (lysis extraction), enter the sample count number followed by the word "Lysate" (e.g., "87Lysate"). This extraction type accommodates an 800 uL starting sample volume and 75 uL elution volume.

5.8.6.3.4. The protocol will run for approximately 1 hour 45 minutes, depending on how many samples are being processed.

5.8.6.3.5. When the run is finished, reservoirs may be carefully rinsed, labeled, and reused. Discard partial tip box from position P11. Remaining partial tip boxes may be used again. Discard processing plates (both 1.2 ml Round-Bottom Deep Well Plates). Turn off the heating block.

5.8.6.3.6. Label and retain the original sample plate (2.2 ml Square-Well Deep Well Plate) containing the lysate, as back-up until the samples have been completely processed. Cover and store at room temperature.

5.8.6.3.7. Cover the elution plate (1.1 ml Square-Well V-Bottom Deep Well Plate) containing the extracted samples with a temporary seal and refrigerate if the plate is not to be quantified immediately.

5.8.7. REAL-TIME PCR QUANTIFICATION USING QUANTIFILER® TRIO Prepared on the Biomek NX^P Laboratory Automation Workstation

5.8.7.1. Instrument Deck Setup

5.8.7.1.1. In the Biomek Software, open the "Trio Quant" method contained in the ABI project.

5.8.7.1.2. Click on the "User Interface" line within the method.

5.8.7.1.2.1. To the right, check that "Quantifiler Trio" is selected.

5.8.7.1.2.2. Enter the number of samples to process (equal to the number of elution plate wells that contain extract).

5.8.7.1.2.3. Do not count the NTC or quantification standards.

5.8.7.1.2.4. Do not add extra samples for excess volume; it is already calculated into the program.

5.8.7.1.3. Place the following labware on the deck as shown in the figure below:

5.8.7.1.3.1. New box of Beckman Coulter Biomek P50 Barrier Tips (Cat. no. A21586) or Axygen 30 µl Filtered Tips (Cat. no. FXF-50-R-S) on position P2. Alternatively, a partial box may be used by selecting the number and position of remaining tips in the box under the "Properties" tab of the "Instrument Setup" step.

5.8.7.1.3.2. Box of P250 Span-8 Barrier Tips on position P1. A partial box may be used (as above) if at least two columns are available.

5.8.7.1.3.3. New Applied Biosystems (Foster City, CA) MicroAmp® Optical 96-Well reaction plate (Cat. no. N801-0560) on position P11.

5.8.7.1.3.4. The elution plate containing extracted samples on position P12. This plate must be centrifuged prior to quantification to prevent bubbles in the sample wells.

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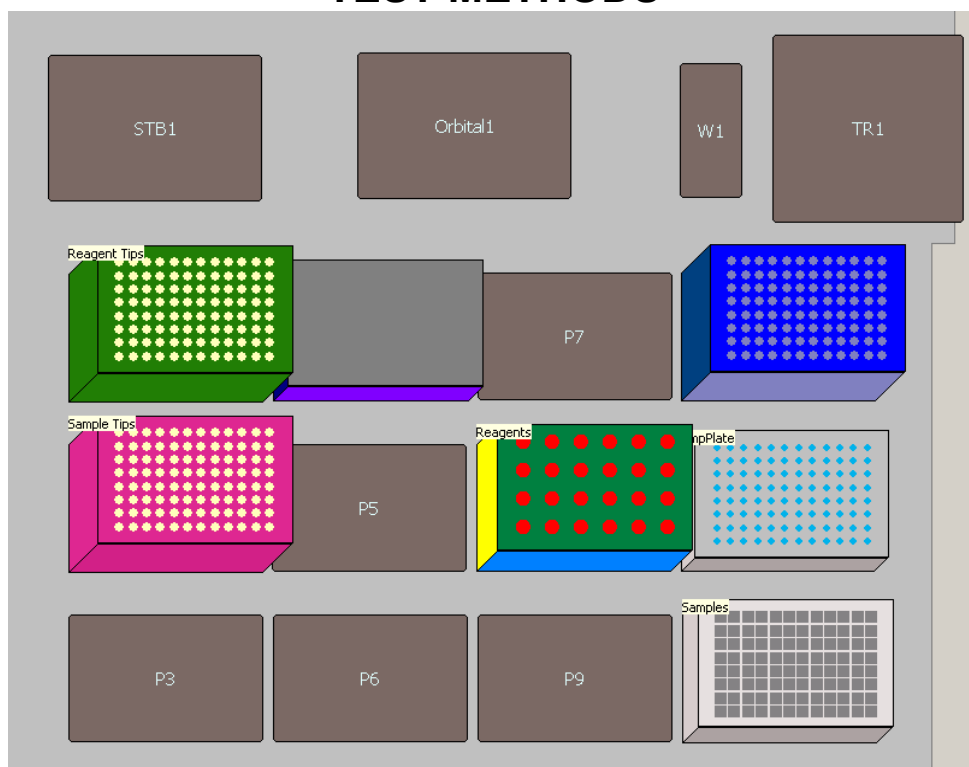
5.8.7.2. Reagent Preparation

5.8.7.2.1. Open the Automation Workbook from the "Robot Team" network folder.

5.8.7.2.2. The "Reagents" tab has a description of the reagents and supplies needed to run automated quantification setup.

5.8.7.2.2.1. Enter the number of samples on the plate, the same as entered into the "User Interface" above.

5.8.7.2.3. When a new Quantifiler® Trio kit is opened, Quantification Enhancer may be added to the reaction mix as follows:

5.8.7.2.3.1. Using a 2.5 µl pipettor, carefully add 1 µl of Quantification Enhancer to each fresh tube of reaction mix. Vortex each reaction mix tube briefly.

5.8.7.2.3.2. Mark each tube of reaction mix with "QE" to indicate that Quantification Enhancer has been added.

5.8.7.2.3.3. Record the lot number of the Quantification Enhancer on the Automated Extraction/Quantification Worksheet.

5.8.7.2.3.4. Quantification Enhancer shall be stored at room temperature.

5.8.7.2.4. Prepare the master mix as described in section 2 of the Forensic Biology Section Casework Test Method, using the volumes indicated on the Reagents tab. Note that for 40 or more samples, the master mix must be divided evenly into two tubes. Vortex the Master Mix 3 to 5 seconds, then centrifuge briefly.

5.8.7.2.5.   Place the master mix tube(s) in wells A6 (and B6) of the Reagent Tube Rack as indicated below.

5.8.7.2.6.   Prepare the standard dilution series as described in Quantifiler® Trio section of the Biology Section Casework Test Method or use a previously prepared dilution series. Each tube must contain at least 20 µl of standard.

5.8.7.2.7.   Place the standard tubes in column 2 of the Reagent Tube Rack as indicated below.

5.8.7.2.8.   Open and fold back all tube caps and place the Reagent Tube Rack on deck position P8.
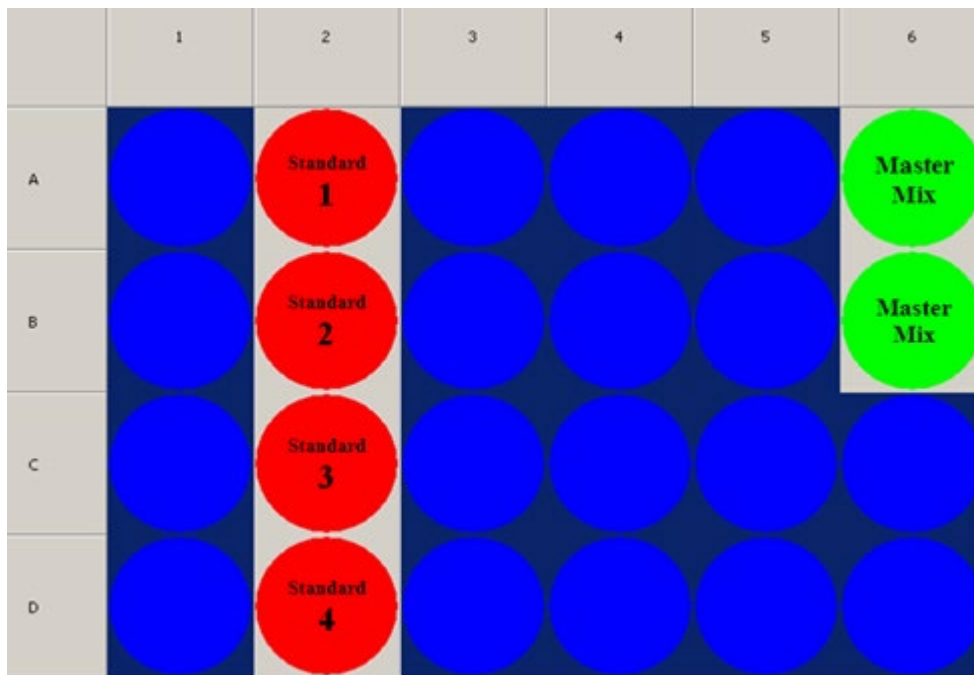
5.8.7.3.   Running the Automated Quant Trio Setup Method

5.8.7.3.1.   With the "Quant Trio" method open and the number of samples specified, click the "Run" button at the top of the screen.

5.8.7.3.2.   A window will appear to confirm the amount and placement of the master mix tubes. Click "OK".

5.8.7.3.3.   A window will appear to confirm the deck layout as shown by the software. Click "OK".

5.8.7.3.4.   A window will appear to confirm the total amount of master mix, the amount of standard in each of the standard tubes, the sample count, and the quantification kit. Click "OK".

5.8.7.3.5.   The protocol will begin. When the run is finished, remove the elution plate with extracts and cover with a temporary seal until ready to proceed with amplification.

5.8.7.3.6.   Seal the optical plate with an optical adhesive cover. Run the edge of the cover applicator between the rows and columns of the wells to ensure that all wells are sealed properly.

5.8.7.3.7.   Spin the plate using either a centrifuge or a salad spinner to remove any bubbles and force samples into the bottom of each well.

5.8.7.3.8.   Place the plate in the real-time PCR instrument, turn on the instrument, and open the 7500 System software.

5.8.7.4.   Create A Plate Document

5.8.7.4.1.   Open the Automation Workbook file containing the batch of samples being run, and click on the "Extraction-Quant" tab. Ensure that the corresponding plate document contains sample information, otherwise, add and save that information now. Also ensure that the batch number has been entered into the yellow box on the "Samples" tab.

5.8.7.4.2.   Press the "Create Quant Setup File" button. A new text file named "Batch#_QuantSetup_Date" will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.7.4.2.1.   If there are duplicate sample names in the batch, an error message will list them and the Quant Setup file will not be created until the duplicate names are fixed.

5.8.7.4.3.   On the computer connected to the real-time PCR instrument, create a new plate document as described in the Quantifiler® Trio section of the Biology Section Casework Test Method.

5.8.7.4.4.   Import the text file you just created. The wells should populate with the names of the samples and standards.

5.8.7.4.5.   Enter the plate name under "Experiment". Save the plate document and proceed with real-time PCR analysis as described in the Quantifiler® Trio section of the Biology Section Casework Test Method.

5.8.8.   PowerPlex® Fusion 6C Amplification
Prepared on the Biomek NX^P Laboratory Automation Workstation

5.8.8.1.   Vortexing Extracts

5.8.8.1.1.   Prior to amplification, the extract plate (the elution plate from the DNA IQ™ procedure) shall be vortexed briefly at low speed to ensure uniform pipetting of sample.

5.8.8.1.2.   Spin down the extract plate again before proceeding to amplification set-up. (CAUTION: Ensure that there are no bubbles in the wells.) Remove temporary seal.

5.8.8.2.   Normalization Data Setup

5.8.8.2.1.   Open the Automation Workbook file containing the batch to be amplified.

5.8.8.2.2.   On the "Extraction-Quant" tab, click the "Import & Analyze Quant Results" button.

5.8.8.2.3.   Browse for the appropriate text import file containing quantification data for this batch. Click "Open".

5.8.8.2.4.   The Automation Workbook will analyze the batch, create the Quantification Results and Standard Curve worksheets, create the Amplified Samples summary, create the Samples Not Amplified summary, and fill the samples on the Amplification Setup/CE Plate Record.

5.8.8.2.4.1.   If any standard curve parameter fails, or if the NTC contains a value, or if a standard curve well has an undetected IPC, an error message will appear and the batch will not be analyzed until the standard curve is fixed. Points should be omitted in the Real-Time PCR software and a new results file exported.

5.8.8.2.5.   A new .csv file named "Batch#_AmpSetup" (the AmpSetup file) will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.8.2.6.   Move or copy this AmpSetup file to the folder //IN PROGRESS/Amp Setups on the "Robot Team" drive.

5.8.8.3.   Instrument Deck Setup

5.8.8.3.1.   In the Biomek Software, open the "Normalize-Amp_Fusion6C" method contained in the Promega project.

5.8.8.3.2.   Place the following labware on the deck as shown in the figure below:

5.8.8.3.2.1. Box of P250 Span-8 Barrier Tips on the Rapid-Shuck module.

5.8.8.3.2.2. Box of P50 Barrier Tips on position P1. A second box of P50 Barrier Tips may be required on position P3.

5.8.8.3.2.3. 1.1 ml square-bottom deepwell plate on position P5. A second 1.1 ml square-bottom deepwell plate may be required on position P8.

5.8.8.3.2.4. New 96-well optical amplification plate on position P6.

5.8.8.3.2.5. Extract plate containing samples on position P4.

5.8.8.3.2.5.1. CAUTION: ensure that the MagnaBot module has been removed.
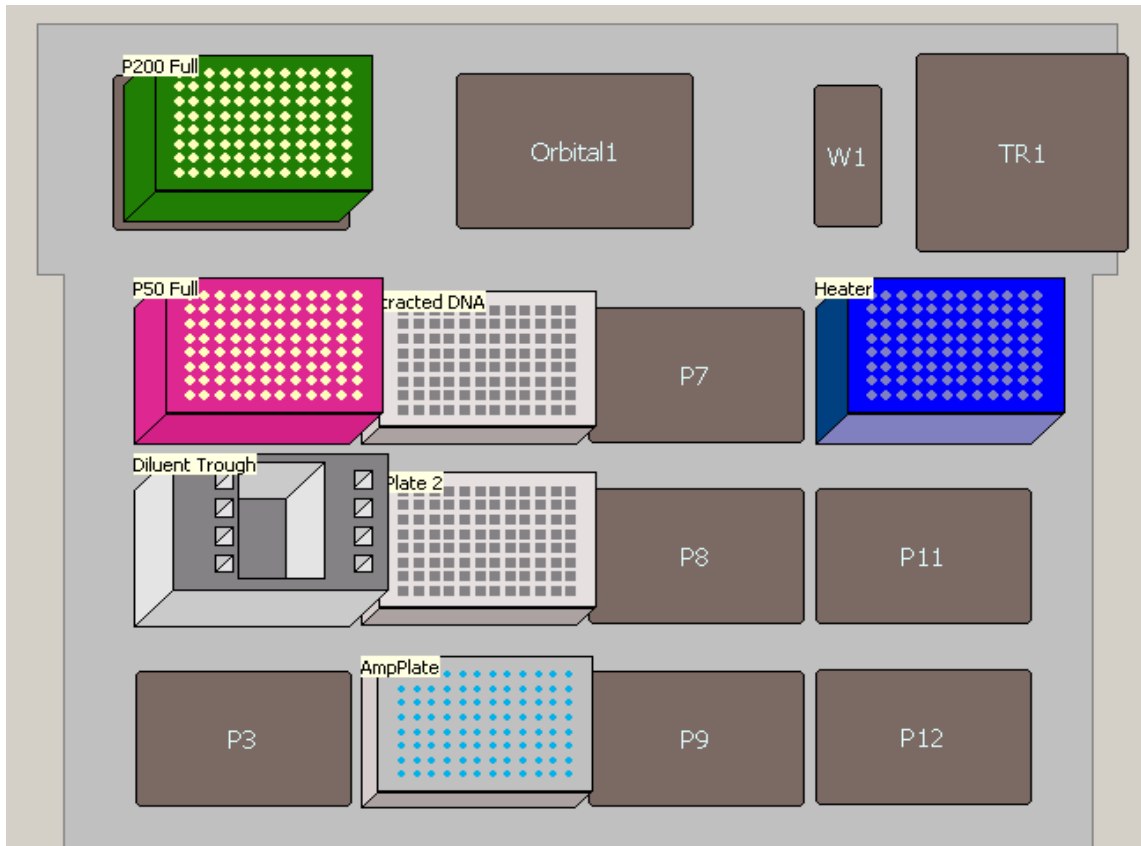
5.8.8.3.3.   Click "Run".

5.8.8.3.4.   The program will prompt for three values:

5.8.8.3.4.1.   BatchNumber = The batch number being amplified, in the format Year-XXX. This directs the Biomek software to import the correct Amp Setup file from the //IN PROGRESS/Amp Setups folder.

5.8.8.3.4.2.   First_P250_Tip = the number of the first unused tip in the 250 µl tip box on the Rapid-Shuck module, numbering down columns and then across. For a new box, enter 1.

5.8.8.3.4.2.1.   *Example: If two and a half columns (twenty tips) of P250 tips have been used, enter "First_P250_Tip" as 21.*

5.8.8.3.4.3.   First_P50_Tip = the number of the first unused tip in the 50 µl tip box on position P1, numbering down columns and then across. For a new box, enter 1.

5.8.8.4.   Reagent Preparation

5.8.8.4.1.   Follow the prompts within the Biomek Software. Note that depending on the dilution of samples within the batch, the protocol may require an additional 1.1 ml square-bottom deepwell plate on position P8.

5.8.8.4.2.   Place the STR Master Mix tube in position 4 (front-most) of the Left Tube Holder on the reagent rack.

5.8.8.4.3.   Place the tube containing diluted positive control (at least 20 µl) in position 1 (rear-most) of the Right Tube Holder on the reagent rack. Click "OK".

5.8.8.4.4.   Pipet the indicated amount of amplification-grade water or nuclease-free water into the center trough of the reagent rack. Place the reagent rack on deck position P2.

5.8.8.5.   Running the Normalization/Amp Set-up Method

5.8.8.5.1.   Check that the deck layout matches the layout indicated in the next prompt. Click "OK".

5.8.8.5.2.   The run will begin, performing normalization and amplification setup in one step.

5.8.8.5.3.   When the run is finished, remove the amplification plate and cover with adhesive foil. Press the foil well using a roller tool.

5.8.8.5.4.   Remove the elution plate containing extracted samples and cover with a temporary seal and refrigerate.

5.8.8.6.   Create CE Plate Record

5.8.8.6.1.   The Amp-CE Plate Record in the Automation Workbook is automatically populated with the amplified samples when the batch is analyzed with quantification results.

5.8.8.6.2.   Open the Automation Workbook containing the batch of interest and click on the "Amp-CE" tab.

5.8.8.6.3.   Click the "Export CE Setup File" button. A new text file named "Batch#_CESetup_Date" will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.8.6.3.1.   If no plate name has been filled in, the file will contain a default plate name in the format "Batch#_Date_01". This can be changed on the "Amp-CE" tab or in the genetic analyzer software.

5.8.8.6.4.   Samples may be added manually to the CE plate record and "CE Setup" tab, as needed, before clicking the "Export CE Setup File" button. Only samples listed on the "CE Setup" tab will be included in the exported CE Setup file.

5.8.8.6.5.   Fill out the rest of the worksheet as designated, including dates, reagent lot numbers, amplification reaction type, and injection protocols.

5.8.9. PowerPlex® Y23 Amplification
   Prepared on the Biomek NX^P Laboratory Automation Workstation

5.8.9.1.   NOTE: PowerPlex® Y23 amplification via Biomek automation is not validated for casework unknowns and shall only be performed on known samples.

5.8.9.2. Normalization Data Setup

5.8.9.2.1. Open the Automation Workbook file containing the batch to be amplified.

5.8.9.2.2. Assuming the quantitation data for the batch has already been imported and processed (see preceding section for Fusion 6C amplification), go to the Data Extract tab.

5.8.9.2.2.1. The "Amp YSTR" column on the right contains TRUE/FALSE designations for each sample. This controls whether a sample will be included in a Y-STR amplification. Samples are automatically marked "TRUE" for Y-STR amplification if they both:

1) Have a F:M ratio ≤ 5
2) Are marked as a standard (Code S)

5.8.9.2.2.2. Y-STR amplification may be manually turned on/off per sample by editing the Amp YSTR column values.

5.8.9.2.2.3. If the Amp YSTR column reflects the samples that should be amplified, click the "Amp Y-STR" button above it.

5.8.9.2.3. The Automation Workbook will analyze the batch according to the Y quant values and create a Y-STR version of the following worksheets: Amplified Samples Summary, Amplification Setup/CE Plate Record, and CE Setup. These worksheets are normally hidden in the Workbook but will appear as blue tabs.

5.8.9.2.4. A new .csv file named "Batch#_YSTR_AmpSetup" (the AmpSetup file) will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.9.2.5. Move or copy this YSTR_AmpSetup file to the folder //IN PROGRESS/Amp Setups on the "Robot Team" drive.

5.8.9.2.6. Proceed with Instrument Deck Setup, Reagent Preparation, Running the Normalization/Amp Setup Method, and Create CE Plate Record as described in the previous section, *except*:

5.8.9.2.6.1. For Instrument Deck Setup, open the "Normalize-Amp_PPY23" method contained in the Promega project.

5.8.9.2.6.2. For Reagent Preparation, be sure to use the PowerPlex® Y23 amplification kit. Follow the master mix preparation recipe as prompted in the Biomek software.

5.8.9.2.6.3. For Create CE Plate Record, click on the "Export CE Setup File" on the "Y-Amp-CE" worksheet.

5.8.10. Biomek i5 Preparation

5.8.10.1. Wipe down the deck with 70% ethanol, 3-6% hydrogen peroxide (with 70% ethanol rinse) or 10% bleach.

5.8.10.2. Ensure that the workstation trash is not full.

5.8.10.3. Ensure that the waste carboy is not full.

5.8.10.4. Ensure that there is enough distilled water in the intake carboy (the valve must be underwater).

5.8.10.5. Ensure that the software is connected to the instrument and not in Simulate mode:

> 5.8.10.5.1.   "Utilities" tab → "Hardware Setup".

> 5.8.10.5.2.   Set "Name" to "Biomek".

> 5.8.10.5.3.   Click "Accept".

5.8.10.6. Run "Home All Axes" under the "Method" tab.

> 5.8.10.6.1.   Let the tips flush until there are no bubbles created when the pistons move.

> 5.8.10.6.2.   There may be a large bubble stationary at the tops of the syringes; this is normal.

5.8.10.7. Ensure that ethanol and isopropyl alcohol have been added to the alcohol wash buffer in the DNA IQ™ kit (35 mL of each).

5.8.11. Initial Tube Transfer

5.8.11.1. Place sample tubes in the tube racks (4x6 with white adapters), arranging the tubes according to the order shown in the Automation Workbook. Do not skip columns or spaces between samples.

5.8.11.2. Ensure that the sample tube lid hinges have been cut.

5.8.11.3. Spin down the tube racks (containing the sample tubes) in the centrifuge. Carefully remove the sample tube lids and discard.

> 5.8.11.3.1. CAUTION: Once the lids are removed, the Automation Workbook is the only record of the order and identity of each sample.

5.8.11.4. Arrange the deck layout as shown in the figure below:

> 5.8.11.4.1. Place sample tube racks on positions P8, P9, P11, and P12, again paying attention to sample order according to the Automation Workbook.

5.8.11.4.2. Place a new Promega (Madison, WI) 2.2 ml Square-Well Deep Well Plate (Cat. no. V6781) on position P5. Orient the plate so that the clipped corner is on the bottom left.

5.8.11.4.3. Place a new box of Beckman Coulter (Fullerton, CA) Biomek i-Series 190 µl Barrier tips (Cat. no. B85911) on the Rapid-Shuck module (top right).
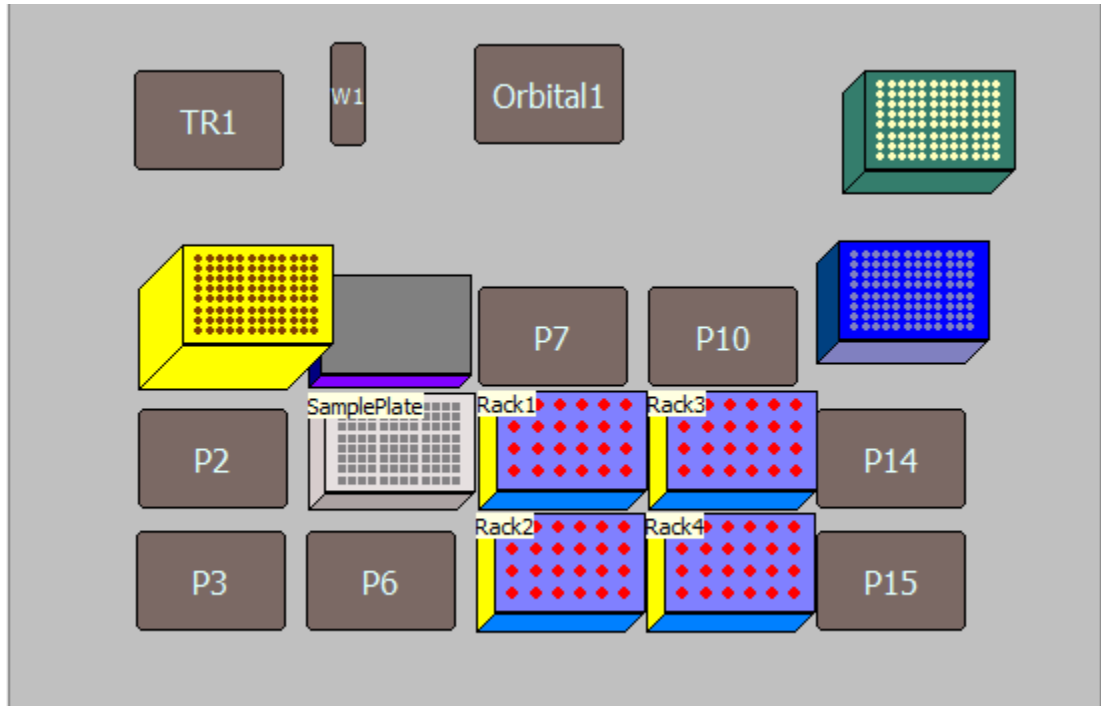
5.8.11.5. Open the "Initial Tube Transfer LyseSpin" method contained in the Promega project.

5.8.11.6. Click the green "Run" button at the top of the screen.

5.8.11.7. The method will prompt for two values:

5.8.11.7.1.   SampleColumns = the number of full or partial columns of sample on the extraction plate.

5.8.11.7.1.1.   *Example: 20 samples/blanks will use two full columns and half of a third. Enter "SampleColumns" as 3.*

5.8.11.7.2.   SampleVolume = the approximate volume of liquid in the sample tubes, in microliters. Round to the next highest hundred microliters, to a maximum of 400 µl.

5.8.11.7.2.1. *Example: Most of the sample tubes contain 300 µl, but one tube contains 340 µl. Enter "SampleVolume" as 400.*

5.8.11.8. When the instrument has finished transferring sample from the tubes to the sample plate, remove the tube racks from the deck and discard the empty sample tubes.

5.8.11.9. Leave the used Biomek i-Series 190 µl Barrier Tips on the Rapid-Shuck module for subsequent use in DNA IQ™ automated extraction. Each tip is now associated with a single sample and will only be used to pipet that sample.

5.8.12. DNA IQ™ Automated Extraction Procedure

5.8.12.1. Instrument Deck Setup

5.8.12.1.1. Turn on the heating block and ensure that temperature is set to 85^0C.

5.8.12.1.2. Place the 2.2 ml Square-Well Deep Well Plate sample plate [containing sample transferred during Initial Tube Transfer (5.8.4)] on position P5.

5.8.12.1.3. The box of Biomek 190 µl Barrier Tips used in the Initial Tube Transfer (5.8.4) should be unchanged on the Rapid-Shuck module. DO NOT change the orientation of this box.

5.8.12.1.4. Place the following labware on the deck as shown in the figure below:

5.8.12.1.4.1. Box of Beckman Coulter Biomek i-Series 1025 µl Barrier tips (Cat. no. B85955) on position P1. If there are fewer than five columns of tips available, the program will prompt for a full box of 1025 µL tips on position P3, otherwise, a partial box may be used.

5.8.12.1.4.2. Box of new i-Series 190 µl Barrier tips on position P10.

5.8.12.1.4.3. Promega 1.2 ml Round-Bottom Deep Well Plates (Cat. no. V6771) on positions P6 and P4 (aligned on top of the MagnaBot module in P4). Orient each plate so that the clipped corner is on the bottom left.

5.8.12.1.4.4. Promega 1.1 ml Square-Well V-Bottom Deep Well Plate (Cat. no. V6821) on position P15. Orient the plate so that the clipped corner is on the bottom left.
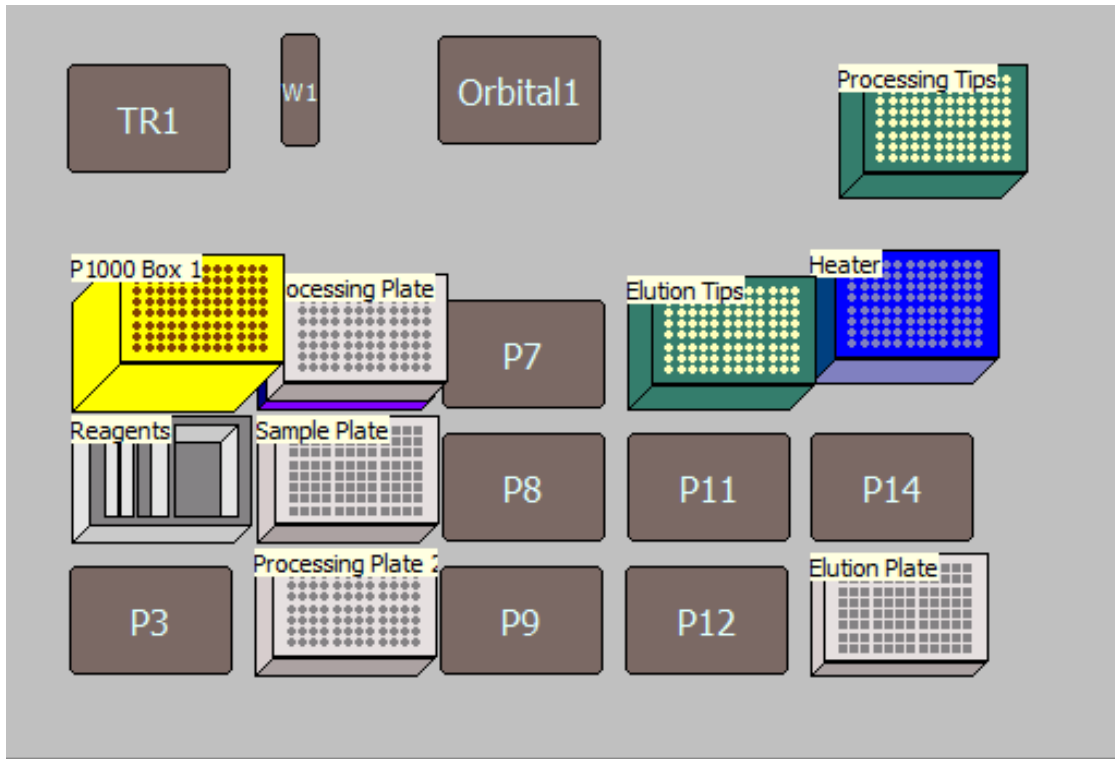
5.8.12.2. Reagent Setup

5.8.12.2.1.   Open the Automation Workbook from the "Robot Team" network folder.

5.8.12.2.2.   The "Samples" tab has a description of the reagents and supplies needed to run automated DNA IQ™ extraction.

    5.8.12.2.2.1.   Enter the number of samples on the plate, including reagent blanks.

    5.8.12.2.2.2.   Enter the desired elution volume (between 50-100 µl per sample).

    5.8.12.2.2.3.   Do not count the No Template Control (NTC) or quantification standards.

    5.8.12.2.2.4.   Do not add extra samples for excess volume; it is already calculated into the program.

5.8.12.2.3.   In a reservoir holder, place the following clean reservoirs in order from left to right: one Quarter Module reservoir (Beckman Coulter, Fullerton CA, cat. no. 372790) divided by length, one additional Quarter Module reservoir, and one Half Module reservoir (Beckman Coulter, cat. no 372786).

5.8.12.2.4.   Load reagents in each reservoir as indicated in the "Samples" tab, adding elution buffer last.

5.8.12.2.5.   Place the reservoir holder on deck position P2.

5.8.12.3. Running the DNA IQ™ Method

5.8.12.3.1.   Open the "Extraction_v2.1.0.ISP" method, contained in the Promega project.

5.8.12.3.2.   Click the "Run" button at the top of the screen.

5.8.12.3.3.   The method will prompt for three values:

  5.8.12.3.3.1.   UsedP1000TipCols = the number of **used** full or partial columns in the 1000 µl tip box on position P1. For a new box, enter 0.

    5.8.12.3.3.1.1.   *Example: If two and a half columns of P1000 tips have been used, enter "UsedP1000TipCols" as 3.*

  5.8.12.3.3.2.   UsedP200TipCols = the number of **used** full or partial columns in the 190 µl tip box on the Rapid-Shuck module. Assuming a new box was used during the Initial Tube Transfer (5.8.4), the value should be 0.

  5.8.12.3.3.3.   SampleCount = the number of total samples to be extracted, up to 87. Wells may not be skipped; this number corresponds to the last well of the plate containing a sample, counting down columns then across rows (e.g., well G11 = 87th well)

    5.8.12.3.3.3.1.   For a default (aqueous) extraction, simply enter the sample count number (e.g., "87"). This extraction type accommodates a 400 µL starting sample volume and 75 µL elution volume.

    5.8.12.3.3.3.2.   To re-extract/purify a previously extracted lysate plate (lysis extraction), enter the sample count number followed by the word "Lysate" (e.g., "87Lysate"). This extraction type accommodates an 800 µL starting sample volume and 75 µL elution volume.

5.8.12.3.4.   The protocol will run for approximately 1 hour 45 minutes, depending on how many samples are being processed.

5.8.12.3.5.   When the run is finished, reservoirs may be carefully rinsed, labeled, and reused. Discard partial tip box from position P10. Remaining partial tip boxes may be used again. Discard processing plates (both 1.2 ml Round-Bottom Deep Well Plates). Turn off the heating block.

5.8.12.3.6.   Label and retain the original sample plate (2.2 ml Square-Well Deep Well Plate) containing the lysate, as back-up until the samples have been completely processed. Cover and store at room temperature.

5.8.12.3.7.   Cover the elution plate (1.1 ml Square-Well V-Bottom Deep Well Plate) containing the extracted samples with a temporary seal and refrigerate if the plate is not to be quantified immediately.

5.8.13. **Combined Initial Tube Transfer/DNA IQ™ Extraction**

5.8.13.1. If desired, the Initial Tube Transfer and Extraction steps may be completed in a single method.

5.8.13.2. Instrument Deck Setup

5.8.13.2.1. In the Biomek Software, open the "TubeTransfer_And_Extraction" method contained in Promega projects.

5.8.13.2.2. Follow the deck setup for both the Initial Tube Transfer and Extraction methods described above. Both will fit concurrently on the deck.
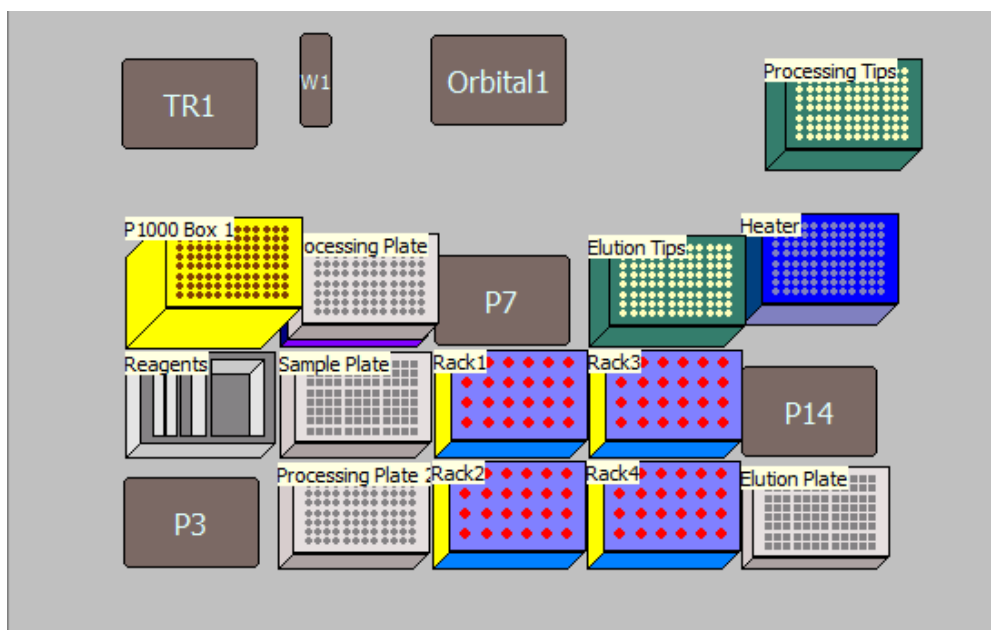
5.8.13.2.3. Click the "Run" button at the top of the screen.

5.8.14. **REAL-TIME PCR QUANTIFICATION USING QUANTIFILER® TRIO Prepared on the Biomek i5 Laboratory Automation Workstation**

5.8.14.1. Instrument Deck Setup

5.8.14.1.1. In the Biomek Software, open the "Trio Quant" method contained in the ABI projects.

5.8.14.1.2.   Click on the "User Interface" line within the method.

5.8.14.1.2.1.   To the right, check that "Quantifiler Trio" is selected.

5.8.14.1.2.2.   Enter the number of samples to process (equal to the number of elution plate wells that contain extract).

5.8.14.1.2.3.   Do not count the NTC or quantification standards.

5.8.14.1.2.4.   Do not add extra samples for excess volume; it is already calculated into the program.

5.8.14.1.3.   Place the following labware on the deck as shown in the figure below:

5.8.14.1.3.1.   New box of Beckman Coulter Biomek i-Series 50 µl Barrier tips (Cat. no. B85888) on position P2. Alternatively, a partial box may be used by selecting the number and position of remaining tips in the box under the "Properties" tab of the "Instrument Setup" step.

5.8.14.1.3.2.   Box of i-Series 190 µl Barrier tips on position P1. A partial box may be used (as above) if at least one column is available.

5.8.14.1.3.3.   New Applied Biosystems (Foster City, CA) MicroAmp® Optical 96-Well reaction plate (Cat. no. N801-0560) on position P11.

5.8.14.1.3.4.   The elution plate containing extracted samples on position P12. This plate must be centrifuged prior to quantification to prevent bubbles in the sample wells.
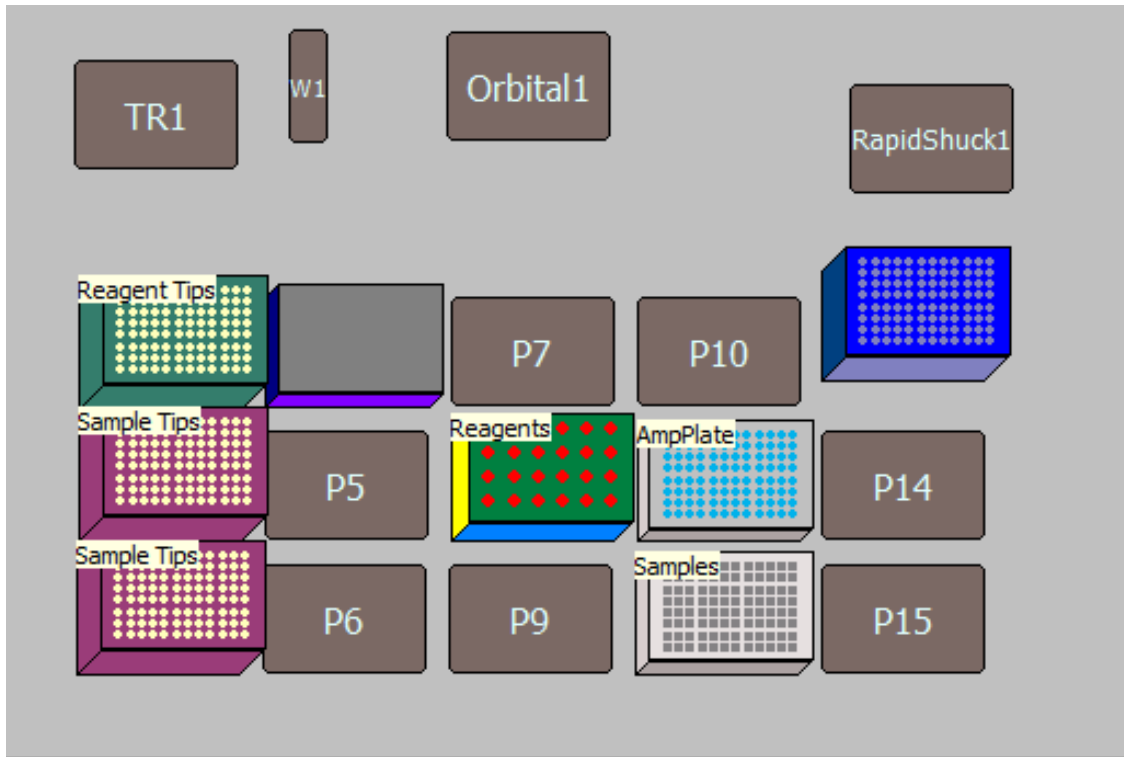
5.8.14.2. Reagent Preparation

5.8.14.2.1. Open the Automation Workbook from the "Robot Team" network folder.

5.8.14.2.2. The "Samples" tab has a description of the reagents and supplies needed to run automated quantification setup.

5.8.14.2.2.1. Enter the number of samples on the plate, the same as entered into the "User Interface" above.

5.8.14.2.3. When a new Quantifiler® Trio kit is opened, Quantification Enhancer may be added to the reaction mix as follows:

5.8.14.2.3.1. Using a 2.5 µl pipettor, carefully add 1 µl of Quantification Enhancer to each fresh tube of reaction mix. Vortex each reaction mix tube briefly.

5.8.14.2.3.2. Mark each tube of reaction mix with "QE" to indicate that Quantification Enhancer has been added.

5.8.14.2.3.3. Record the lot number of the Quantification Enhancer on the Automated Extraction/Quantification Worksheet.

5.8.14.2.3.4. Quantification Enhancer shall be stored at room temperature.

5.8.14.2.4. Prepare the master mix as described in section 2 of the Forensic Biology Section Casework Test Method, using the volumes indicated on the

Reagents tab. Note that for 40 or more samples, the master mix must be divided evenly into two tubes. Vortex the Master Mix 3 to 5 seconds, then centrifuge briefly.

5.8.14.2.5. Place the master mix tube(s) in wells A6 (and B6) of the Reagent Tube Rack as indicated below.

5.8.14.2.6. Prepare the standard dilution series as described in Quantifiler® Trio section of the Biology Section Casework Test Method or use a previously prepared dilution series. Each tube must contain at least 20 µl of standard.

5.8.14.2.7. Place the standard tubes in column 2 of the Reagent Tube Rack as indicated below.

5.8.14.2.8. Open and fold back all tube caps and place the Reagent Tube Rack on deck position P8.
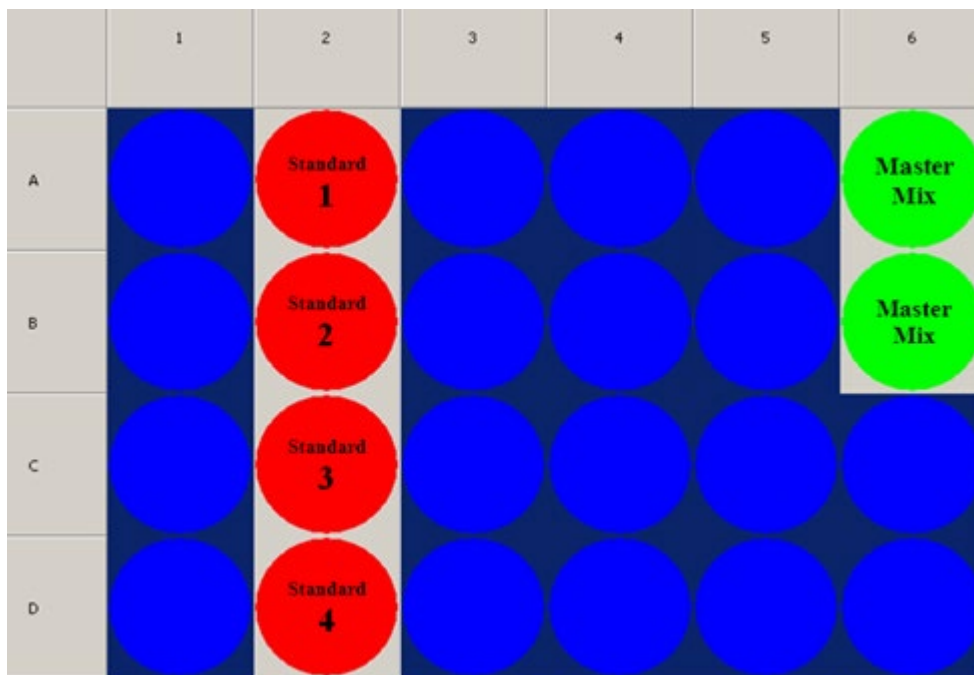
5.8.14.3. Running the Automated Quant Trio Setup Method

5.8.14.3.1. With the "Trio Quant" method open and the number of samples specified, click the "Run" button at the top of the screen.

5.8.14.3.2. A window will appear to confirm the amount and placement of the master mix tubes. Click "OK".

5.8.14.3.3. A window will appear to confirm the deck layout as shown by the software. Click "OK".

5.8.14.3.4. A window will appear to confirm the total amount of master mix, the amount of standard in each of the standard tubes, the sample count, and the quantification kit. Click "OK".

5.8.14.3.5. The protocol will begin. When the run is finished, remove the elution plate with extracts and cover with a temporary seal until ready to proceed with amplification.

5.8.14.3.6. Seal the optical plate with an optical adhesive cover. Run the edge of the cover applicator between the rows and columns of the wells to ensure that all wells are sealed properly.

5.8.14.3.7. Spin the plate using either a centrifuge or a salad spinner to remove any bubbles and force samples into the bottom of each well.

5.8.14.3.8. Place the plate in the real-time PCR instrument, turn on the instrument, and open the 7500 System software.

5.8.14.4. Create A Plate Document

5.8.14.4.1. Open the Automation Workbook file containing the batch of samples being run, and click on the "Extraction-Quant" tab. Ensure that the corresponding plate document contains sample information, otherwise, add and save that information now. Also ensure that the batch number has been entered into the yellow box on the "Samples" tab.

5.8.14.4.2. Press the "Create Quant Setup File" button. A new text file named "Batch#_QuantSetup_Date" will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.14.4.2.1. If there are duplicate sample names in the batch, an error message will list them and the Quant Setup file will not be created until the duplicate names are fixed.

5.8.14.4.3. On the computer connected to the real-time PCR instrument, create a new plate document as described in the Quantifiler® Trio section of the Biology Section Casework Test Method.

5.8.14.4.4. Import the text file you just created. The wells should populate with the names of the samples and standards.

5.8.14.4.5. Enter the plate name under "Experiment". Save the plate document and proceed with real-time PCR analysis as described in the Quantifiler® Trio section of the Biology Section Casework Test Method.

5.8.15. PowerPlex® Fusion 6C Amplification
Prepared on the Biomek i5 Laboratory Automation Workstation

5.8.15.1. Vortexing Extracts

5.8.15.1.1. Prior to amplification, the extract plate (the elution plate from the DNA IQ™ procedure) shall be vortexed briefly at low speed to ensure uniform pipetting of sample.

5.8.15.1.2. Spin down the extract plate again before proceeding to amplification set-up. (CAUTION: Ensure that there are no bubbles in the wells.) Remove temporary seal.

5.8.15.2. Normalization Data Setup

5.8.15.2.1. Open the Automation Workbook file containing the batch to be amplified.

5.8.15.2.2. On the "Extraction-Quant" tab, click the "Import & Analyze Quant Results" button.

5.8.15.2.3. Browse for the appropriate text import file containing quantification data for this batch. Click "Open".

5.8.15.2.4. The Automation Workbook will analyze the batch, create the Quantification Results and Standard Curve worksheets, create the Amplified Samples summary, create the Samples Not Amplified summary, and fill the samples on the Amplification Setup/CE Plate Record.

5.8.15.2.4.1. If any standard curve parameter fails, or if the NTC contains a value, or if a standard curve well has an undetected IPC, an error message will appear and the batch will not be analyzed until the standard curve is fixed. Points should be omitted in the Real-Time PCR software and a new results file exported.

5.8.15.2.5. A new .csv file named "Batch#_AmpSetup" (the AmpSetup file) will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.15.2.6. Move or copy this AmpSetup file to the folder //IN PROGRESS/Amp Setups on the "Robot Team" drive.

5.8.15.3. Instrument Deck Setup

5.8.15.3.1. In the Biomek Software, open the "Normalize-Amp_Fusion6C" method contained in the Promega project.

5.8.15.3.2. Place the following labware on the deck as shown in the figure below:

5.8.15.3.2.1. Box of i-Series 190 µl Barrier tips on the Rapid-Shuck module.

5.8.15.3.2.2.   Box of i-Series 50 µl Barrier tips on position P1. A second box of P50 Barrier tips may be required on position P3.

5.8.15.3.2.3.   1.1 ml square-bottom deepwell plate on position P5. A second 1.1 ml square-bottom deepwell plate may be required on position P8.

5.8.15.3.2.4.   New 96-well optical amplification plate on position P6.

5.8.15.3.2.5.   Extract plate containing samples on position P4.

    5.8.15.3.2.5.1.   CAUTION: ensure that the MagnaBot module has been removed.
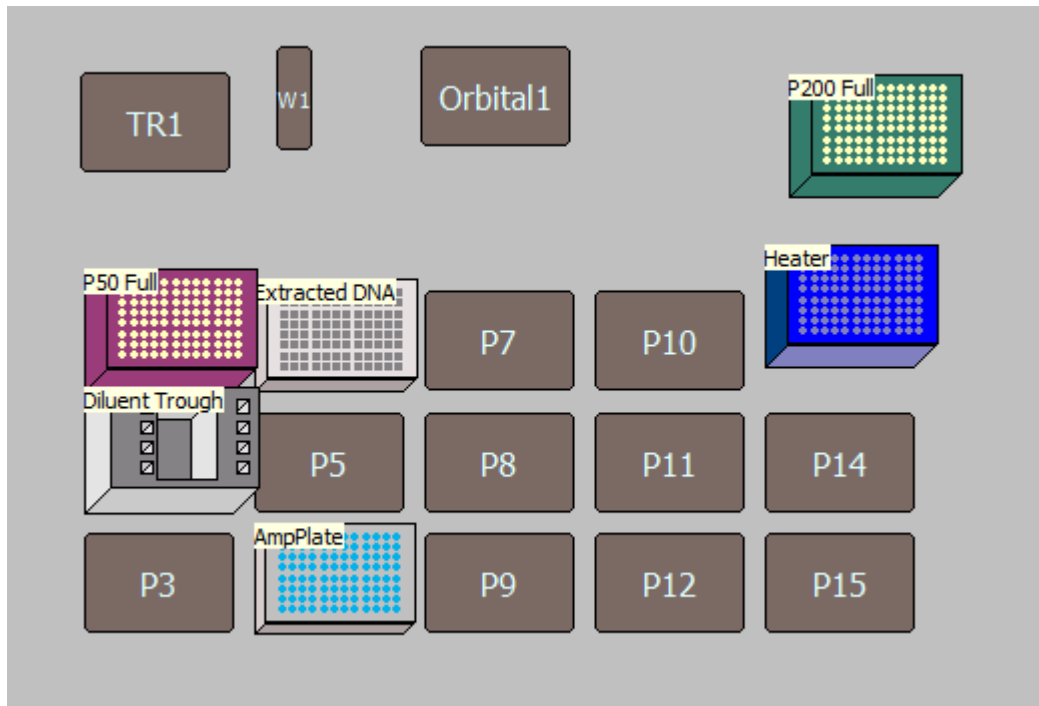
5.8.15.3.3. Click "Run".

5.8.15.3.4. The program will prompt for three values:

5.8.15.3.4.1.   BatchNumber = The batch number being amplified, in the format Year-XXX. This directs the Biomek software to import the correct Amp Setup file from the //IN PROGRESS/Amp Setups folder.

5.8.15.3.4.2.   First_P250_Tip = the number of the first unused tip in the 190 µL tip box on the Rapid-Shuck module, numbering down columns and then across. For a new box, enter 1.

5.8.15.3.4.2.1. *Example: If two and a half columns (twenty tips) of P250 tips have been used, enter "First_P250_Tip" as 21.*

5.8.15.3.4.3. First_P50_Tip = the number of the first unused tip in the 50 µl tip box on position P1, numbering down columns and then across. For a new box, enter 1.

5.8.15.4. Reagent Preparation

5.8.15.4.1. Follow the prompts within the Biomek Software. Note that depending on the dilution of samples within the batch, the protocol may require an additional 1.1 ml square-bottom deepwell plate on position P8.

5.8.15.4.2. Place the STR Master Mix tube in position 4 (front-most) of the Left Tube Holder on the reagent rack.

5.8.15.4.3. Place the tube containing diluted positive control (at least 20 µl) in position 1 (rear-most) of the Right Tube Holder on the reagent rack. Click "OK".

5.8.15.4.4. Pipet the indicated amount of amplification-grade water or nuclease-free water into the center trough of the reagent rack. Place the reagent rack on deck position P2.

5.8.15.5. Running the Normalization/Amp Set-up Method

5.8.15.5.1. Check that the deck layout matches the layout indicated in the next prompt. Click "OK".

5.8.15.5.2. The run will begin, performing normalization and amplification setup in one step.

5.8.15.5.3. When the run is finished, remove the amplification plate and cover with adhesive foil. Press the foil well using a roller tool.

5.8.15.5.4. Remove the elution plate containing extracted samples and cover with a temporary seal and refrigerate.

5.8.15.6. Create CE Plate Record

5.8.15.6.1. The Amp-CE Plate Record in the Automation Workbook is automatically populated with the amplified samples when the batch is analyzed with quantification results.

5.8.15.6.2. Open the Automation Workbook containing the batch of interest and click on the "Amp-CE" tab.

5.8.15.6.3. Click the "Export CE Setup File" button. A new text file named "Batch#_CESetup_Date" will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.15.6.3.1. If no plate name has been filled in, the file will contain a default plate name in the format "Batch#_Date_01". This can be changed on the "Amp-CE" tab or in the genetic analyzer software.

5.8.15.6.4. Samples may be added manually to the CE plate record and "CE Setup" tab, as needed, before clicking the "Export CE Setup File" button. Only samples listed on the "CE Setup" tab will be included in the exported CE Setup file.

5.8.15.6.5. Fill out the rest of the worksheet as designated, including dates, reagent lot numbers, amplification reaction type, and injection protocols.

5.8.16. PowerPlex® Y23 Amplification
Prepared on the Biomek i5 Laboratory Automation Workstation

5.8.16.1. NOTE: PowerPlex® Y23 amplification via Biomek automation is not validated for casework unknowns and shall only be performed on known samples.

5.8.16.2. Normalization Data Setup

5.8.16.2.1. Open the Automation Workbook file containing the batch to be amplified.

5.8.16.2.2. Assuming the quantitation data for the batch has already been imported and processed (see preceding section for Fusion 6C amplification), go to the Data Extract tab.

5.8.16.2.2.1. The "Amp YSTR" column on the right contains TRUE/FALSE designations for each sample. This controls whether a sample will be included in a Y-STR amplification. Samples are automatically marked "TRUE" for Y-STR amplification if they both:

1) Have a F:M ratio ≤ 5
2) Are marked as a standard (Code S)

5.8.16.2.2.2. Y-STR amplification may be manually turned on/off per sample by editing the Amp YSTR column values.

5.8.16.2.2.3. If the Amp YSTR column reflects the samples that should be amplified, click the "Amp Y-STR" button above it.

5.8.16.2.3. The Automation Workbook will analyze the batch according to the Y quant values and create a Y-STR version of the following worksheets: Amplified Samples Summary, Amplification Setup/CE Plate Record, and CE Setup.

These worksheets are normally hidden in the Workbook but will appear as blue tabs.

5.8.16.2.4.   A new .csv file named "Batch#_YSTR_AmpSetup" (the AmpSetup file) will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.16.2.5.   Move or copy this YSTR_AmpSetup file to the folder //IN PROGRESS/Amp Setups on the "Robot Team" drive.

5.8.16.2.6.   Proceed with Instrument Deck Setup, Reagent Preparation, Running the Normalization/Amp Setup Method, and Create CE Plate Record as described in the previous section, *except*:

5.8.16.2.6.1.   For Instrument Deck Setup, open the "Normalize-Amp_PPY23" method contained in the Promega project.

5.8.16.2.6.2.   For Reagent Preparation, be sure to use the PowerPlex® Y23 amplification kit. Follow the master mix preparation recipe as prompted in the Biomek software.

For Create CE Plate Record, click on the "Export CE Setup File" on the "Y-Amp-CE" worksheet.

5.8.17. Final Tube Transfer

5.8.17.1.   The Automation Workbook consolidates samples that are to be returned to tubes and creates an output for the robot to cherry-pick those samples. The remaining samples will remain in the elution plate. Samples returned to tubes will include those given code "T" by the case analyst, samples with more than 50 ng/uL of small autosomal DNA, and any samples that meet ALL of the following criteria:

5.8.17.1.1.   The sample is code "M".

5.8.17.1.2.   The sample is not a standard.

5.8.17.1.3.   The sample contains male DNA >0.006 ng/uL.

5.8.17.1.4.   The sample contains <0.01 ng/uL of small autosomal DNA *or* has a female:male ratio >50.

5.8.17.2.   Open the Automation Workbook containing the set of samples that have been run, and click on the "FTT Layout" tab. Click the "Consolidate Samples" button on the upper right.

5.8.17.2.1.   The layout worksheet will tell you how many tubes/labels you will need. If you wish to print labels on a partially used sheet, you may designate how many labels are missing from the sheet in the "# of used labels" box. A map of a label sheet is included on the right for reference.

5.8.17.2.1.1.   You cannot use a partial sheet if the # of used labels + # of tubes required is greater than 80.

5.8.17.3. Click the "Export FTT File" button. A new .csv file named "<batch ID>_FTT Setup" will appear in the same folder that contains the Automation Workbook. Click "OK" to proceed.

5.8.17.4. Move or copy the .csv file to the folder //IN PROGRESS/FTT Setups on the "Robot Team" drive.

5.8.17.5. Place new, empty, labeled Molecular BioProducts (San Diego, CA) 1.5 ml screw-top tubes (Cat. no. 3474) in the tube racks (4x6 with white adapters), arranging the tubes according to the order shown in the "Consolidated Extracts in Tubes" layout on the "FTT Layout" tab of the Automation Workbook. Do not skip columns or spaces between samples.

5.8.17.6. Arrange the deck layout as shown in the figure below:

    5.8.17.6.1. Place sample tube racks on positions P5, P6, P8, and P9, again paying attention to sample order according to the FTT Layout.

    5.8.17.6.2. Place the plate containing extracted samples on position P4.

    5.8.17.6.3. Place a new box of Beckman Coulter Biomek P250 Span-8 Barrier Tips (for the Biomek NXp) or Biomek i-series 190 μl Barrier Tips (for Biomek i5) on position P1.
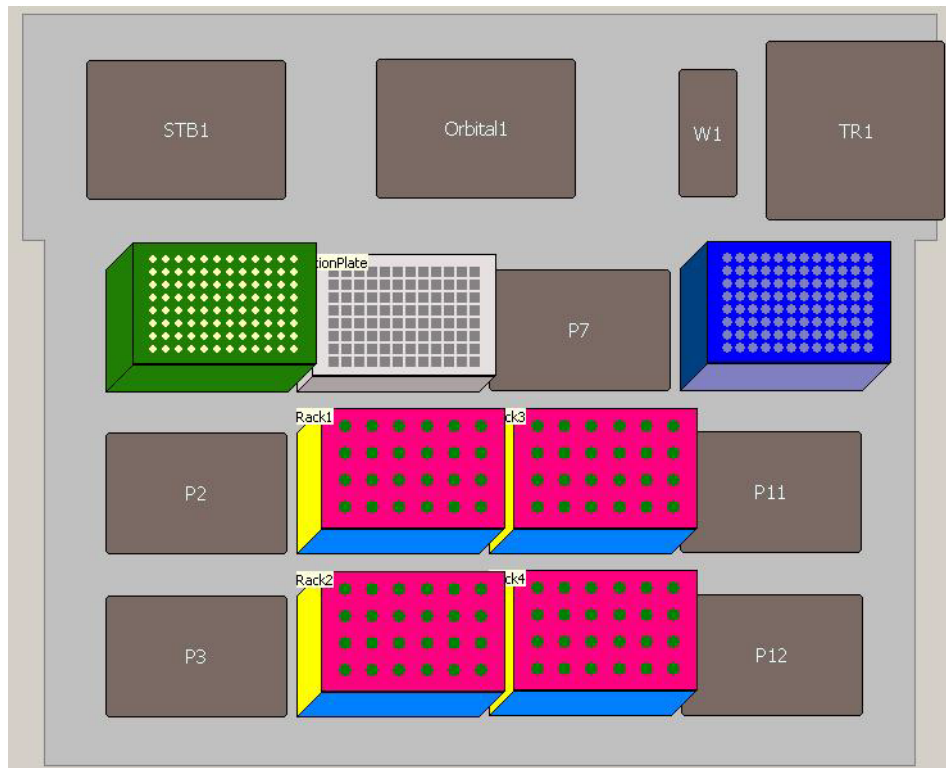
Biomek NX-p
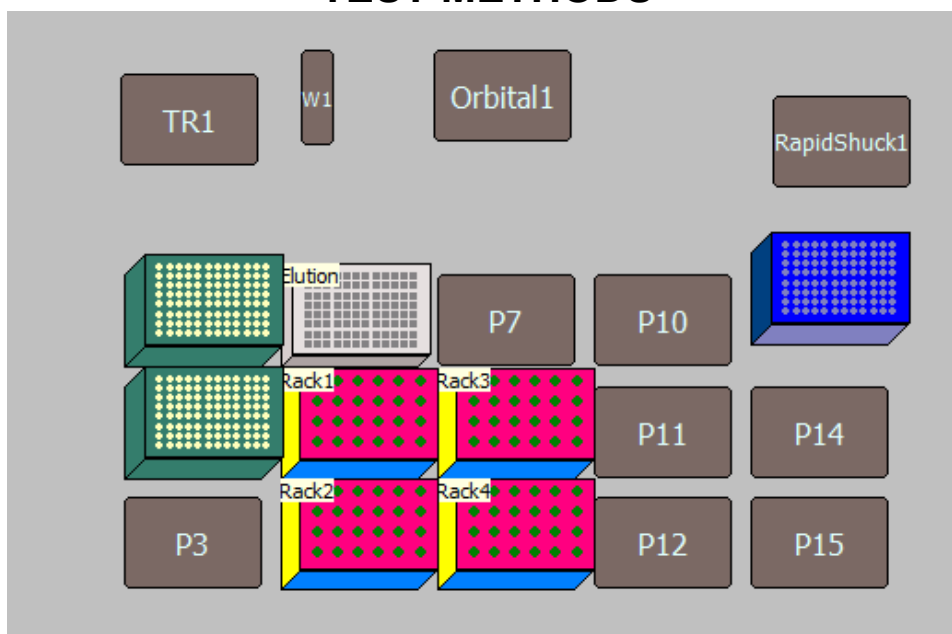
Biomek i5

5.8.17.7. Open the "Final Plate to Tube Transfer" method contained in the Promega project.

5.8.17.8. Click the green "Run" button at the top of the screen.

    5.8.17.8.1. The method will prompt for the Batch ID. Type the Batch ID in the format "Year-000" (e.g., "2017-001"). This tells the software which .csv file to use in the folder "FTT Setups" mentioned above.

5.8.17.9. When the instrument has finished transferring sample from the extraction plate to the tubes, remove the tube racks from the deck and cap the screw-top tubes.

    5.8.17.9.1. The extraction plate, which now contains only extracts of reference samples and property crime samples, shall be covered with a temporary seal, refrigerated, and saved for a minimum of 60 days, after which it may be discarded.

5.8.18. When processing is completed, the submitting analyst will be notified by e-mail that the data is available on the DNA server and the extracted DNA samples can be retrieved from a designated location.

5.9. **Records:**

5.9.1. **Sample Tracking for Automated Processing of Casework Samples**

5.9.1.1. The below documentation steps are unnecessary if an automation analyst is processing their own case samples throughout the entire automation process.

5.9.1.2. As part of the process of preparing samples for automated processing, the analyst shall complete the most current version of the Robot Submission Form, then email the form to DAutomation@isp.in.gov.

5.9.1.3. This form, as well as the email that included it, shall be retained by the analyst in the case record.

5.9.1.4. This documents a step in the chain of custody and denotes the point of divergence of extracts from their associated parent samples' chain of custody if the extract is later requested to be retained as a subitem. The extracts leave the analyst's custody and are transferred to a storage area where they will be retrieved for automated processing.

5.9.1.5. At IRL - Within the same workday that the Robot Submission Form is submitted, the analyst shall deliver all associated samples to the IRL S201A robot room storage area. ERL, LRL, and FWRL samples will be delivered with the next evidence appointment.

5.9.1.6. The robot operator shall collect samples from the storage area for automated processing. The transfer shall be documented for the case record on the Biomek NX Automated Extraction/Quantitation Worksheet.

5.9.1.7. The name (or initials) of robot operator receiving custody of samples, as well as the date of transfer, shall be marked on the worksheet.

5.9.1.8. Following extraction, the lysis portion of the samples shall be retained (sealed in plate) in the IRL S201 robot room storage area. The lysis plate shall be retained for approximately 60 days.

5.9.1.9. If the lysis plate is required for reprocessing, the robot operator shall assume custody of the lysis plate and associated samples. This transfer shall be documented in the case record to illustrate a clear, linear chain of custody and shall include, at a minimum, the name (or initials) of robot operator and date of transfer.

5.9.1.10. After approximately 60 days post-processing, the lysis plate shall be discarded.

5.9.1.11. Each subsequent step of automated processing (quantitation, amplification, and CE Injection) may be performed by the initial operator, or by another robot operator. For each step, name (or initials) of the robot operator, as well as the date of the step performed, shall be documented in the case record on the corresponding automation worksheets.

5.9.1.12. When different operators perform downstream steps of automated processing, the worksheets serve to document the dates of transfer from operator to operator at each point of automated processing.

5.9.1.13. If processing requires repeating steps or changes that result in additional transfers between operators and/or analysts, this shall be documented in the case record to illustrate a clear, linear chain of custody and shall include, at a minimum, the name (or initials) of individuals and date of transfer.

5.9.1.14. Following CE data generation, the robot operator shall send an email to the analyst informing them that data (and, if applicable, extracts) are available for collection.

5.9.1.15. If any associated extracts are designated to be retained for preservation or additional manual processing (e.g., Y-STR), a robot operator shall transfer those extracts into individual tubes and return these extracts to the IRL S201A robot room storage area within the same workday that the notification email is delivered.

5.9.1.16. This email shall be retained by the analyst in the case record. This documents a step in the chain of custody and denotes the point where the extract is transferred from the operator's custody to the storage location.

5.9.1.17. An analyst may transfer extracts destined for additional manual processing from the plate into individual tubes. This shall be clearly documented by the analyst in the case record. This documents a step in the chain of custody from robot operator to analyst, and shall include, at a minimum, the name (or initials) of analyst and the date of transfer.

5.9.1.18. If an extract designated to be retained is completely consumed (or if the extract has completely evaporated prior to tube transfer), this shall be clearly documented by the robot operator in the automation case worksheets, and no tube shall be generated.

5.9.1.19. The extracts not designated to be retained (i.e., remaining in the sample plate) shall be transferred (sealed in plate) to the IRL S201A robot room storage area. These samples will be retained for approximately 60 days, in the event that the samples are required for retention or retesting (see above).

5.9.1.20. After approximately 60 days post-processing, any remaining extracts not designated for retention shall be discarded.

5.9.1.21. The analyst shall retrieve any retained extracts from the robot room storage area to return them to their custody. The date of the transfer shall be recorded by the analyst in the case record.

5.9.1.22. This documents a step in the chain of custody and denotes the point where the extract re-enters the analysts' custody and adopts the associated parent samples' chain of custody.

5.10.   Interpretation of Results:

5.10.1. N/A

5.11.   Report Wording:

5.11.1. N/A

5.12.   References:

5.12.1. N/A

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

6. **Next-Generation Sequencing Test Methods:**

   6.1. **Scope**: Next-generation sequencing (NGS) may be performed in cases involving missing persons and unidentified human remains where information beyond what is obtained with traditional STR analysis may assist in resolution of the case. DNA analysis performed prior to NGS analysis shall be in accordance with the test methods outlined in the DNA Methods section of this document.

   6.2. **Precautions/Limitations:** Samples that may be suitable for NGS analysis should be evaluated by a member of the NGS team to determine the most appropriate sample(s) to analyze in a case. The following should be considered:

      6.2.1. Cases that have not provided meaningful or conclusive autosomal results should be considered for analysis.

      6.2.2. Cases with previously determined unidentified human remains or missing person profiles may be considered for analysis.

      6.2.3. Due to the limitations of the technology, items which demonstrate mixed DNA profiles with autosomal STR analysis will not be considered for NGS. Exceptions require Technical Leader approval.

      6.2.4. For optimal results, samples from various cases/analysts may be pooled to achieve desired flow cell load.

   6.3. **Related Information:**

      6.3.1. Worksheet Manual

   6.4. **Instruments:**

      6.4.1. Verogen MiSeq FGx® Sequencing System – NGS platform that delivers multiplex results of an array of STR and SNP loci from a prepared library while also providing interpretation functions using the fully integrated Universal Software Analysis (UAS) feature.

   6.5. **Reagents/Materials:**

      6.5.1. See DNA Test Methods Section 2.5

      6.5.2. Ethanol

      6.5.3. Nuclease Free Water (NFH$_2$O)

      6.5.4. Amp Grade Water

      6.5.5. ForenSeq™ Signature Prep Kit

      6.5.6. MiSeq FGx® Reagent Kit

   6.6. **Hazards/Safety:**

      6.6.1. See DNA Test Methods Section 2.6

      6.6.2. Two components of the ForenSeq™ DNA Signature Prep Kit, LNA1 and LNW1, have hazardous properties. **CAUTION:** Both reagents contain formamide and B-mercaptoethanol. Formamide is a suspected carcinogenic and a reproductive toxicant. B-mercaptoethanol is a toxin and irritant that can cause nervous system and organ damage. Therefore, universal precautions shall be used when working with these samples. In addition, appropriate waste receptacles shall be utilized when disposing of these reagents after use.

      6.6.3. The cartridge of the MiSeq FGx® Reagent Kit contains formamide in reservoir 8. After a sequencing run using this cartridge, the contents of reservoir 8 shall be removed using universal precautions inside a fume hood and properly disposed.

6.6.4. In addition, the waste bottle within the MiSeq instrument contains potentially hazardous chemicals after a sequencing run. The contents shall be disposed of in the appropriate waste receptacle using universal precautions.

6.7. Reference Materials/Controls/Calibration Checks:

6.7.1. See DNA Test Methods Section 2.7

6.7.2. An assessment of the run quality metrics shall serve as a check of the accuracy and specificity of test results in addition to the testing of controls.

6.8. Procedures/Instructions:

6.8.1. **See DNA Test Methods Section 2.8 for extraction and quantification procedures**

6.8.1.1. Performing NGS analysis before or after autosomal STR analysis will be evaluated on a case-by-case basis.

6.8.1.2. NGS analysis may be performed on extracts previously used for autosomal STR testing or may require re-extraction of a sample.

6.8.1.3. Quantification results from previous autosomal STR testing may be used for amplification of the sample.

6.8.2. **ForenSeq™ DNA Signature Prep**

6.8.2.1. **Introduction**

6.8.2.1.1. The Verogen ForenSeq™ DNA Signature Prep Kit is a sequencing multiplex containing two primer sets that amplifies Short Tandem Repeats (STRs) and Single Nucleotide Polymorphisms (SNPs). Primer Set A amplifies 27 autosomal STRs, 7 X-STRs, 24 Y-STRs, and 94 identity informative SNPs. Primer Set B amplifies the same markers as Primer Set A plus 56 ancestry informative SNPs and 22 phenotype informative SNPs. Only Primer Set B is currently in use in the laboratory.

6.8.2.1.2. The ForenSeq™ DNA Signature Prep Kit locus-specific information is available at https://verogen.com/resources/product-documentation/?_products=forenseq-dna-signature-prep-kit in the ForenSeq™ DNA Signature Prep Reference Guide VD2018005.

6.8.2.1.3. The ForenSeq™ DNA Signature Prep Kit reagents come in three boxes. Box 1 should be stored at -25°C to -15°C. After first use, the 2800M positive control should be stored at 2°C to 8°C. Box 2 should be stored at -25°C to -15°C. LNS2 should be stored at room temperature and LNW1 should be stored at 2°C to 8°C after opening of Box 2. Box 3 should be stored at 2°C to 8°C.

6.8.2.1.4. The preparation of the Amplify and Tag Targets step (PCR1) shall be performed in the PCR amplification set-up area. All further steps shall be performed in a post-PCR area.

6.8.2.2. **Sample Preparation**

6.8.2.2.1. Samples shall be extracted and quantified using procedures in DNA Test Methods section 2.8.

6.8.2.2.2. The ideal target concentration is 1ng, however samples may be amplified in a range from 0.05-2ng. The Normalization Dilution Worksheet in the Sequencing Workbook is recommended for this purpose.

6.8.2.2.3. An attempt should be made to maximize samples on a flow cell. For Primer Set B, the capacity of a standard flow cell is 32 samples, and the capacity of a micro flow cell is 12 samples.

6.8.2.2.4. No fewer than 8 samples containing DNA shall be run on a flow cell. If fewer than 8 samples are available, additional positive control samples or standards may be added to the run to reach a minimum of 8 samples.

6.8.2.2.5. The maximum number of samples per flow cell may be exceeded if the overage is comprised of negative controls.

6.8.2.2.6. A ForenSeq Plate Record worksheet shall be used to track library prep. It is recommended to use the Sequencing Workbook for this purpose.

6.8.2.2.6.1. Add samples to plate record, balancing the number of samples in each column and row.

6.8.2.2.6.2. Assign i5 and i7 indexes to the sample setup, ensuring unique combinations are used. Libraries may be re-run as necessary as long as the index assignments don't overlap with those for the new libraries.

6.8.2.3. Amplify and Tag Targets (PCR1)

6.8.2.3.1. For initial use, thaw one tube each of PCR1 reaction mix, DPMB primer mix, FEM enzyme, and 2800M positive control at room temperature. After first use, all reagents should be returned to -25°C to -15°C except the 2800M which should be stored at 2°C to 8°C.

6.8.2.3.2. Label a 96-well plate FSP (for ForenSeq Sample Plate) to use for amplification. Crosslink the plate.

6.8.2.3.3. Determine the number of samples to be amplified including controls. Add approximately 10% extra reagent for overage to compensate for the loss that occurs during reagent transfer. Calculate the required amount of each component of the PCR master mix. Multiply the volume (µl) per sample by the total number of reactions to obtain the final volume (µl).

Components of Master Mix/sample:
4.7 µl PCR1
0.3 µl FEM
5.0 µl DPMB

6.8.2.3.4. Do not vortex the FEM enzyme. Pipette to mix.

6.8.2.3.5. Vortex PCR1 and DPMB tubes and centrifuge briefly.

6.8.2.3.6. Add the calculated volume of each component to a 1.5 ml tube. Pipette to mix and centrifuge briefly.

6.8.2.3.7. Add 10 µl master mix to each well of the FSP plate. Optionally, the master mix may be evenly distributed into each well of an eight-strip tube, then a multichannel pipette may be used to dispense.

6.8.2.3.8. Dilute 2 µl 2800M with 98 µl nuclease-free water in a 1.5 ml tube. Gently flick and centrifuge briefly. A new dilution shall be made for each run performed.

6.8.2.3.9. Add 5 µl sample, positive amplification control, or negative amplification control to the appropriate well in the FSP plate. Pipette to mix.

6.8.2.3.10. Seal the plate with a foil seal. Centrifuge plate briefly and place in a Veriti™ Thermal Cycler.

6.8.2.3.11. Select the **Sig Prep PCR1** protocol with the following amplification procedure. Approximately 3.5 hour cycling time.

1 Cycle	8 Cycles	10 Cycles	1 Cycle	Hold

98°C
3 minutes

96°C
45 seconds

80°C
30 seconds

54°C
2 minutes

68°C
2 minutes

96°C
30 seconds

68°C
3 minutes

68°C
10 minutes

10°C
∞

(All ramp rates set to 4%)
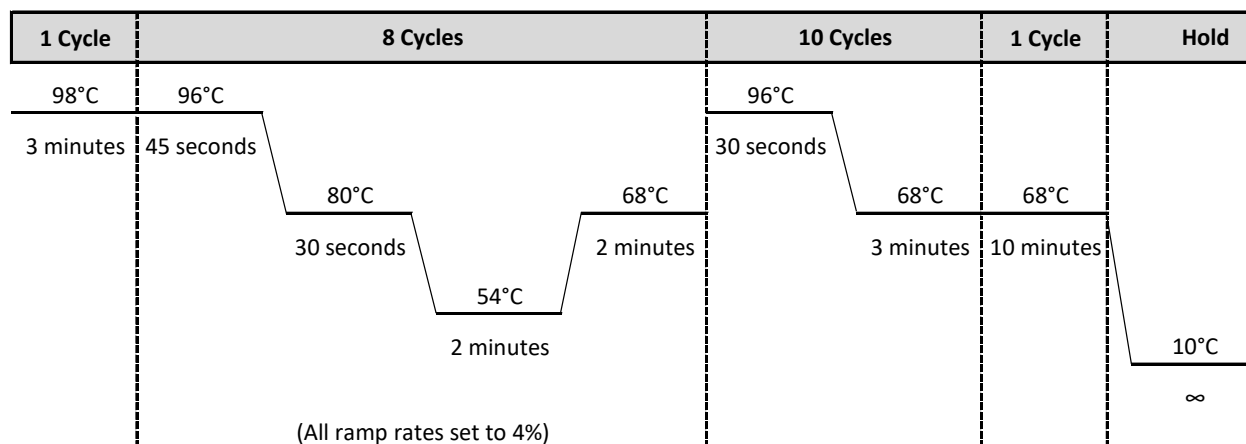
6.8.2.3.12. SAFE STOPPING POINT. Remove samples after the amplification process is completed and proceed immediately or the plate may be stored at 2°C to 8°C for up to two days. The plate may also be left on the thermal cycler overnight.

6.8.2.4. Enrich Targets (PCR2)

6.8.2.4.1. Thaw index adapters and PCR2 reaction mix at room temperature for approximately 20 minutes. Only thaw index adapters that are being used.

6.8.2.4.2. Vortex the index adapters and centrifuge briefly.

6.8.2.4.3. Centrifuge the FSP at 1000 x g (rcf) for 30 seconds.

6.8.2.4.4. Arrange i7 adapters in columns 1-12, or as many columns as are needed, of the ForenSeq Index Plate Fixture.

6.8.2.4.5. Arrange i5 adapters in rows A-H, or as many rows as are needed, of the ForenSeq Index Plate Fixture.

6.8.2.4.6. Place the FSP on ForenSeq Index Plate Fixture and remove the foil seal.

6.8.2.4.7. Add 4 µl i7 adapters to each column. Using a multichannel pipette is recommended. Replace the caps on the i7 tubes with new orange caps.

6.8.2.4.8. Add 4 µl i5 adapters to each row. Using a multichannel pipette is recommended. Replace the caps on the i5 tubes with new white caps.

6.8.2.4.9. Vortex PCR2 reaction mix and centrifuge briefly.

6.8.2.4.10. Add 27 µl PCR2 reaction mix to each well. If more than 8 samples are being processed, PCR2 reaction mix may be evenly distributed into each well of an eight-strip tube, then a multichannel pipette may be used to dispense.

6.8.2.4.11. Seal the plate with a foil seal and centrifuge at 1000 x g (rcf) for 30 seconds.

6.8.2.4.12. Place plate in a Veriti™ Thermal Cycler.

6.8.2.4.13. Select the **Sig Prep PCR2** protocol with the following amplification procedure. Approximately 46 minute cycling time.

1 Cycle	15 Cycles	1 Cycle	Hold

98°C
30 seconds

98°C
20 seconds

66°C
30 seconds

68°C
90 seconds

68°C
10 minutes

10°C
∞

(All ramp rates set to 4%)
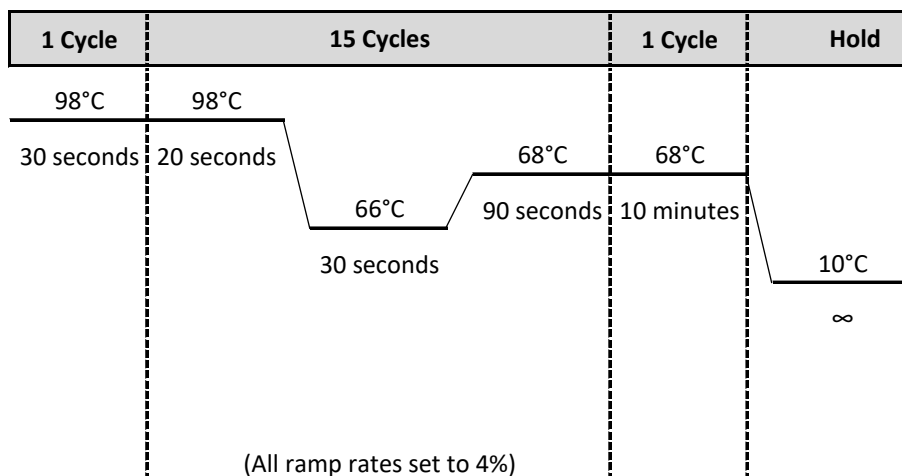
6.8.2.4.14. SAFE STOPPING POINT. Remove samples after the amplification process is completed and proceed immediately. Alternatively, the plate may be stored at 2°C to 8°C for up to seven days or left on the thermal cycler overnight.

6.8.2.5. **Purify Libraries**

6.8.2.5.1. Let RSB and SPB stand for approximately 30 minutes to bring to room temperature.

6.8.2.5.1.1. Vortex SPB before use and frequently during use to make sure that the beads are evenly distributed. Aspirate and dispense SPB slowly due to its viscosity.

6.8.2.5.2. Label a midi plate PBP (for Purification Bead Plate) and a PCR plate as PLP (for Purified Library Plate).

6.8.2.5.3. Prepare SPB according to the number of libraries you are preparing.

Number of libraries	Procedure
<16	Add 50 µl SPB x number of libraries to a 1.5 ml tube.
16-96	Add [50 µl SPB x (number of libraries/8)]+5 µl SPB to each well of a column of a new midi plate or a reagent reservoir.
>96	Add (50 µl SPB x number of libraries) + 200 µl SPB to a reagent reservoir.

6.8.2.5.4. Add 45 µl SPB to each well of the PBP plate according to the plate record.

6.8.2.5.5. Centrifuge the FSP plate at 1000 x g (rcf) for 30 seconds and remove the foil seal.

6.8.2.5.6. Transfer 45 µl sample from the each well of the FSP to the corresponding well of the PBP. Discard the FSP plate.

6.8.2.5.7.   Seal the PBP plate with a plastic adhesive seal and shake at 1800 rpm for 2 minutes.

6.8.2.5.8.   Incubate at room temperature for 5 minutes.

6.8.2.5.9.   Place the plate on a magnetic stand and wait until the liquid is clear (~2 minutes).

6.8.2.5.10.   Remove the clear adhesive seal.

6.8.2.5.11.   Remove and discard all supernatant from each well.

6.8.2.5.12.   On the magnetic stand, wash two times as follows:

6.8.2.5.12.1.   Add 200 µl freshly prepared 80% EtOH to each well.

6.8.2.5.12.2.   Incubate on the magnetic stand for 30 seconds.

6.8.2.5.12.3.   Remove and discard all supernatant from each well.

6.8.2.5.13.   Centrifuge at 1000 x g (rcf) for 30 seconds.

6.8.2.5.14.   Place the plate on the magnetic stand.

6.8.2.5.15.   Remove residual EtOH from each well. A 20 µl pipette is recommended.

6.8.2.5.15.1.   All EtOH must be removed from each well. Remaining EtOH can inhibit future steps.

6.8.2.5.16.   Remove the plate from the magnetic stand.

6.8.2.5.17.   Add 52.5 µl RSB to each well.

6.8.2.5.18.   Seal the plate with a plastic adhesive seal and shake at 1800 rpm for 2 minutes.

6.8.2.5.19.   Make sure after shaking that beads are in solution. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 2 minutes.

6.8.2.5.20.   Incubate at room temperature for 2 minutes.

6.8.2.5.21.   Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

6.8.2.5.22.   Remove the clear adhesive seal.

6.8.2.5.23.   Transfer 50 µl from each well of the PBP to the corresponding well of the PLP plate.

6.8.2.5.24.   Seal the plate with a plastic adhesive seal and centrifuge at 1000 x g (rcf) for 30 seconds.

6.8.2.5.25.   SAFE STOPPING POINT. The procedure may be continued immediately, or the plate can be sealed with a clear adhesive seal and stored at -25°C to -15°C for up to a year.

6.8.2.6.   **Normalize Libraries**

6.8.2.6.1.   This set of reagents contains formamide. LNA1 and LNW1 contain β-mercaptoethanol. These reagents, tips, and waste from these steps shall be disposed of in the appropriate hazardous waste container.

6.8.2.6.2.   Thaw HP3 and LNA1 at room temperature for at least 30 minutes. Let LNB1 and LNW1 stand for approximately 30 minutes to bring to room temperature.

6.8.2.6.3.   Vortex LNA1 with intermittent inversion. Hold LNA1 up to light to make sure no crystals are present after vortexing.

6.8.2.6.4.   Vortex LNB1 for at least 1 minute, stopping every 15 seconds to invert tube 5 times. Pipette to mix until the bead pellet at the bottom is resuspended.

6.8.2.6.5.   Label a midi plate NWP (for Normalization Working Plate) and a PCR plate NLP (for Normalization Library Plate).

6.8.2.6.6.   Create a master mix of LNA1 and LNB1. The amounts listed account for overage.

> **Components of Master Mix/sample:**
> 46.8 µl LNA1
> 8.5 µl LNB1

6.8.2.6.7.   Vortex then invert the tube several times to mix.

6.8.2.6.8.   Pour into a reagent reservoir.

6.8.2.6.9.   Transfer 45 µl to each well of the NWP that will contain a library.

6.8.2.6.10.   To clear any beads that may have aspirated, place the PLP on the magnetic stand and wait until the liquid is clear (~2 minutes).

6.8.2.6.11.   Remove the clear adhesive seal from the PLP plate.

6.8.2.6.12.   Transfer 20 µl from each well of the PLP plate to the corresponding well of the NWP plate.

6.8.2.6.13.   Seal the plate with a clear adhesive seal and shake at 1800 rpm for 30 minutes.

6.8.2.6.14.   If sequencing immediately after library prep, begin thawing the MiSeq FGx® Reagent Kit Box 1 (stored -25°C to -15°C) as described in 7.8.3.2.

6.8.2.6.15.   While the plate is shaking, perform the following:

6.8.2.6.15.1.   Prepare 0.1N HP3 in a new 1.5 ml tube. Amounts account for overage.

6.8.2.6.15.1.1. Add 33.3 µl nuclease-free water per sample.

6.8.2.6.15.1.2. Add 1.8 µl HP3 per sample.

6.8.2.6.15.1.3. Invert the tube several times to mix.

6.8.2.6.15.1.4. Set aside.

6.8.2.6.15.2.   Add 30 µl LNS2 to each well of the NLP plate that will contain library.

6.8.2.6.16.   Immediately after the NWP has finished shaking, place the NWP on the magnetic stand and wait until the liquid is clear (~2 minutes).

6.8.2.6.17.   Remove the clear adhesive seal.

6.8.2.6.18.   Remove and discard all supernatant from each well.

6.8.2.6.19.   Remove the plate from the magnetic stand.

6.8.2.6.20.   Wash two times with 45 µl LNW1 as follows:

6.8.2.6.20.1.   Add 45 µl LNW1 to each well.

6.8.2.6.20.2.   Seal with a clear adhesive seal and shake at 1800 rpm for 5 minutes.

6.8.2.6.20.3. Make sure after shaking that beads are in solution. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 5 minutes.

6.8.2.6.20.4. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

6.8.2.6.20.5. Remove the clear adhesive seal.

6.8.2.6.20.6. Remove and discard all supernatant from each well.

6.8.2.6.21. Remove the plate from the magnetic stand.

6.8.2.6.22. Seal with clear plastic adhesive seal and centrifuge at 1000 x g (rcf) for 30 seconds.

6.8.2.6.23. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

6.8.2.6.24. Remove the clear adhesive seal.

6.8.2.6.25. Remove residual supernatant from each well. A 20 µl pipette is recommended.

6.8.2.6.26. Remove the plate from the magnetic stand.

6.8.2.6.27. Add 32 µl freshly prepared 0.1 N HP3 to each well.

6.8.2.6.28. Seal the plate with a clear plastic adhesive seal and shake at 1800 rpm for 5 minutes. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 5 minutes.

6.8.2.6.29. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

6.8.2.6.30. Remove the clear adhesive seal.

6.8.2.6.31. Transfer 30 µl from each well of the NWP to the corresponding well of the NLP plate. Pipette to mix.

6.8.2.6.32. Seal with clear plastic adhesive seal and centrifuge at 1000 x g (rcf) for 30 seconds.

6.8.2.6.33. SAFE STOPPING POINT. The procedure may be continued immediately, or the plate can be sealed with a clear adhesive seal and stored at -25°C to -15°C for up to 30 days.

6.8.2.7. **Pool Libraries**

6.8.2.7.1. Determine which libraries to pool for sequencing.

6.8.2.7.2. Label a 1.5 ml tube PNL (for Pooled Normalized Libraries).

6.8.2.7.3. Remove clear plastic seal.

6.8.2.7.4. Transfer 5 µl of each library from NLP plate to an eight-tube strip.

6.8.2.7.5. The NLP plate should be sealed and stored at -25°C to -15°C for up to 30 days.

6.8.2.7.6. Transfer the contents of each well of the eight-tube strip to the PNL tube.

6.8.2.7.7. Vortex the PNL tube and centrifuge briefly.

6.8.2.7.8. SAFE STOPPING POINT. The procedure may be continued immediately, or the tube can be stored at -25°C to -15°C for up to 30 days.

6.8.2.8.   **Prepare the Human Sequencing Control**

    6.8.2.8.1.   Remove the HSC (Human Sequencing Control) and the HP3 (2N NaOH) from the ForenSeq™ DNA Signature Prep Kit and allow to thaw completely at room temperature.

    6.8.2.8.2.   Flick to mix the HSC and centrifuge briefly to pull contents to the bottom of the tube. Vortex the HP3 and centrifuge briefly.

    6.8.2.8.3.   Combine in a 1.5ml tube:

                2 µl of HSC

                2 µl of HP3

                36 µl nuclease free water

    6.8.2.8.4.   Vortex and centrifuge briefly.

    6.8.2.8.5.   Incubate at room temperature for 5 minutes.

6.8.2.9.   **Dilute and Denature Libraries**

    6.8.2.9.1.   Label a 1.5 ml tube DNL for denatured normalized libraries.

    6.8.2.9.2.   If not previously thawed, remove the Hybridization Buffer (HT1) from the MiSeq FGx® Reagent Kit Box 1 (stored -25°C to -15°C) and thaw completely at room temperature.

    6.8.2.9.3.   Combine 591 µl of HT1 with 7 µl of the pooled normalized libraries (PNL). Pipette to mix.

    6.8.2.9.4.   The PNL can be stored at -25°C to -15°C for up to 30 days.

    6.8.2.9.5.   Add 4 µl of the prepared HSC mixture. Pipette to mix.

    6.8.2.9.6.   Vortex and centrifuge briefly.

    6.8.2.9.7.   Denature at 96°C for 2 minutes in a pre-heated thermomixer. Invert several times to mix.

    6.8.2.9.8.   Place on ice or into a cold block for 5 minutes.

6.8.3.   **Verogen MiSeq FGx® Sequencing System**

6.8.3.1.   **Introduction**

    6.8.3.1.1.   The Verogen MiSeq FGx® Sequencing System is a sequencing instrument that performs sequencing by synthesis, a type of targeted DNA sequencing. The system uses instrument-specific reagents and flow cells to measure fluorescence signals of labeled nucleotides. The system also includes internal imaging hardware and data analysis software.

    6.8.3.1.2.   The data produced by the Verogen MiSeq FGx® Sequencing System is analyzed with an internal software that performs base calling, demultiplexes, aligns data to targeted regions and reports STR and SNP calls. Analyst data analysis occurs in Verogen's Universal Analysis Software (UAS).

    6.8.3.1.3.   The MiSeq FGx® Reagent Kit materials come in two boxes. Box 1 should be stored at -25°C to -15°C. Box 2 should be stored at 2°C to 8°C.

6.8.3.2.   **Reagent Cartridge Preparation**

6.8.3.2.1. Thaw the MiSeq FGx® Reagent Kit (Box 1). The MiSeq FGx® Reagent Kit (Box 1) contains the reagent cartridge and the hybridization buffer (HT1). Thaw the HT1 at room temperature. Thaw the reagent cartridge in a room temperature water bath for approximately 60-90 minutes. Do not submerge the cartridge above the maximum water line. Alternatively, thaw the reagent cartridge overnight in the refrigerator.

6.8.3.2.1.1. The thawed reagent cartridge may be stored on ice for up to 6 hours if necessary. The MiSeq FGx® Reagent Kit may not be thawed and refrozen.

6.8.3.2.2. Once thawed, invert the cartridge at least ten times to mix. Ensure all reagent reservoirs are completely thawed and there are no precipitates. Remove bubbles and excess water by firmly tapping the cartridge on the benchtop. Dry the bottom of the cartridge with a lab wipe.

6.8.3.2.3. In a fume hood, pierce the foil of the highlighted sample position on the reagent cartridge, using a clean pipette tip.

6.8.3.2.4. Load the entire volume of the diluted denatured libraries (DNL) into the highlighted position on the reagent cartridge, taking care to avoid bubbles.

6.8.3.2.5. Tap the cartridge to ensure contents move to the bottom of the well.

6.8.3.3. **Create a Run**

6.8.3.3.1. Log in to the ForenSeq™ Universal Analysis Software.

6.8.3.3.2. Select **Create New Run** in the upper right corner.

6.8.3.3.3. Select the appropriate type of flow cell for the run (micro or standard)

6.8.3.3.4. The run name shall be the batch ID and the date the run is started (ex. 001-2023_01Jan23).

6.8.3.3.5. An optional description may be entered if desired.

6.8.3.3.6. Ensure that **Forensic Genomics** is selected in the Application box.

6.8.3.4. **Import or Enter Samples**

6.8.3.4.1. To import samples from the Sequencing Workbook (recommended) or other text file, select **Import Samples**.

6.8.3.4.2. Drag and drop or click to select the appropriate file for upload.

6.8.3.4.3. To manually enter sample details, select **Add New Samples** or **Add Existing Samples**.

6.8.3.4.3.1. Enter sample names, index combinations, and sample type into the appropriate fields.

6.8.3.4.3.2. Ensure Mix Type B is selected.

6.8.3.4.3.3. Select **Add New Sample**. Repeat for all samples in the run, then select **Save Run**. The run will now be added to the queue.

6.8.3.5. **Start the Sequencing Run**

6.8.3.5.1. On the Verogen MiSeq FGx®, ensure server 1.3 is selected and log in with a valid username and password.

6.8.3.5.2. Select **Forensic Genomics** as the run type.

6.8.3.5.3. Select the run previously created in the Universal Analysis Software.

6.8.3.5.4. Remove the flow cell and the PR2 from the MiSeq FGx® Reagent Kit Box 2 (store at 2°C to 8°C.)

6.8.3.5.5. Load the flow cell, PR2, waste bottle and previously prepared reagent cartridge as prompted by the software.

6.8.3.5.5.1. The flow cell must be thoroughly rinsed with water to remove excess salts. Dry the flow cell, taking care not to damage the gasket.

6.8.3.5.5.2.   Use an alcohol wipe or a lab wipe moistened with ethanol to clean the flow cell glass. Do not use alcohol on the flow cell gasket. Ensure flow cell glass is clean and dry and that the gasket is properly seated before loading.

6.8.3.5.6. The instrument will perform a pre-run check, verifying all the RFID tags for the consumables. When complete, select **Start Run**.

6.8.3.5.7. If a RFID tag cannot be read, it can be entered manually using the keyboard. If necessary, contact Verogen technical support for assistance.

6.8.3.6. **Evaluate the Sequencing Run**

6.8.3.6.1. When the run is complete, select **Next**. Ensure the sequencing run completed successfully. The sequencing run status may be viewed on the sequencer in real time or evaluated in the Universal Analysis Software after the run completes. Run Quality Metrics should be evaluated as described in 7.8.4.1.

6.8.4. **ForenSeq™ Universal Analysis Software Data Analysis**

6.8.4.1. **ForenSeq™ Universal Analysts Software Settings are detailed in [Appendix 17](#).**

6.8.4.2. **Introduction**

6.8.4.2.1. Run information generated by the MiSeq FGx® Sequencing System is stored on the ForenSeq server. Upon run completion, files are transferred to the Universal Analysis Software (UAS) which initiates the analysis of data using the default analysis settings.

6.8.4.2.2. UAS performs analysis processes including demultiplexing, sequence alignment, allele counting, genotype calling, stutter determination, noise filtering, and quality assessment.

6.8.4.2.3. Analytical thresholds (AT) and interpretation thresholds (IT) are percent-based values of the total read count at each locus.

6.8.4.2.4. The internally determined and validated AT and IT are 1.5% and 4.5%, respectively, for all STR and SNP loci, with the exception of DYS389II at 5.0% and 15% and DYS448 and DYS635 both at 3.3% and 10%.

6.8.4.2.5. For instances of low read coverage, a minimum read count of 650 reads is used to establish the AT and IT which correlates to an AT of 10 reads and IT of 30 reads.

6.8.4.2.6. Stutter is filtered by the UAS for back, forward, and double back stutter using internally determined and validated back (n-3/n-4/n-5/n-6) stutter parameters. For forward (n+3/n+4/n+5/n+6) and double back (n-6/n-8/n-10/n-12) stutter, the square of the back stutter value is applied for filtering.

6.8.4.2.7. The ISP default analysis method has been set within the UAS incorporating the AT and IT thresholds and stutter percentages listed in [Appendix 18](#).

6.8.4.2.8. Stochastic thresholds (ST) have been internally determined and validated. For all STR loci, a stochastic threshold of 450 reads shall be applied. A concentration based stochastic threshold shall be applied to SNP loci. Samples with less than 0.1ng of target DNA shall have a stochastic of 200 reads. Samples between 0.1ng and less than 1ng shall have a stochastic of 100 reads. Samples with 1ng or greater target DNA require no stochastic application.

6.8.4.2.9. ST are not incorporated into the ISP default settings within the UAS. ST must be applied during interpretation either within the UAS (by toggling or untyping) or by using SeqLite.

6.8.4.3. **Evaluating Run Quality Metrics**

6.8.4.3.1. To open the analysis of a project (plate), select the Project name.

6.8.4.3.2. Analysis methods are then listed in the left column of the Project page. Some basic run information is also available in this left column including the number of samples in project and review of controls.

6.8.4.3.3.   To access the results of the quality metrics and controls for a project, select the appropriate analysis method which will then open the Analysis Page. Note: projects analyzed with the ISP Default Analysis Settings will be listed as **1.0 – Batch ID**.

6.8.4.3.4.   Before evaluating the quality metrics and controls, ensure that the analysis state is listed as completed.

6.8.4.3.5.   The preliminary results of the positive controls, negative controls and quality metrics are listed in a box in the middle of Analysis Page with color-coded run quality indicator icons. Each icon can be clicked to expand for additional details.

6.8.4.3.5.1.   The Positive Control icon includes the results for the typing of the 2800M Control and the Human Sequencing Control (HSC).

6.8.4.3.5.2.   The Negative Control icon includes the results for all reagent blanks and amplification negative controls.
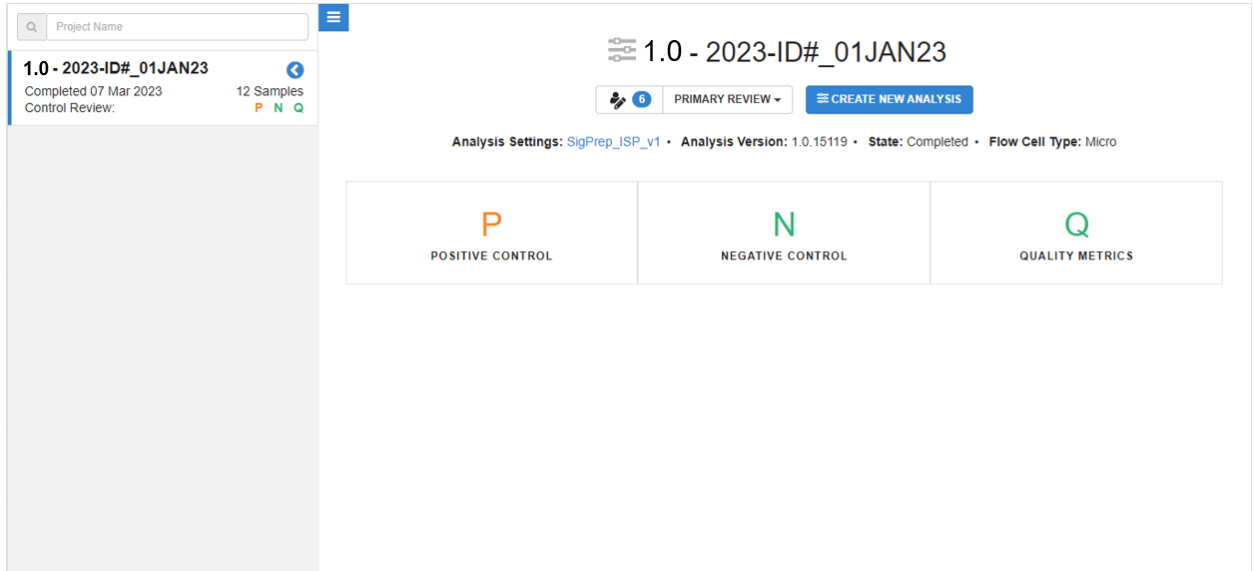
6.8.4.3.6. Quality Indicator Results for Controls and Quality Metrics

Color Coded Indicator	Positive Control	Human Sequencing Control	Negative Control	Quality Metrics
Green	All STR and SNP loci are typed and concordant with 2800M DNA Control.	Concordant genotype determined with sufficient coverage for the sample.	All designated reagent blanks or negative controls have no typed STR or SNP alleles.	All metrics are in recommended ranges.
Orange	At least 1 locus is untyped or disconcordant with 2800M DNA Control. Total number of reads for sample may be less than 85,000 reads.	Discordant genotype determined or overall intensity not met.	At least 1 STR or SNP has demostrated typed results.	At least 1 metric is not within recommended ranges.

6.8.4.3.7. Controls or quality metrics highlighted in green pass recommended parameters for samples. If highlighted in orange, further evaluation or interpretation of the sample is required.

6.8.4.3.7.1. Incomplete coverage or dropout may occur in the typing of a positive control and shall not be considered a failed result. A positive control is considered a failed control if incorrect genotypes are determined for the sample.

6.8.4.3.7.2. The Human Sequencing Control is an internal sequencing control that presents either pass or fail results. The actual sequencing result is not viewable in the UAS; however, any discordant results will be listed. Consult with the Technical Leader if a failed result is obtained.

6.8.4.3.7.3. Negative controls should have no typed results as no template DNA should be present in the samples. A negative control which demonstrates read counts above IT at more than 5 loci shall be considered a failed control.

6.8.4.3.7.4. Ensure that Quality Metrics meet the following guidelines under the Run Metrics tab.

Run Quality Metric	Recommended Ranges
Cluster Density	400 - 1650 k/mm^2
Cluster Passing Filter	≥80%
Phasing	≤0.25%
Pre-phasing	≤0.15%

6.8.4.3.8. Evaluate additional metrics, including Index CV and number of read counts across samples, under the Sample Representation tab, to further assess the quality of the run and samples.

6.8.4.3.8.1. Samples demonstrating less than 85,000 read counts are considered to have low coverage and should be interpreted with caution.

6.8.4.4.   **Interpreting, Comparing and Reporting DNA Results Associated with Failed Controls and Contamination Events**

    6.8.4.4.1.   The Technical Leader shall be notified if a positive or negative control fails and/or if sample contamination occurs. Sample contamination is defined as additional peaks present in an unknown or reference sample that can be conclusively identified to not have originated in the sample (e.g., case to case contamination or a mixture in a reference sample).

    6.8.4.4.2.   Assessment of the integrity of the associated DNA results shall be done in consultation with the Technical Leader. This shall include the possible cause and effect of the failed control or contamination. Additionally, an assessment of the risk associated with moving forward with interpretation shall be documented, as well as the risks associated with retesting (e.g., unnecessary consumption of evidence).

        6.8.4.4.2.1.   If retesting is performed, the reasons for doing so shall be documented.

        6.8.4.4.2.2.   If results are determined to be suitable for interpretation without retesting, the appropriate interpretations, comparisons, and statistical results and a description of the failed control or contamination event shall be reported per the Report Writing Guidelines.

        6.8.4.4.2.3.   If results are determined to be not suitable for interpretation and retesting cannot be performed, the results shall be reported as not suitable for comparison per the Report Writing Guidelines.

    6.8.4.4.3.   Documentation in the case record shall include at a minimum: the forensic sample, reference or control test results that failed or was contaminated, the likely or known cause of the failed control or contamination, the likely or known source of the contamination, the impact on the integrity of the DNA results, and the determination if the test result is suitable or unsuitable for interpretation.

6.8.4.5.   **General Sample Detail Information**

    6.8.4.5.1.   Navigate to sample results by selecting the blue arrow in the left column of the Project page to expand the sample list. Specific samples can then be opened for review in a Sample Details page.

    6.8.4.5.2.   Within each Sample Details page, results for STR and iiSNPs are viewable for interpretation. To access the piSNP and aiSNP results, navigate to Phenotype Estimation page as outlined in Section 6.12.

    6.8.4.5.3.   Sample and locus level quality indicators may be present which may aid in sample interpretation at the locus level in the Sample Details page.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

6.8.4.5.4.   Information about the sample includes a graph with read intensities of each typed STR and SNP locus, color-coded indicator used to identify single source samples, color-coded indicator highlighting interlocus balance performance, gender determination summary, and the typing results across the loci.
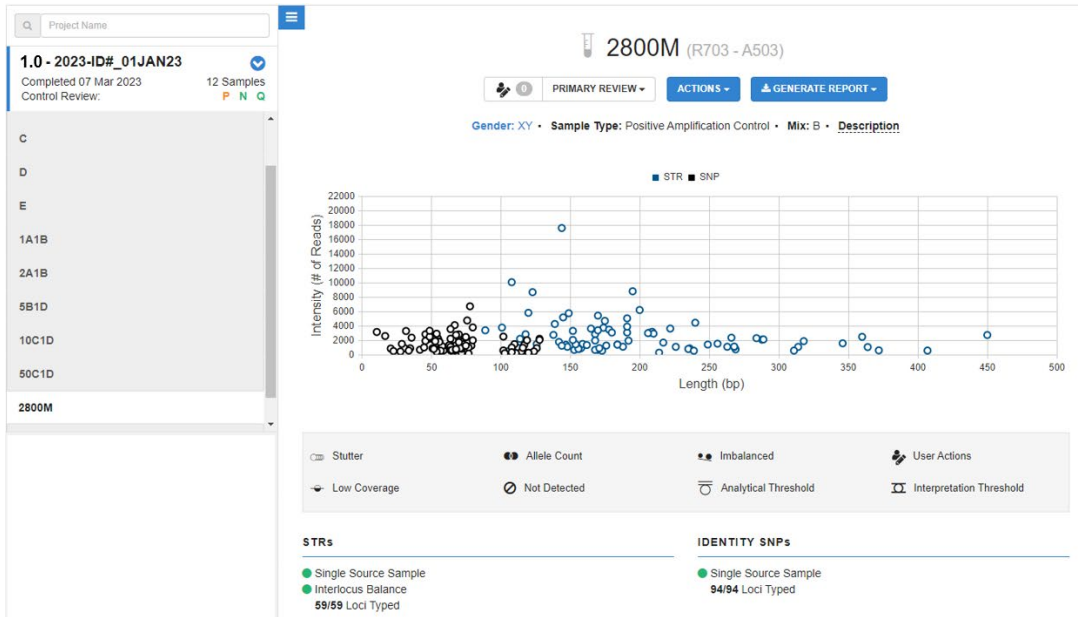
6.8.4.5.4.1.   UAS utilizes sample results to indicate if a sample appears to be single source or may be a mixture.

6.8.4.5.4.1.1.   If no or few locus level quality indicators are flagged, then the sample will have a green color-coded indicator for single source.

6.8.4.5.4.1.2.   If greater than 5 STR loci and/or greater than 10 iiSNP loci yield locus level quality indicators, then an orange color-coded indicator will be displayed for single source sample indicating the possible presence of a mixture.

6.8.4.5.4.2.   Interlocus balance of the STR loci is assessed internally within the UAS. A green indicator shows that the balance is within the recommended range while an orange indicator shows that the balance falls outside of optimal range.

6.8.4.5.5.   Locus level quality indicators shall be evaluated and can aid in determination of single source contributor or mixture. Locus level quality indicators are listed in the image above.

6.8.5. **Interpretation Guidelines for ForenSeq™ Signature Prep Data Analysis**

6.8.5.1.   **Sample Evaluation of Results**

6.8.5.1.1.   Each locus with quality indicator flags shall be reviewed.

6.8.5.1.2. It shall be at the analyst's discretion, based on experience and training, to determine if read counts are true alleles, stutter, noise, or artifacts. If read counts are determined to not be true alleles, then the analyst shall untype the read counts and indicate a reason in the comment field.

6.8.5.1.2.1. **Stutter:** Read counts that fall in a stutter position that exceed the stutter filters applied by the UAS software may be removed by the analyst. The sequence of the possible stutter shall be reviewed to further assess the association to the allele before removal.

6.8.5.1.2.2. **Sequence-Based Noise:** Read counts that exceed the filter range for noise may be removed by the analyst. The sequence of the possible noise shall be reviewed to further assess the association to the allele before removal. Sequence-Based Noise that differs by more than 1 bp can also be present and removed by the analyst.

6.8.5.1.2.3. **Artifacts:** A documented artifact has been observed at STR locus D7S820 with the addition of a single T bp at the end of the sequence resulting in +.1 base pair allele call. This artifact typically occurs at a much lower intensity than the primary allele read count. An artifact has also been observed at the piSNP locus rs201326893_Y152OCH which shows as read counts for the allele T (an unexpected genotype allele at this locus) typically under IT for the locus. However, this artifact may show as a typed allele in a low read count sample.

6.8.5.1.3. Single source, mixture, and gender determination

6.8.5.1.3.1. The overall profile, including results of all STR and SNP loci, shall be evaluated to determine if a sample is single source or a mixture.

6.8.5.1.3.2. Untyped read counts under AT or IT may indicate the presence of an additional contributor.

6.8.5.1.3.3. If there are no typed genotype results indicating the presence of a mixture, then the number of contributors does not need to be increased due to the presence of untyped read counts. Analyst's discretion may be used to

determine if the untyped read counts interfere with the ability to interpret the typed component.

6.8.5.1.3.4. Generally, a single source profile should contain no more than two alleles at each autosomal STR or SNP locus. For gender specific markers, profiles from female individuals should contain no more than two alleles for the X-STR loci and no alleles for the Y-STR loci. Profiles from male individuals should contain only one allele for each X-STR and Y-STR locus, with the exception of DYS385a-b and DYF387S1 which may have up to two alleles.

6.8.5.1.3.4.1. Three allele patterns in autosomal STR loci as well as unexpected two allele patterns in the Y-STR loci have been observed and may not necessarily indicate the presence of a mixture. A sample with only one autosomal STR locus demonstrating this pattern could be an indication of a tri-allelic locus. A sample with only one Y-STR locus demonstrating this pattern could be an indication of a duplication event.

6.8.5.1.3.4.2. Other patterns outside of the general expectations for a single source profile may be observed. Consultation with the Technical Leader is required to determine the necessary action for the locus.

6.8.5.1.3.4.3. Biological anomalies such as tri-alleles or duplications should be confirmed through concordance with an additional sample contained within the case or re-amplification. Alternatively, amplification with PowerPlex® Fusion 6C or PowerPlex® Y23, for applicable loci, may be performed.

6.8.5.1.3.5. Analysis algorithms within UAS based on allele counts are used for preliminary gender determination for a sample. However, gender determination shall be established by the analyst and then gender information can be used to aid in interpretation and/or typing of gender specific markers.

6.8.5.1.3.6. Profiles with typed alleles in excess of the expectations of a single source profile or of tri-allelic or duplication patterns shall be defined as mixtures. Additionally, a profile shall be defined as a mixture if the only additional alleles present are typed in the gender specific markers.

6.8.5.1.3.7. Determination of the number of contributors in a mixture shall be done at the analyst's discretion, based on experience and training, and should take into account number of alleles present as well as allele count ratios and possible genotype pairings.

6.8.5.2. **Single Source Profile Interpretation**

6.8.5.2.1. Due to stochastic effects, low coverage, and intralocus imbalance issues, careful evaluation of untyped additional components shall be performed using the established Analytical, Interpretation, and Stochastic Thresholds.

6.8.5.2.2. The following guidelines shall be used to aid in genotype determinations for read counts below thresholds if present.

6.8.5.2.2.1. See also Interpretation Guide (Appendix 19).

6.8.5.2.3. A profile shall be evaluated for stutter and/or artifacts prior to the interpretation of untyped additional components.

6.8.5.2.4. **STR Loci Genotype Determinations**

6.8.5.2.4.1.   Read counts below analytical threshold shall not be typed or interpreted.

6.8.5.2.4.2.   Read counts below interpretation threshold may be used in genotype determinations in some instances.

6.8.5.2.4.2.1. If no typed alleles are present, then read counts below IT shall not be used for interpretation. Therefore, there shall not be any toggling/typing of read counts below IT.

6.8.5.2.4.2.2. If there is one typed allele, then additional untyped read counts (i.e., an additional component) below IT shall be further analyzed to determine if typing is warranted. Read counts below IT that become typed (toggled) can be used for interpretation.

6.8.5.2.4.2.2.1. If the additional component is in a stutter position and not previously identified as stutter, then no additional typing shall be done, and the genotype shall be considered inconclusive due to ambiguity for the sister allele determination.

6.8.5.2.4.2.2.2. If the additional component is not in stutter position, then the Allele Count Ratio (ACR) between the typed allele and the additional component shall be determined.

6.8.5.2.4.2.2.2.1.   ACR shall be calculated by dividing the read counts of the additional component by the read counts of the typed allele.

6.8.5.2.4.2.2.2.2. If the ACR is >0.30, the additional component shall be typed resulting in a heterozygous genotype determination for the locus.

6.8.5.2.4.2.2.2.3. If the ACR is not greater than 0.30, then no additional typing shall be done, and the genotype shall be considered inconclusive due to ambiguity for the sister allele determination.

6.8.5.2.4.2.3. If there are two typed alleles, with only one greater than the ST, the ACR should be evaluated in loci with an imbalance quality indicator.

6.8.5.2.4.2.3.1. If the ACR is >0.15 between the two typed alleles, then the heterozygous genotype shall be called.

6.8.5.2.4.2.3.2. If the ACR is <0.15 between the two typed alleles, then the heterozygous genotype shall be called if the two alleles are not in a stutter position of each other.

6.8.5.2.4.2.3.3. If the ACR is <0.15 between the two typed alleles, then the genotype shall be considered inconclusive due to ambiguity for the sister allele determination if the alleles are in a stutter position of each other.

6.8.5.2.4.2.4. If there are two typed alleles and an additional component, more than two typed alleles present at a locus, or one typed allele and multiple additional components, then the locus shall be considered inconclusive.

6.8.5.2.4.2.5. Gender determination may be used in assessing additional components in the X-STR and Y-STR loci. The presence of additional components outside of the gender expectations may warrant calling a locus inconclusive.

6.8.5.2.4.2.6. If a locus is inconclusive for genotype determination, then all alleles for that locus shall be toggled to untyped if the alleles are greater than stochastic.

6.8.5.2.4.2.7. Any homozygous alleles less than stochastic will be filtered from genotype determination using the SeqLite tool.

6.8.5.2.5. SNP Loci Genotype Determinations

6.8.5.2.5.1. Read counts below analytical threshold shall not be typed or interpreted.

6.8.5.2.5.2. Read counts below interpretation threshold may be used in genotype determinations in some instances.

6.8.5.2.5.2.1. If no typed alleles are present, then read counts below IT shall not be used for interpretation. Therefore, there shall not be any toggling/typing of read counts below IT.

6.8.5.2.5.2.1.1. **Exception:** If there are two untyped alleles (both below IT) in a piSNP or aiSNP locus, both may be typed to aid in phenotype and ancestry estimations.

6.8.5.2.5.2.2. If there is one typed allele, then additional untyped read counts (i.e., an additional component) below IT shall be further analyzed to determine if typing is warranted. Read counts below IT that become typed (toggled) can be used for interpretation.

6.8.5.2.5.2.2.1. The Allele Count Ratio (ACR) between the typed allele and the additional component shall be determined.

6.8.5.2.5.2.2.2. If the typed allele is above the ST for the sample and the ACR is between 0.10 to 0.20, then no additional typing shall be done. The genotype shall be considered inconclusive due to ambiguity for the sister allele determination.

6.8.5.2.5.2.2.3. If the typed allele is above the ST for the sample and the ACR is <0.10, then no additional typing shall be done of the additional component. The genotype shall be considered as homozygous for the typed allele at the locus.

6.8.5.2.5.2.2.4. If the typed allele is not above the ST for the sample and the ACR is >0.20, then the additional component shall be typed resulting in a heterozygous genotype determination for the locus.

6.8.5.2.5.2.2.5. If the typed allele is not above the ST for the sample and the ACR is <0.20, then no additional typing shall be done. The genotype shall be considered inconclusive due to ambiguity for the sister allele determination.

6.8.5.2.5.2.3. If there are two typed alleles with an ACR of <0.10, then at the analyst's discretion the locus may be called inconclusive.

6.8.5.2.5.2.4. If a locus is inconclusive for genotype determination, then all alleles for that locus shall be toggled to untyped if the alleles are greater than ST.

6.8.5.2.5.2.5. Any homozygous alleles less than ST will be filtered from genotype determination using the SeqLite tool.

6.8.5.3. Mixture Profile Interpretation

6.8.5.3.1.   Mixture profiles may be suitable for interpretation. Additional sampling, extraction, or amplification should be considered before interpreting a mixture result.

6.8.5.3.2.   A profile shall be evaluated for stutter and/or artifacts prior to the genotype determinations and no untyped additional components shall be typed for interpretation.

6.8.5.3.3.   The following guidelines shall be used to determine the genotype of the major profile of the mixture. The minor profile shall not be utilized for interpretation.

6.8.5.3.3.1.   A major profile shall be determined at 12 or more autosomal STR loci in order for the profile to be suitable for interpretations.

6.8.5.3.3.2.   If a major profile is unable to be determined based off the guidelines, then the mixture will be deemed to be an indistinguishable mixture and no conclusions shall be drawn.

6.8.5.3.4.   **Two Person Mixture Genotype Determinations**

6.8.5.3.4.1.   An assessment of the STR loci (autosomal, X, and Y) shall be performed to determine the gender of the contributors to the mixture, if possible.

6.8.5.3.4.2.   The allele count ratios of STR loci (autosomal, X, and Y) will be used to determine the major profile accordingly. Typed alleles are designated A-D in descending read count order. SNP loci in mixture samples will not be interpreted or utilized for phenotype or ancestry estimations.

6.8.5.3.4.3.   Allele count ratios shall be calculated by dividing the read counts of an allele (B) by the read counts of another allele (A).

6.8.5.3.4.4.   Loci with 4 alleles (A-D)

6.8.5.3.4.4.1.   If the allele count ratio of the two highest alleles (A and B) is ≥ 0.60 and the allele count ratio of the lowest high allele (B) and the highest low allele (C) is ≤ 0.30, then a heterozygous major profile (A, B) will be determined.

6.8.5.3.4.5.   Loci with 3 alleles (A-C)

6.8.5.3.4.5.1.   If the allele count ratio of the two highest alleles (A and B) is ≥ 0.60 and the allele count ratio of the lowest high allele (B) and the lowest allele (C) is ≤ 0.30, then a heterozygous major profile (A, B) will be determined.

6.8.5.3.4.5.2.   If the allele count ratio of the two highest alleles (A and B) is ≤ 0.30 and the allele count ratio of the lowest high allele (B) and the lowest allele (C) ≥ 0.50, then a homozygous major profile (A) will be determined.

6.8.5.3.4.5.3.   If the allele count ratio of the two highest alleles (A and B) is ≤ 0.15, then a homozygous major profile (A) will be determined.

6.8.5.3.4.6.   Loci with 2 alleles (A and B)

6.8.5.3.4.6.1.   If the allele count ratio of the two alleles (A and B) is ≥ 0.60, then a heterozygous major profile (A, B) will be determined.

6.8.5.3.4.6.2.   If the allele count ratio of the two alleles (A and B) is ≤ 0.20, then a homozygous major profile (A) will be determined.

6.8.5.3.4.7.   Loci with 1 allele (A)

6.8.5.3.4.7.1. If the allele is greater than stochastic, then a homozygous major profile (A) will be determined.

6.8.5.3.4.7.2. A major profile (A) may be determined when the allele is below stochastic, only for X and Y STR loci, if the major profile is deemed to be from a male individual. **Exception**: one allele below stochastic at DYS385a-b or DYF387S1 may not be used for interpretation.

6.8.5.3.5. **Three Person Mixture Genotype Determinations**

6.8.5.3.5.1. An assessment of the STR loci (autosomal, X, and Y) shall be performed to determine the gender of the contributors to the mixture, if possible.

6.8.5.3.5.2. The allele count ratios of STR loci (autosomal, X, and Y) will be used to determine the major profile accordingly. Typed alleles are designated A-F is descending read counts order. SNP loci in mixture samples will not be interpreted or utilized for phenotype or ancestry estimations.

6.8.5.3.5.3. Allele count ratios shall be calculated by dividing the read counts of an allele (B) by the read counts of another allele (A).

6.8.5.3.5.4. Loci with 5-6 alleles

6.8.5.3.5.4.1. If the allele count ratio of the two highest alleles (A and B) is ≥0.60 and the allele count ratio of the lowest high allele (B) and the highest low allele (C) is ≤0.30, then a heterozygous major profile (A, B) will be determined.

6.8.5.3.5.4.2. If the allele count ratio of the two highest alleles (A and B) is ≤0.30, then a homozygous major profile (A) will be determined.

6.8.5.3.5.5. All remaining loci

6.8.5.3.5.5.1. If the allele count ratio of the two highest alleles (A and B) is ≥0.60 and the allele count ratio of the lowest high allele (B) and the highest low allele (C) is ≤0.20, then a heterozygous major profile (A, B) will be determined.

6.8.5.3.5.5.2. If the allele count ratio of the two highest alleles (A and B) is ≤0.20, then a homozygous major profile (A) will be determined.

6.8.5.3.5.5.3. If only 2 alleles are typed and the allele count ratio is ≥0.60, then a heterozygous major profile (A, B) will be determined.

6.8.5.3.5.5.4. If only 1 allele is typed and the allele is greater than stochastic, then a homozygous major profile (A) will be determined.

6.8.5.4. **General Interpretation Considerations for UAS**

6.8.5.4.1. If a locus is inconclusive for genotype determination for a single source profile, then all alleles for that locus shall be toggled to untyped if the alleles are greater than stochastic. The reason for the change shall be documented in the comment section.

6.8.5.4.2. Any homozygous alleles less than stochastic will be filtered from genotype determination using the SeqLite tool in a single source profile.

6.8.5.4.3. Only alleles determined as the major profile of a mixture shall be typed for a mixture sample. The reason for any changes to the remaining portion of the profile shall be documented in the comments section.

6.8.5.5. **Exporting Sample Details**

6.8.5.5.1. After all typing and genotype determinations, a Sample Details Report shall be generated from the UAS.

6.8.5.5.2. Sample Details Reports can be generated in two different ways.

6.8.5.5.2.1. At the top of a sample details page select **Generate Report** to create for individual samples.
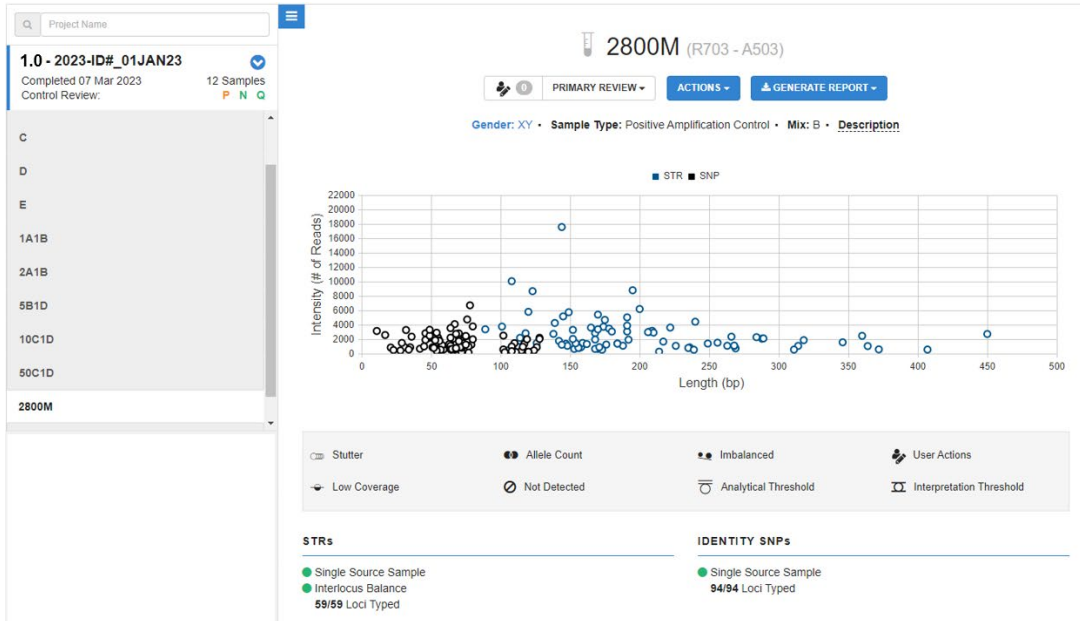
6.8.5.5.2.2. To populate reports for more than one sample, go the Project page, navigate to the Sample Genotype tab, and select **Generate Report(s)**. Select **Sample Details** and then multiple samples can be toggled on for generation of Sample Details Reports.

6.8.5.5.3.   Sample Details Reports shall be exported from the UAS to be utilized for SeqLite.

6.8.5.5.4.   The Sample Details Reports shall be saved under each associated laboratory case number and request folder located in the analyst's folders on the server.

6.8.5.6.   **SeqLite**

6.8.5.6.1.   Sample Details report shall be imported into the Seqlite tool, an Excel program used to apply ST, document final allele calls for a sample, and generate additional interpretation files.

6.8.5.6.2.   The appropriate concentration-based ST shall be selected within Seqlite.

6.8.5.6.3.   For CODIS eligible samples, a CMF file may be created and saved in the appropriate CMF server file for upload.

6.8.5.6.4.   Additionally, a.txt file can be created to be used for statistical calculations within DBLR. The .txt file shall be saved under each associated laboratory case number and request folder located in the analyst's folders on the server.

6.9. Records

6.9.1. The appropriate worksheets as contained in the Sequencing Workbook or the equivalent working document shall be used to record all procedures.

6.9.2. All data sheets, notes, and other information generated from the laboratory examination shall be retained in the case record.

6.9.3. The batch technical review of a NGS project/run shall be recorded on the appropriate batch review worksheet. The technical review of all data for the NGS analysis shall be recorded on the appropriate technical review worksheet.

6.9.4. Sequencing run data shall be exported using the data management tool within the Universal Analysis Software. The export file shall include all projects, samples and reports generated from the run and shall be retained in the appropriate batch folder on the network drive. All files stored on the server shall be routinely backed up to ensure proper security of data.

    6.9.4.1. Do not edit the compressed export file generated by the Universal Analysis Software.

6.9.5. Any case data not retained in the batch folder shall be retained in the case record and/or in a case folder on the network drive. All files stored on the server shall be routinely backed up to ensure proper security of data.

6.9.6. Sequencing run data may be purged from the Universal Analysis Software as necessary to maintain database space.

6.9.7. Electronic records shall be retained as indicated in Appendix 9.

6.10. Interpretations of Results

6.10.1. See Section 7.8.4 for NGS interpretation guidelines and Biology Casework Test Methods Section 3 for Relationship Comparison guidelines.

6.10.2. If it has been determined that a sample is unsuitable for interpretation, an explanation as to why the sample is uninterpretable should be given. Examples include:

    6.10.2.1. No results: Insufficient read counts were detected.

    6.10.2.2. Inconclusive: Read counts were detected; however, due to limited data, poor quality data, or an indistinguishable mixture, no conclusions were drawn.

6.10.3. Test results from an unknown sample that are suitable for comparison to a reference sample may have the following conclusions reported:

    6.10.3.1. Included: The DBLR™ calculated Likelihood Ratio favors inclusion.

    6.10.3.2. Excluded: The profile obtained from the unknown had discrepant genotypes as compared to the profile of the reference sample (i.e., was not the same) and can be visually excluded or the DBLR™ calculated Likelihood Ratio favors exclusion.

    6.10.3.3. Uninformative: The DBLR™ calculated Likelihood Ratio falls in the uninformative range.

6.10.4. If conclusive results are obtained from a sample, appropriate CODIS eligible profiles shall be entered into CODIS for searching. The analyst and technical reviewer shall evaluate CODIS eligibility before submitting profiles for entry.

6.11. Report Writing for NGS Analysis

6.11.1. Rules for Forensic Relationship Comparisons are listed in Forensic Biology Casework Test Methods Section 3.

    6.11.1.1. Possible mutations at X-STR and Y-STR loci shall be considered. The analyst shall consult with the Technical Leader to determine an appropriate course of action.

6.11.2. See Appendix 7 for formatting, general rules, and specific reporting examples.

6.11.3. **Direct Comparisons Statistics using DBLR™**

    6.11.3.1. Statistics shall only be performed with single source samples or autosomal STR major profiles determined from mixtures.

6.11.3.2.   Y-STR, X-STR, aiSNP and piSNP loci shall only be used for interpretation purposes and not for statistical evaluations.

6.11.3.3.   Direct comparison statistics shall be calculated using the Kinship module of DBLR™.

6.11.3.3.1.   In the H1 screen, construct the pedigree by leaving a single individual.

6.11.3.3.1.1.   The symbol used is not required to reflect the sex of the corresponding sample.

6.11.3.3.2.   Add H2 by clicking the "**+**" symbol next to H1.

6.11.3.3.2.1.   Add the unknown, unrelated individual by choosing either the male or female symbol from the toolbar, as appropriate.

6.11.3.4.   Choose **Numeric Kinship** in the bottom, right corner.

6.11.3.5.   Add the profiles to the box marked **Load Samples**. This may be done by dragging and dropping the appropriate files, or by selecting **Browse** and navigating the file tree.

6.11.3.5.1.   DBLR™ does not have an option to omit a locus; therefore, NGS loci indicating potential drop-out or in which a major profile could not be determined shall be omitted using the SeqLite tool.

6.11.3.6.   Under **Configuration**, add the appropriate Analysis Name. At a minimum, the name shall include the case number, followed by an underscore and the DBLR run number, increasing sequentially (e.g., 23L1234_DBLR1, 23L1234_DBLR2). If additional characters are added to the "Analysis Name" designation, those characters shall be added after the specified naming convention.

6.11.3.7.   Under **Parameters**, choose the appropriate Allele Frequencies file. Verify that "Model Fst" is checked and set to 0.01. Linkage can be left on as a default as it will not be applied by the program for this comparison.

6.11.3.7.1.   Direct comparisons shall be performed with the appropriate NIST 2017 population data using the racial information provided by the contributing agency. If racial information is unknown or unobtainable, calculations shall be performed using the Combined population data.

6.11.3.8.   Select **Confirm Inputs** once all profiles are loaded and settings are verified.

6.11.3.9.   Link the unknown and reference both to the node in H1. In H2, link the unknown to one node and the reference to the second node. This may be accomplished by dragging the sample name to the appropriate position in the pedigree, or by clicking **Add Relationship** and selecting the person in the pedigree and its corresponding sample from the drop-down menus.

6.11.3.9.1.   Verify that the correct Contributor Position is selected if a STRmix deconvolution is used as a sample input.

6.11.3.9.2.   When F_{ST} is enabled, a warning message will show that there are unexplained contributor positions until all loaded samples are linked to the pedigree. This must be resolved before a likelihood ratio can be calculated.

6.11.3.10.   Click **Compute Likelihood Ratios** and review the data.

6.11.3.11.   Save a copy of the Kinship Reports in the casefile and the run folder on the DNA server (See Appendix 9).

6.11.3.11.1.   The report will list linked loci together whenever Linkage is enabled. However, the model is only applied when needed based on the pedigree and sample profiles.

6.11.3.12.   If the analyst cannot clearly exclude an individual, DBLR™ may be used to assist in the comparison. If DBLR™ is used to determine an exclusion, the appropriate exclusion statement shall be included in the report for that individual.

6.11.3.12.1.   LR values less than 1 favor the defense proposition (H2). LR values less than 1 will be evaluated for the verbal equivalent as 1/LR.

6.11.3.12.1.1. 1/LR values between 2 and 99 will be reported as limited support for exclusion and will include the hypotheses and calculated 1/LR value in the report as stated in the report wording guidelines.

6.11.3.12.1.2.1/LR values above 99 shall use the general exclusion statement as stated in the report wording guidelines.

6.11.3.12.1.3.An LR value of zero indicates no scientific support for the prosecution proposition (H1) and shall use the general exclusion statement as stated in the report wording guidelines.

6.11.3.13.   DBLR™ LR values shall be rounded to two significant figures for reporting.

6.11.3.13.1.   When the LR is in the support of H1, it shall be rounded down to two significant figures.

6.11.3.13.1.1. When the LR in support of H1 is less than 2, it shall be rounded down to 1.0 (uninformative).

6.11.3.13.2.   When the LR is in support of H2, the 1/LR value shall be rounded up to two significant figures.

6.11.3.13.2.1. If the 1/LR is less than 2, it shall be rounded up to 2.0 (limited support for exclusion).

6.12. Investigative Information on Phenotype and Ancestry Estimations

6.12.1. At the analyst's discretion, the piSNPs and aiSNPs may be utilized for phenotype and ancestry estimations in order to provide investigative information.

6.12.2. A full piSNP profile shall be developed for phenotype estimations to be performed. At least 27 complete aiSNP loci are required in order to utilize an ancestry estimation.

6.12.3. From the Sample Details page, click the **Actions** drop-down button and select **Phenotype Estimation**.
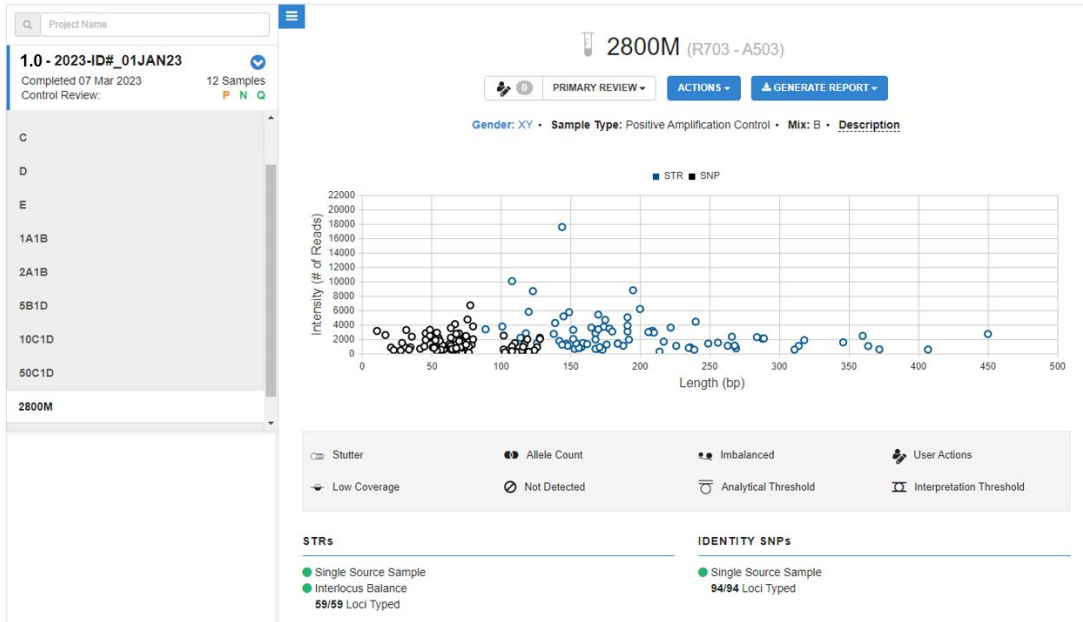
6.12.4. A table of piSNP and aiSNP results will be displayed. Data for the piSNPs and aiSNPs shall be interpreted using the Interpretations Guidelines outlined in Section 6.8.5 before generating a phenotype estimation report. Select **Estimate Phenotype** to generate phenotype estimation from the SNP information.
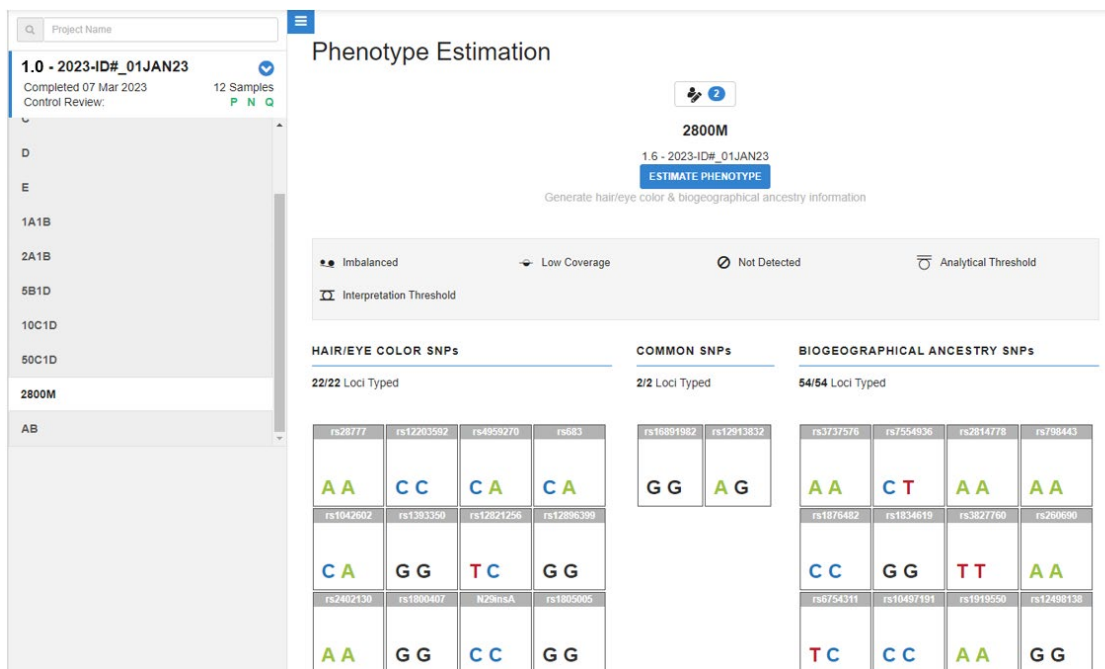
6.12.5. Hair and eye color phenotype percentages and the ancestry result as generated from the estimation shall be issued to the investigating agency via a memo as shown in Appendix 7.

6.12.6. The Phenotype Report shall be exported and saved under each associated laboratory case number and request folder located in the analyst's folders on the server.

6.13. References:

6.13.1. Verogen, Inc. *MiSeq FGx® Sequencing System Reference Guide*. Document# VD2018006, Rev. F (or current version). San Diego, CA, 2021. https://verogen.com/resources/product-documentation/?_products=miseq-fgx-system

6.13.2. Verogen, Inc. *ForenSeq™ DNA Signature Prep Reference Guide*. Document# VD2018005, Rev. D (or current version). San Diego, CA, 2022. https://verogen.com/resources/product-documentation/?_products=forenseq-dna-signature-prep-kit

6.13.3. Verogen, Inc. *ForenSeq™Universal Analysis Software v1 Guide*. Document# VD2018007, Rev. A (or current version). San Diego, CA, 2018. https://verogen.com/resources/product-documentation/?_products=forenseq-universal-analysis-software-v1-3

6.13.4. *Verogen MiSeq FGx® and ForenSeq™ DNA Signature Prep Kit Primer Set B Validation for Missing Person and Unidentified Human Remains Applications*. Indiana State Police Laboratory, 2023. Indianapolis, IN.

6.14. Definitions:

6.14.1. Allele Count Ratio – A ratio of read counts of two alleles, determined by dividing the lower read count allele by the higher read count allele.

6.14.2. Cluster – A distinct spot on a flow cell made up of ~1000 copies of a library containing one amplicon.

6.14.3. DNA Library – A DNA sample that is ready for sequencing.

6.14.4. Flow Cell – A MiSeq FGx® component where the libraries are amplified and sequenced.

6.14.5. Index Adapters – A DNA sequence added to a sample for sample multiplexing and flow cell binding.

6.14.6. Isoallele – An allele that has the same number of repeats as another allele, but a different sequence.

6.14.7. Sequencing by Synthesis – A sequencing process that establishes the DNA sequence as the strand is being synthesized.

6.14.8. Single Nucleotide Polymorphism (SNP) – A single-base sequence variation at a particular point in the genome.

APPENDIX 1

Automation Sample Triage

<u>Sample Property Codes</u>:

S	Standard	Allows standards from different cases to share a reagent blank (RB must also be marked S). As usual, standards will be placed last in a robot plate.
M	Stop if no Male	Marks a sample for triage based on total amplifiable male DNA (see below). Has no effect on reagent blanks or Standards (S).
L	Locked	Combined with code M, causes a sample to be amplified if it has sufficient male DNA (Priority 3 or better) regardless of triage. Will not prevent better samples from being amplified. No effect unless combined with code M, and no effect on reagent blanks.
T	Return to Tube	Marks any sample or reagent blank to be returned to a tube. No effect on other codes.

<u>Triage Process</u>:
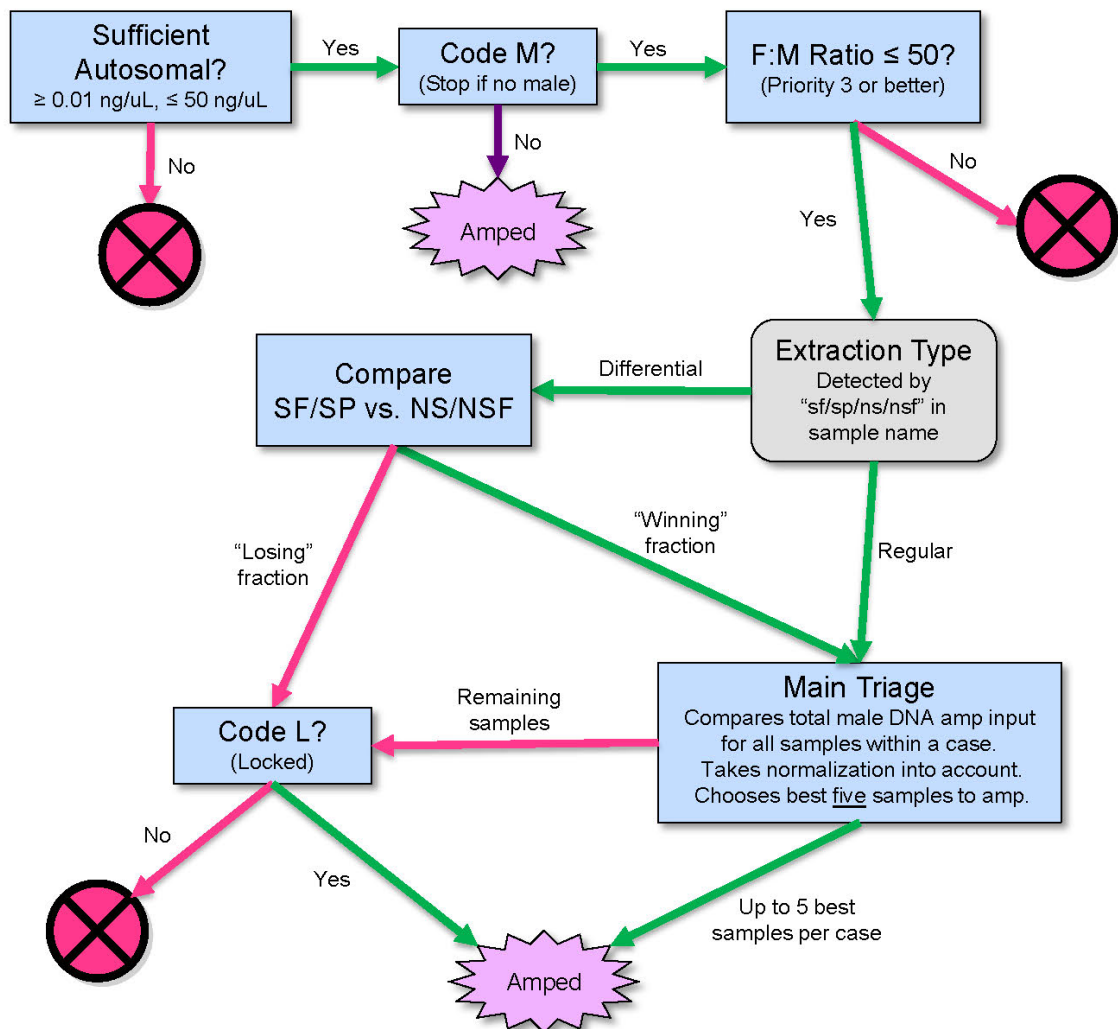
APPENDIX 2

HID Real-Time PCR Analysis Software v1.2 and Quantifiler® Trio template set-up

1.1 Create a "CASEWORK" folder on the AB_SW&DATA D: drive and a shortcut on the desktop.

1.2 Create an "IMPORT" folder on the desktop.

1.3 Open the HID Real-Time PCR Analysis Software v1.2.

1.4 Select **Tools → Preferences** and select the "Defaults" tab.

    1.4.1 Change the default Data Folder and Export Folder destinations to the "CASEWORK" folder found on the D: drive. Change the default Import Folder destination to the "IMPORT" folder on the desktop.

    1.4.2 Select the "Startup" tab and deselect "User name required". Mark only "FAM" and "VIC" for calibration status.
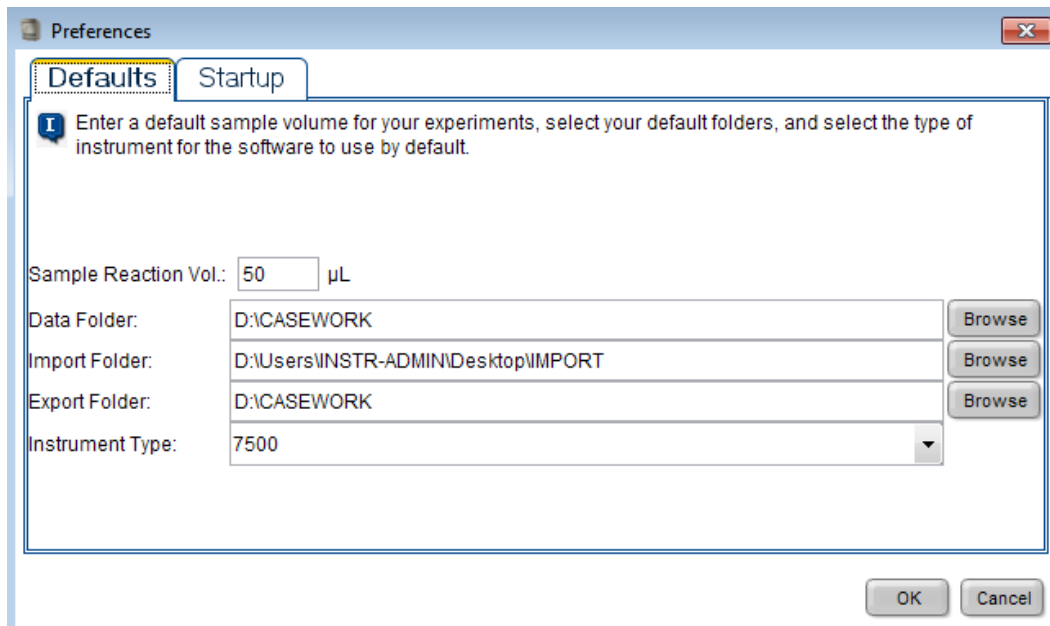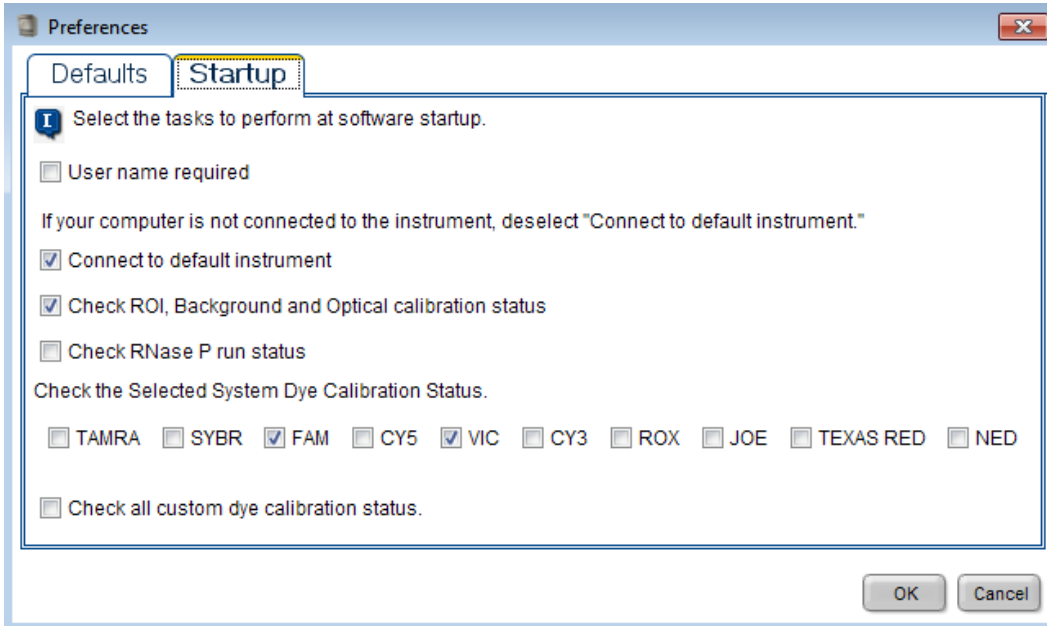
1.5 Select the **Quantifiler® Trio** icon on the Home screen.

1.6 Select **Analysis → Analysis Settings** to remove all flags from the "HID Settings" and "Flag Settings" tabs.
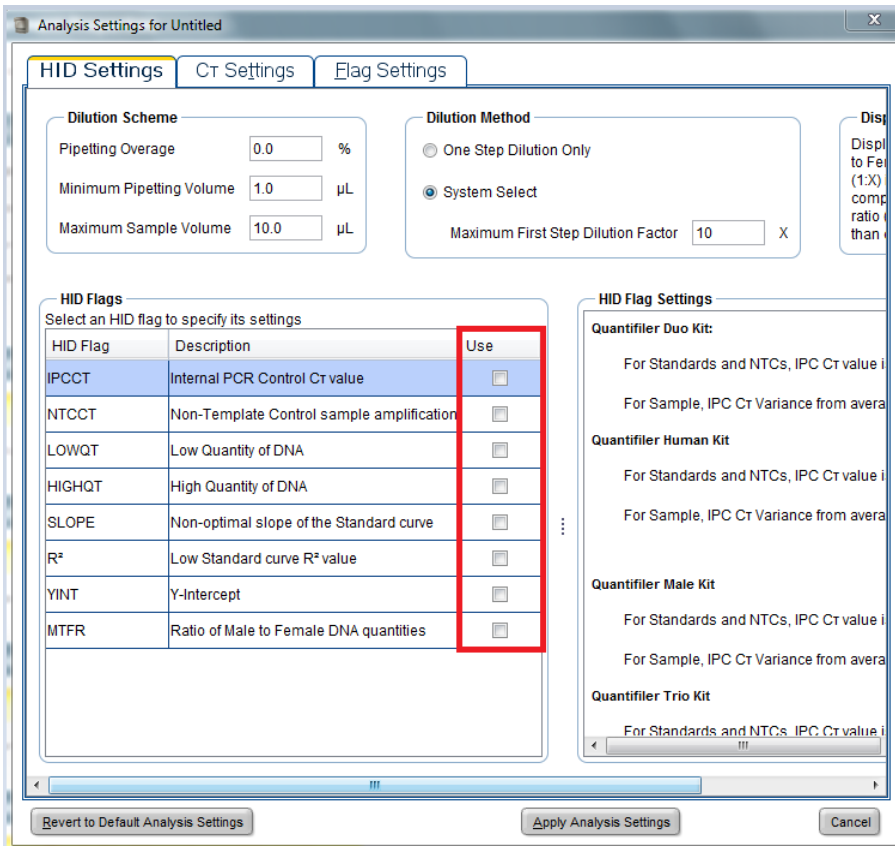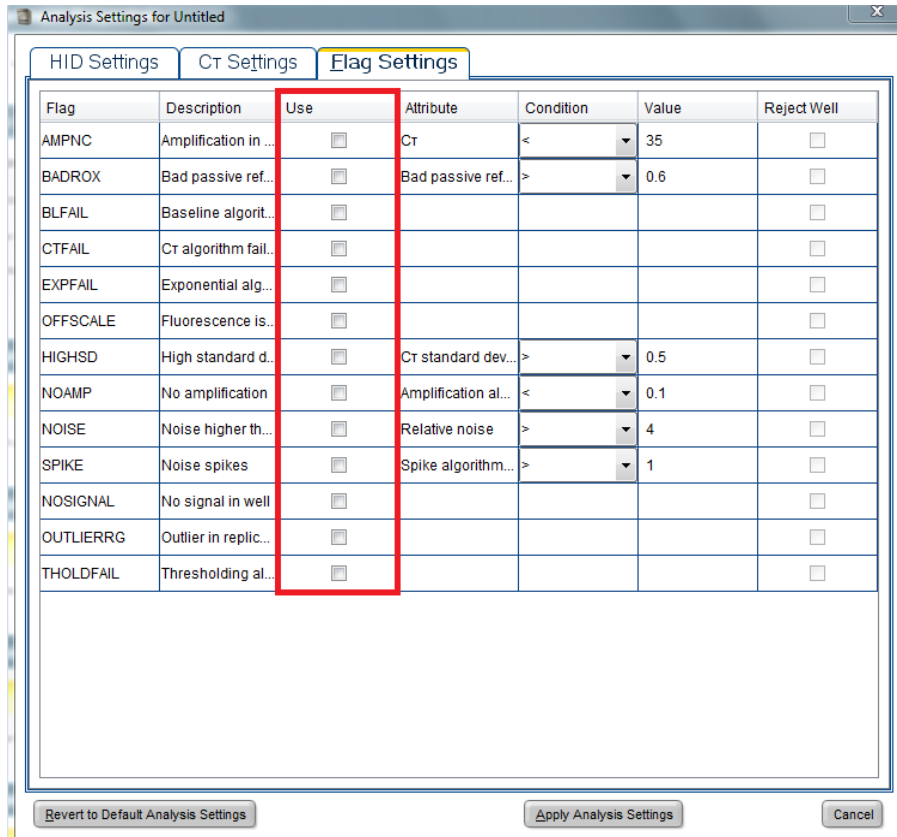
1.7 Verify the CT Settings in the image below.
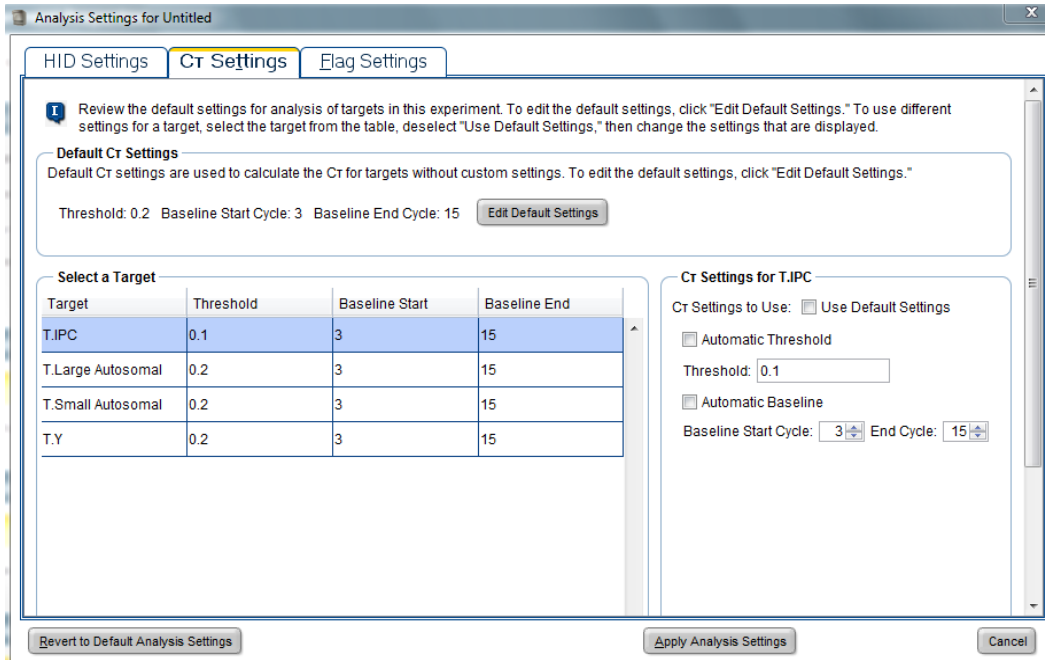
1.8 Select **Plate Setup** from the left navigation pane and assign the 4 standard curve points to column 12 in duplicate.

1.8.1 The task for the Lg. Auto, Sm. Auto, and Y targets for each standard curve point shall be designated as "Standard". The task for the IPC target shall be designated as "Unknown".

1.8.2 Enter the appropriate quantity for each Lg. Auto, Sm. Auto, and Y targets. (See the Standard Curve Prep Table above)

1.9 Assign the NTC to well H11 and mark the task for the Lg. Auto, Sm. Auto, and Y targets as "Negative control". The task for the IPC target shall be designated as "Unknown".

1.10 All remaining wells shall be left blank.

1.11 Verify the **Run Method**.

Holding Stage: 95.0°C for 2.00 minutes.

Cycling stage: 40 cycles of 95°C for 9 seconds, 60.0°C for 30 seconds.
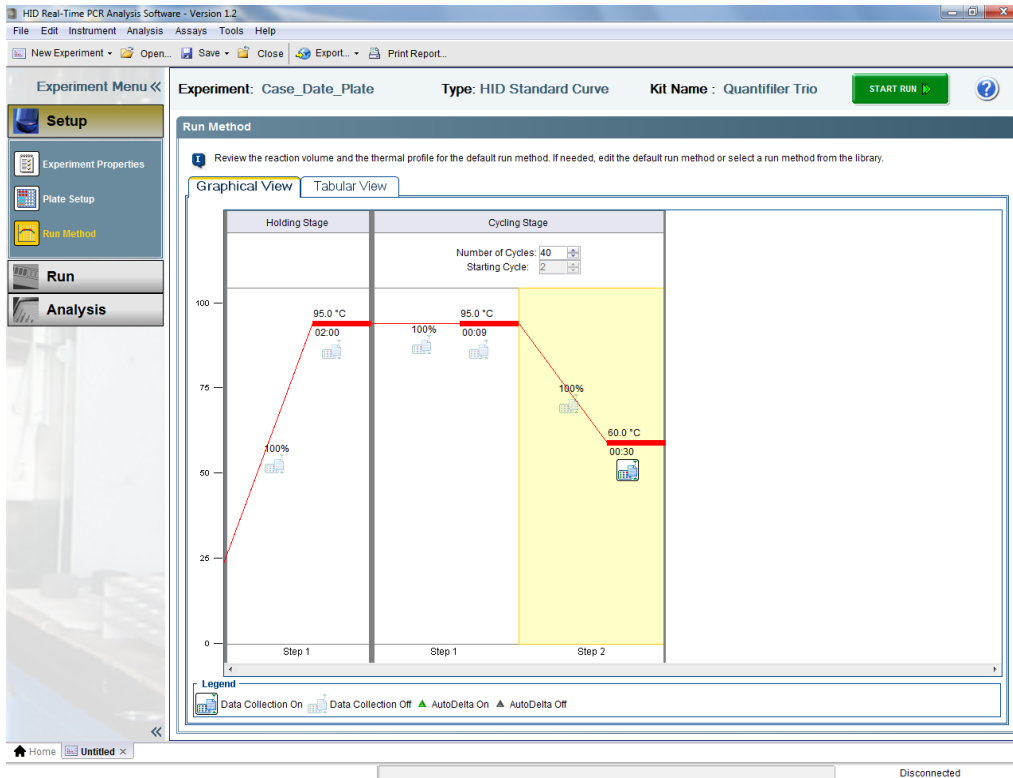
1.12 Select **Export** and change the options to match the following two images. Ensure the Results Export are in the following order: Well, Sample Name, Task, Target Name, C_T, and Quantity. Select **Save as default** at the bottom and **Start Export**.
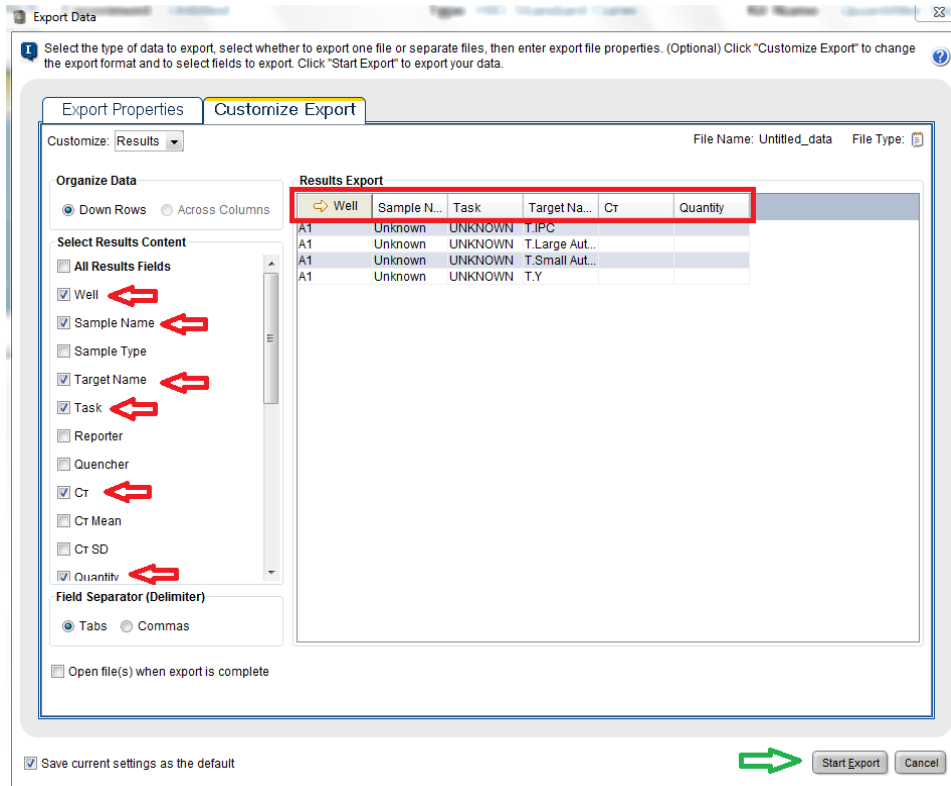
1.13 Overwrite the QuantifilerTrio.edt template by selecting **Save → Save changes as template**. Navigate to **AB_SW&DATA (D:) → Applied Biosystems** folder → **7500** folder → **config** folder → **templates** folder and select **QuantifilerTrio.edt.**

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 3

Instrument setup for Applied Biosystems® 3500 Genetic Analyzer - Data Collection Software version 2.0

(only necessary the first time PowerPlex® Fusion 6C samples are run or if the parameters change)

1.0 Configure the Security Settings

1.1 Navigate to **Tools** and select "Security".

1.2 Change Screen settings to those depicted below.

1.3 Click "Save Settings".

Security Screen:

2.0 Edit User Roles

2.1 Click the "Users" button in the left navigation pane.

2.2 Open the "Roles" tab.

2.3 Set preferences to those depicted below.

2.4 Select "Scientist" and click "Edit".

2.5 Click "Save Role".

Scientist User Role:

Edit Role ⌧

Instruction

Enter the name and description for your role, then select the permission(s) to assign to this role. When you are done select the "Save Role" button.   * = Required

1. Define New Role

* Role Name: `Scientist`

Description: `_____`

2. Select Permission(s)

- ▷ ☐ Locking
- ▲ ◼ Preferences
  - ☑ Edit System Preferences
  - ☐ Export System Preferences
  - ☐ Import System Preferences
  - ☐ Export User Preferences(All)
- ▲ ☑ Calibrations
  - ☑ Perform Spatial Calibration Run
  - ☑ Perform Spectral Calibration
- ▲ ☑ Performance Check
  - ☑ Running Performance Check Install Standards
- ▲ ☑ Archiving
  - ☑ Archive Datastore Objects
  - ☑ Purge Datastore Objects
  - ☑ Restore Datastore Objects
- ▷ ☐ SAE Configuration

[Close]   [Save Role]

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

3.0 Create User Accounts

3.1 Click on the "Users" tab.

3.2 Click "Create" to access a New User window.

3.3 Enter a unique User Name (ex. jdoe8251), set "Password" to lowercase "password" and re-enter. Enter user's first and last name and change settings to those depicted below. Ensure the "User Role" is either set as an "Administrator" or "Scientist".

3.4 Click Save.

New User Window:

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

4.0 Manage Audit Settings

      4.1 Navigate to **Tools** and Select "Audit".

      4.2 Turn Auditing off by ensuring the "Disable Audit" is grayed out and the "Enable Audit" appears black as depicted below.

      4.3 Audit settings may be altered depending on current need.

Disable Audit Settings:

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

5.0 Manage Electronic Signature Settings

5.1 Navigate to **Tools** and Select "E-Signature".

5.2 Turn E-Signature off by ensuring that the "Disable E-Sig" is grayed out and the "Enable E-sig" is black as depicted below.

5.3 E-Signature settings may be altered depending on need.

Disable E-Signature Settings:

6.0 Create an Instrument Protocol

6.1   Navigate to the **Library** and select "Instrument Protocols".

6.2   Select "Create".

6.3   Select "HID" for the Application Type.

6.4   Select "36" for the Capillary Length.

6.5   Select "POP4" for the Polymer.

6.6   Select "Promega_J6" for the Dye Set.

6.7   Select "HID36_POP4" for the Run Module on the 8-capillary 3500. Select "HID36_POP4xl" on the 24-capillary 3500xl.

6.8   Enter "13.0" for the Run Voltage (kVolts).

6.9   Enter "1.2" for Injection Voltage (kVolts).

6.10   Enter "15" seconds for the Injection Time on the 8-capillary 3500. Enter "20" seconds on the 24-capillary 3500xl.

6.11   Change the Run Time (sec) to "1500".

6.12   Name the protocol with the kit name and the injection voltage and time (i.e., Fusion6C_1.2kv15s).

6.13   Mark "Locked" and click "Save".

Instrument Protocol:

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

7.0 Create a Size Standard

7.1 Navigate to the **Library** and select "Size Standard".

7.2 Select "Create".

7.3 Assign the name "WEN ILS 500".

7.4 Select "Orange" for the Dye Color.

7.5 Type the following into the left column: 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500. Click "Add Size(s) >>".

7.6 Mark "Locked" and click "Save".

Size Standard:

8.0 Create a QC Protocol

8.1 Navigate to the **Library** and select "QC Protocols".

8.2 Select "Create".

8.3 Assign the name "WEN ILS 500".

8.4 Change settings to match the figure below.

8.5 Mark "Locked" and click "Save".

QC Protocol:

9.0 Create an Assay

9.1 Navigate to the **Library** and Select "Assays".

9.2 Select "Create".

9.3 Title the assay name "Fusion6C_1.2kv15s" on the 8-capillary 3500, and "Fusion6C_1.2kv20s" on the 24-capillary 3500xl.

9.4 Select "HID" for the Application Type.

9.5 Select "Fusion6C (WEN ILS 500)" as the QC Protocol.

9.6 Select the appropriate instrument protocol.

9.7 Mark "Locked" and click "Save".

Assay:

10.0 Create a Naming Convention

10.1 Navigate to the **Library** and Select "File Name Convention".

10.2 Select "Create".

10.3 Title the name convention "Fusion6C_FNC".

10.4 Select the attributes and delimiters in the image below, click "Add". The order of the attributes and delimiters can be changed using the "Move Up" and "Move Down" buttons.

10.5 Leave file location as the default.

10.6 Look at the "Preview of File Name" field for accuracy.

10.7 Mark "Locked", and Click "Save".

File Name Convention:

11.0   Create a Results Group

11.1   Navigate to the **Library** and Select "Results Group".

11.2   Select "Create".

11.3   Title the results group "Fusion6C_RG".

11.4   Select the attributes and delimiters in the image below, click "Add". The order of the attributes and delimiters can be changed using the "Move Up" and "Move Down" buttons.

11.5   Ensure that "Store reinjection sample file with original sample files (same level)" is selected.

11.6   Leave file location as the default.

11.7   Click so the "Include a Result Group Name folder" box is selected.

11.8   Look at the "Preview of Results Group Name" field for accuracy.

11.9   Mark "Locked" and click "Save".

Results Group:

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 4
GeneMapper^{TM} ID-X

1.   Setting Up GeneMapper^{TM} ID-X Version 1.6 Software User Accounts and Security System

  1.1 **Setting Up Password Policies**

    1.1.1   Open the GeneMapper^{TM} ID-X version 1.6 software.

    1.1.2   Login using an administrator user account.

    1.1.3   Select "Admin" then "Security Manager".

    1.1.4   Login with the same administrator user account.

    1.1.5   Select "Settings" then "Password Policies".

    1.1.6   Change the settings to match the figure below.

Password Policies:

    1.1.7   Save changes.

  1.2 **Creating a User Group**

    1.2.1   Open the Security Manager.

    1.2.2   Select the appropriate default user group in the left navigation pane (e.g., "Casework User Group").

    1.2.3   Select "Edit" then "Duplicate".

1.2.4 In the "General" section, enter a name for the user group (e.g., "ISP Casework User Group").

1.2.5 In the "Default Rights" section, make sure the "Read" and "Update" options are checked.

1.2.6 Select the "Security Groups" tab. Ensure all associated security groups are checked, including the "GeneMapper ID-X" security group.

1.3 Creating a Security Group

1.3.1 Open the Security Manager.

1.3.2 Select the appropriate default security group in the left navigation pane (e.g., "Casework Security Group").

1.3.3 Select "Edit" then "Duplicate".

1.3.4 In the "General" section, enter a name for the security group (e.g., "ISP Casework Security Group").

1.3.5 In the "Associate" column, select the appropriate user groups to associate with that security group (e.g., "ISP Casework User Group"). Make sure the "Read" and "Update" options are checked for the selected user groups.

1.4 Creating a Profile

1.4.1 Open the Security Manager.

1.4.2 Select the appropriate default profile in the left navigation pane (e.g., "Analyst").

1.4.3 Select "Edit" then "Duplicate".

1.4.4 In the "General" section, enter a name for the profile (e.g., "ISP Analyst").

1.4.5 In the "Installed Elements" table, select allowed actions for that particular profile. Allowed actions are not automatically inherited from the default profile and have to be manually entered. For the "ISP Analyst" profile, check the same allowed actions as those for the default GeneMapper™ ID-X "Analyst" profile, with the exception of the "Update Panel" and "Update Size Standard" actions. Change those actions to match the selections in the image below.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
Allowed Actions of the "ISP Analyst" Profile:
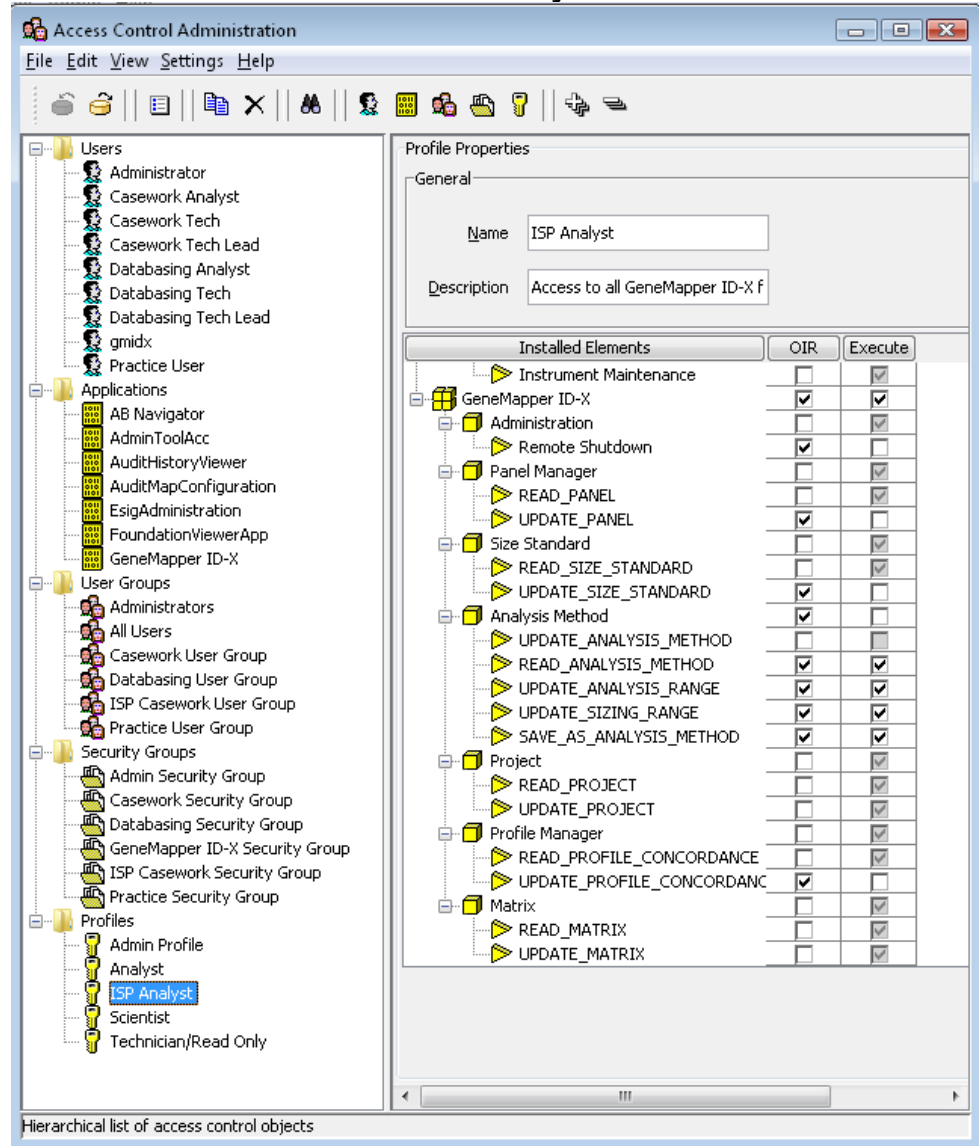
1.5 Setting Up a User Account

1.5.1 Open the Security Manager.

1.5.2 Select the appropriate default user type in the left navigation pane for that particular user (e.g., "Casework Analyst").

1.5.3 Select "Edit" then "Duplicate".

1.5.4 In the "General" section, enter a name. This will be the login name.

1.5.5 In the "User Details" section, enter a full name. Leave the status set to "Active." Uncheck "Show EULA".

1.5.6 Select a profile (e.g., "ISP Analyst").

1.5.7 Select one or more user groups (e.g., "ISP Casework User Group").

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

1.5.8   In the "Password" section, select "Set Password". Enter the same password twice and click "OK". Check "Pre-Expire".

2.0 GeneMapper™ ID-X Version 1.6 Software Settings - PowerPlex® Fusion 6C

2.1 Import Panel and Bin Files

2.1.1   Open the GeneMapper™ ID-X version 1.6 software.

2.1.2   Select **Tools → Panel Manager**.

2.1.3   Highlight the "Panel Manager" icon in the navigation pane.

2.1.4   Select **File → Import Panels**.

2.1.5   Navigate to the saved panel, bin, and stutter files. Select "Fusion6C_v3 _ISP_Panels" and click "Import".

2.1.6   Select the "ISP Casework Security Group". Click "OK".

2.1.7   In the navigation pane, highlight the "Fusion6C_v3_ISP" folder.

2.1.8   Select **File → Import Bin Set**.

2.1.9   Select "Fusion6C_v3_ ISP_Bins" and click "Import".

2.1.10 In the navigation pane, highlight the "Fusion6C_v3_ISP" folder.

2.1.11 Select **File → Import Marker Stutter**. A warning box will appear asking to overwrite the current values. Select "Yes".

2.1.12 Select "Fusion6C_v3_ISP_stutter" and click "Import".

2.1.13 In the Panel Manager window, select "Apply", then "OK".

2.1.14 Repeat this process with the panels, bins and marker stutter files for analysis of unknowns to be run in STRmix™.

   2.1.14.1 Panels: "STRmix_v3_Fusion_6C_ISP_Panels".

   2.1.14.2 Bins: "STRmix_v3_Fusion6C_ISP_Bins".

   2.1.14.3 Marker Stutter: "STRmix_v3_Fusion6C_ISP_stutter".

2.2 Create a Casework Analysis Method

2.2.1   Select **Tools → GeneMapper Manager**.

2.2.2   Select the **Analysis Methods** tab.

2.2.3   Enter the name "Fusion6C_v3".

2.2.4   Select the "ISP Casework Security Group".

2.2.5   Enter "3500" as the instrument.

2.2.6   Select the **Allele** tab. In the "Bin Set" drop-down menu select "Fusion6C_ISP_Bins". Verify that the "Use marker-specific stutter ratio and distance if available" box is checked. All values shall be "0.0".

2.2.7   Select the Peak Detector tab. Change the settings to match those shown in the following figure.

Peak Detector Tab:

Analysis Method Editor

General | Allele | Peak Detector | Peak Quality | SQ & GQ Settings

Peak Detection Algorithm: Advanced

Ranges

Analysis: Full Range
Start Pt: 0
Stop Pt: 10000

Sizing: All Sizes
Start Size: 0
Stop Size: 1000

Peak Detection

Peak Amplitude Thresholds:

B:	150	R:	150
G:	150	P:	150
Y:	150	O:	60

Min. Peak Half Width: 2 pts
Polynomial Degree: 7
Peak Window Size: 11 pts

Slope Threshold

Peak Start: 0.0
Peak End: 0.0

Smoothing and Baselining

Smoothing:
○ None
● Light
○ Heavy

Baseline Window: 51 pts

Size Calling Method

○ 2nd Order Least Squares
○ 3rd Order Least Squares
○ Cubic Spline Interpolation
● Local Southern Method
○ Global Southern Method

Normalization

☐ Use Normalization, if applicable

Factory Defaults

Save As | Save | Cancel | Help

2.2.8   Select the **Peak Quality** tab. Change the settings to match the following figure.

Peak Quality Tab:

2.2.9 Select the **SQ and GQ Settings** tab. Change the settings to match the following figure.

SQ and GQ Settings:

2.2.10 Select "Save".

2.2.11 Repeat these steps to create the "STRmix_Fusion6C_v3" analysis method used to analyze unknown profiles for STRmix™ interpretation. All steps are identical except the following:

2.2.11.1 Enter the name "STRmix_Fusion6C_v3".

2.2.11.2 Select the **Allele** tab. In the "Bin Set" drop-down menu select "STRmix_Fusion6C_ISP_Bins".

2.2.11.3 Select the **Peak Detector** tab. Ensure that the dye channel peak amplitude thresholds are set to the values in the following figure (100 for Blue, Green, and Yellow, 120 for Red, and 60 for Purple and Orange).

Peak Detector Tab:

2.2.11.4 Select the **Peak Quality** tab. Change the settings to match the following figure.

Peak Quality Tab:

2.3 Create a Size Standard

2.3.1 Select **Tools → GeneMapper Manager**.

2.3.2 Select the **Size Standard** tab and click "New".

2.3.3 Name the size standard "WEN ILS 500" in the "Size Standard Editor" screen, choose the "ISP Casework Security Group", and choose "Orange" as the color for the size standard dye. If "WEN ILS 500" is being utilized for both casework and databasing analysis methods on the same GMID-X database, the "GeneMapper ID-X Security Group" should be chosen to allow both the ISP Casework and Database security groups access.

2.3.4 Enter the sizes of the 21 allelic ladder fragments: 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500.

2.3.5 Select "OK".

2.4 Create a Table Setting

2.4.1 Select **Tools → GeneMapper Manager**.

2.4.2 Select the **Table Setting** tab and click "New".

2.4.3　Under the **General** tab name the Table Setting "STRmix_Fusion6C" and select the "ISP Casework Security Group".

2.4.4　Under the **Samples** tab ensure that check marks are located next to only the following: Status, Sample File, Sample Name, Sample Type, Analysis Method, Panel, Size Standard, Sizing Quality Overridden, Sample File Not Found, Sample Off-Scale, and Sizing Quality.

　2.4.4.1 Sort by "Sample Type" and then by "Sample Name". Select the "Ascending" option for both, and leave the final sort option to "None".

　2.4.4.2 Leave the font "Arial" and the size "11".

2.4.5 Under the **Genotypes** tab ensure that check marks are located next to only the following: Sample Name, Marker, Allele, Size, and Height.

　2.4.5.1 Sort by "Sample File", then by "Sample Name", and then by "Marker". Select the "Ascending" option for all three.

　2.4.5.2 Change the "Show number of alleles" to 20 and ensure that "Keep Allele, Size, Height, Area, Data Point, Mutation and Comment together" is NOT checked. (If the option to "Keep Allele, Size, Height…" is not visible in the window, expand the window size until the option appears.)

　2.4.5.3 Leave the font "Arial" and the size "11".

　2.4.6　Select "OK".

2.5　**Create a Plot Setting – Samples, Controls, and Ladders**

2.5.1　Select **Tools → GeneMapper Manager**.

2.5.2　Select the **Plot Settings** tab and click "New".

2.5.3　Under the **General** tab name the Plot Setting "Fusion6C" and select the "ISP Casework Security Group".

2.5.4　Under the **Sample Header** tab ensure that check marks are located next to the following: Sample File, Sample Name, Panel, Sizing Quality Overridden, Sample Off-Scale, and Sizing Quality.

2.5.5　Under the **Genotype Header** tab ensure that check marks are located next to the following: Sample File, Sample Name, Panel, Marker, Sample Off-Scale, Out of Bin Allele, Peak Height Ratio, Control Concordance, and Genotype Quality.

2.5.6　Under the **Sizing Table** tab ensure that check marks are located next to the following: Dye/Sample Peak, Sample File Name, Marker, Allele, Size, Height, Area, and Data Point. Leave the font "Arial" and the size "11".

2.5.7　Select the **Labels** tab. Change the settings to match the following figure. Alternatively, if the "allele edit comment" function is not being used, "Label 4" for "Assigned Allele", "Custom Allele" and "Artifact" can be changed to "NONE".

Labels Tab:

2.5.8 Under the **Display Settings** tab, change the display settings so that they match the following figure and select "OK".

Display Settings Tab:

2.6 **GeneMapper™ ID-X Maintenance**

2.6.1 The number of audit records on the GeneMapper™ ID-X database should be routinely checked. Audit records should be backed-up, saved to the DNA server, and then deleted from the GeneMapper™ ID-X database routinely or if the number of records exceeds 40,000. If the number of audit records exceeds 60,000, the performance of the software may be affected.

2.6.2 The amount of database space in the GeneMapper™ ID-X software should be routinely checked. If the occupied space exceeds 80%, additional disk space should be allocated.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 5
STRmix™ SETTINGS

1.0 STRmix™ Installation and Set-up

1.1 Install the current version of STRmix™ (v2.7.0).

1.2 Copy and Replace the following files from the BiologyQA drive to the STRmix™ Program files located on the (C:) drive:

1.2.1 AlleleFreq folder, config folder, Databases folder, jre folder, Kits folder, logs folder, Populations folder, Results folder, stutters folder, Default Configuration settings, ISP Lab Logo, and Report Defaults XML document.

1.3 Update to the floating license:

1.3.1 After opening STRmix™, if no prompt comes up with the ability to install a license/validate license server, select **About**, and then select **Remove License**. It will tell you that you won't be able to use STRmix™ again until a license is installed and will close the program.

1.3.2 Reopen the program and follow the prompt to validate the license server.

1.3.3 In the Host name, put the following IP address – 10.16.105.102 and the port# 16090.

1.3.4 Select **Validate**. It should then connect via the floating license server and access a license.

1.4 **Kit Parameters**

1.4.1 To access the run parameter settings from the STRmix™ main menu, select **Administration** (an administrator password shall be required), then select **Profiling Kits**.

1.4.2 Navigating the **General**, **Stutters**, and **Loci** tabs, ensure that the correct kit settings are established. The following settings shall be used for the interpretation of profiles:

STRmix

< MANAGE KITS

Profiling Kits

ISP_Fusion6C

ISP_Fusion6C_v2

ISP_Fusion6C_v3

| GENERAL | STUTTERS | LOCI | IMPORT |

Kit Type
Fusion6C

Size Regression File
Fusion6C_SizeRegression_ISP.csv   Edit

VARIANCE

Allelic Variance
8.249, 1.296

Locus Amplification Variance
0.008

Minimum Variance Factor
0.5

Variance Minimization Parameter
1,000

Cancel   Save

Profiling Kits

ISP_Fusion6C

ISP_Fusion6C_v2

ISP_Fusion6C_v3

| GENERAL | STUTTERS | LOCI | IMPORT |

DROP-IN

Drop-in Cap
200

Drop-in Frequency
0.000371

Drop-in Distribution Parameters
☐ Uniform 2.4, 28.93

ADDITIONAL THRESHOLDS

Maximum Degradation
0.01

Degradation Start Point
☑ Use Smallest Peak

Saturation Threshold
28,000

GENERAL	STUTTERS	LOCI	IMPORT

BACK STUTTER

☑ Stutter Enabled

Position Relative to Parent

-1, 0

Inversely Proportional To

Observed Height of Parent Allele ▾

Maximum Stutter Ratio

☐ No Maximum 0.3

Variance

1.825, 9.576

Stutter Regression File

Indiana_Fusion_6C_backward stutter_v2.csv ▾   [Edit]

Stutter Exceptions File

Indiana_stutter_exception_table_v2.csv ▾   [Edit]

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

FORWARD STUTTER

☑ Stutter Enabled

Position Relative to Parent
1, 0

Inversely Proportional To
Expected Height of Stutter Peak ▾

Maximum Stutter Ratio
☐ No Maximum   0.15

Variance
2.208, 2.915

Stutter Regression File
Indiana_Fusion_6C_forward stutter_v2.csv   ▾   **Edit**

Stutter Exceptions File
Select a value   ▾   **Edit**

SE33 -2 STUTTER

☑ Stutter Enabled

Position Relative to Parent
0, -2

Inversely Proportional To
Expected Height of Stutter Peak ▾

Maximum Stutter Ratio
☐ No Maximum   0.15

Variance
1.867, 0.767

Stutter Regression File
Indiana_Fusion_6C_half-backward stutter_v2.csv   ▾   **Edit**

Stutter Exceptions File
Select a value   ▾   **Edit**

GENERAL	STUTTERS	LOCI	IMPORT

LOCUS NAME	GENDER?	REPEAT LENGTH	IGNORE?	DETECTION THRESHOLD	BACK STUTTER	FORWARD STUTTER	SE33 -2 STUTTER
AMEL	✓						
D3S1358	☐	4	☐	100	✓	✓	☐
D1S1656	☐	4	☐	100	✓	✓	☐
D2S441	☐	4	☐	100	✓	✓	☐
D10S1248	☐	4	☐	100	✓	✓	☐
D13S317	☐	4	☐	100	✓	✓	☐
Penta E	☐	5	☐	100	✓	✓	☐
D16S539	☐	4	☐	100	✓	✓	☐

LOCUS NAME	GENDER?	REPEAT LENGTH	IGNORE?	DETECTION THRESHOLD	BACK STUTTER	FORWARD STUTTER	SE33 -2 STUTTER
D18S51	☐	4	☐	100	✓	✓	☐
D2S1338	☐	4	☐	100	✓	✓	☐
CSF1PO	☐	4	☐	100	✓	✓	☐
Penta D	☐	5	☐	100	✓	✓	☐
TH01	☐	4	☐	100	✓	✓	☐
vWA	☐	4	☐	100	✓	✓	☐
D21S11	☐	4	☐	100	✓	✓	☐
D7S820	☐	4	☐	100	✓	✓	☐

LOCUS NAME	GENDER?	REPEAT LENGTH	IGNORE?	DETECTION THRESHOLD	BACK STUTTER	FORWARD STUTTER	SE33 -2 STUTTER
D5S818	☐	4	☐	100	☑	☑	☐
TPOX	☐	4	☐	100	☑	☑	☐
D8S1179	☐	4	☐	120	☑	☑	☐
D12S391	☐	4	☐	120	☑	☑	☐
D19S433	☐	4	☐	120	☑	☑	☐
SE33	☐	4	☐	120	☑	☑	☑
D22S1045	☐	3	☐	120	☑	☑	☐
DYS391	☑						
FGA	☐	4	☐	60	☑	☑	☐
DYS576	☑						
DYS570	☑						

1.4.3   In the "Gender?" column, AMEL, DYS391, DYS576, and DYS570 shall be checked.

1.4.5   The Detection Threshold for shall be set at 100, 120, or 60, depending on the associated dye.

1.4.6   In the "Back Stutter" and "Forward Stutter" columns, all autosomal loci shall be checked.

1.4.7   In the "SE33 -2" column, SE33 shall be checked.

1.5 Default Settings

1.5.1 To access the default settings, from the STRmix™ main menu, select **Administration** (an administrator password shall be required), then select **STRmix Defaults**.

1.5.1.1 Navigating the **General**, **Likelihood Ratio**, and **User Interface** tabs, ensure that the correct kit settings are established. The following default analysis settings shall be used for the interpretation of profiles:

‹ DEFAULT SETTINGS

GENERAL	LIKELIHOOD RATIO	DATABASE SEARCH	USER INTERFACE

Default Profiling Kit
ISP_Fusion6C_v3 ▾

Results Directory
Results   Browse

MCMC

Number of Chains	Burn-in Accepts (per chain)	Post Burn-in Accepts (per chain)	Random Walk SD
8	10,000	50,000	0.005

Post Burn-in Shortlist	☐ Extended Output
9	

GELMAN-RUBIN

☑ Auto-Continue on GR	Gelman-Rubin Threshold	Extra Accepts
	1.2	50,000

CONTRIBUTOR RANGE

Hyper-Rectangle Percent Accepts
2.5

PERFORMANCE

Number of Threads	☐ Low Memory Mode
8	

GENERAL	LIKELIHOOD RATIO	DATABASE SEARCH	USER INTERFACE

DEFAULT POPULATIONS

NIST_2017_African American
NIST_2017_Asian
NIST_2017_Caucasian
NIST_2017_Hispanic

SUB-SOURCE LR

☑ Assign Sub-Source LR

CONTRIBUTOR RANGE

Priors Method
MLE ▾

SAMPLING VARIATION

☑ Calculate HPD	☑ MCMC Uncertainty	☑ Allele Frequency Uncertainty

Number of HPD Iterations	Probability Interval Quantile	Probability Interval Sides
1000 ▾	99 ▾	1 ▾

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

GENERAL	LIKELIHOOD RATIO	DATABASE SEARCH	USER INTERFACE

Database File
Q:\STRmix Database\STRmix Employee Database.csv   [Browse]

Minimum LR
10,000

Population For Search
NIST_2017_Caucasian ▼

Type of Search
Standard ▼

FST For Search
0.01b(1.0, 1.0)

☑ Assign Sub-Source LR

Priors Method
MLE ▼

☑ Auto Database Search

GENERAL	LIKELIHOOD RATIO	DATABASE SEARCH	USER INTERFACE

Default Text File Directory
C:\ProgramData\STRmix\Results   [Browse]

☐ Allow Text Sample Renaming

1.6 Population Settings

1.6.1   The following four population databases shall be used to perform statistical calculations: NIST_2017_Caucasian, NIST_2017_African_American, NIST_2017_Hispanic, and NIST_2017_Asian.

1.6.2   To access the population parameter settings, from the STRmix™ main menu, select **Administration**, then select **Populations**.

1.6.3   The following population settings shall be used for the interpretation of profiles.

1.6.3.1 F_{ST} Coancestry Coefficient, Theta (θ): corrects for two alleles identical by descent in different individuals. An F_{ST} of 0.01 was selected for the four populations.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

Allele Frequency File
NIST_2017_African American.csv

Edit

Population Proportion	Default FST
0.096	0.01b(1.0, 1.0)

Population Size	Children Per Family
6,500,000	4

Sibling Proportion	Child Proportion	Parent Proportion
4.615384615384616E-7	4.102564102564102E-7	2.051282051282051E-7

Uncle/Aunt Proportion	Niece/Nephew Proportion	Grandparent Proportion
6.153846153846154E-7	1.230769230769231E-6	2.051282051282051E-7

Grandchild Proportion	Cousin Proportion	Unrelated Proportion
8.205128205128205E-7	3.692307692307692E-6	0.9999923589743589

Allele Frequency File
NIST_2017_Asian.csv

Edit

Population Proportion	Default FST
0.021	0.01b(1.0, 1.0)

Population Size	Children Per Family
6,500,000	4

Sibling Proportion	Child Proportion	Parent Proportion
4.615384615384616E-7	4.102564102564102E-7	2.051282051282051E-7

Uncle/Aunt Proportion	Niece/Nephew Proportion	Grandparent Proportion
6.153846153846154E-7	1.230769230769231E-6	2.051282051282051E-7

Grandchild Proportion	Cousin Proportion	Unrelated Proportion
8.205128205128205E-7	3.692307692307692E-6	0.9999923589743589

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

Allele Frequency File
NIST_2017_Caucasian.csv   Edit

Population Proportion
0.8

Default FST
0.01b(1.0, 1.0)

Population Size
6,500,000

Children Per Family
4

Sibling Proportion	Child Proportion	Parent Proportion
$4.615384615384616E-7$	$4.102564102564102E-7$	$2.051282051282051E-7$
Uncle/Aunt Proportion	**Niece/Nephew Proportion**	**Grandparent Proportion**
$6.153846153846154E-7$	$1.230769230769231E-6$	$2.051282051282051E-7$
Grandchild Proportion	**Cousin Proportion**	**Unrelated Proportion**
$8.205128205128205E-7$	$3.692307692307692E-6$	0.9999923589743589

Allele Frequency File
NIST_2017_Hispanic.csv   Edit

Population Proportion
0.067

Default FST
0.01b(1.0, 1.0)

Population Size
6,500,000

Children Per Family
4

Sibling Proportion	Child Proportion	Parent Proportion
$4.615384615384616E-7$	$4.102564102564102E-7$	$2.051282051282051E-7$
Uncle/Aunt Proportion	**Niece/Nephew Proportion**	**Grandparent Proportion**
$6.153846153846154E-7$	$1.230769230769231E-6$	$2.051282051282051E-7$
Grandchild Proportion	**Cousin Proportion**	**Unrelated Proportion**
$8.205128205128205E-7$	$3.692307692307692E-6$	0.9999923589743589

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

1.7 **Report Settings**

 1.7.1 To access the Summary Report settings, from the STRmix™ main menu, select **Administration**, then select **Report Defaults**, and then select **Summary Report.**

 1.7.2 Navigating the **Per Report Settings** and **General Settings** tabs, ensure that the correct settings are established. The following report settings shall be used. Drag the individual **Included Components** to arrange their reporting order.

1.7.5 You may need to browse to your local STRmix™ folder to correctly path to the lab logo.

1.7.6 Select **Save.**

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

Appendix 6
PowerPlex® Fusion 6C Mean and Mean + 3SD Stutter

The average observed percent stutter for each locus and mean +3SD (99.7% confidence level) of stutter observed in the PowerPlex® Fusion 6C validation are listed in the table below. Stutter exception files utilized in STRmix™ analysis are determined through different methods outlined and documented in the STRmix™ validation.

	Backward n-3/n-4/n-5 bp		Double Backward n-6/n-8/n-10 bp		Forward n+3/n+4/n+5 bp	
	Mean %	Mean + 3SD %	Mean %	Mean + 3SD %	Mean %	Mean + 3SD %
D3S1358	9.13	14.1	0.74	1.43	0.64	1.39
D1S1656	8.51	14.3	0.91	1.87	0.97	1.84
D2S441	5.03	8.55	0.38	0.69	0.78	1.49
D10S1248	8.31	13.8	0.73	1.46	0.35	1.04
D13S317	5.05	10.9	0.39	0.65	0.87	2.01
Penta E	2.75	6.99	0.41	0.92	0.37	0.84
D16S539	7.04	12.6	0.61	1.27	1.07	2.37
D18S51	8.08	14.9	0.80	1.59	1.07	3.11
D2S1338	8.23	13.8	0.78	1.49	0.80	1.54
CSF1PO	6.50	12.1	0.51	0.81	1.08	2.49
Penta D	1.90	3.91	0.43	1.10	0.56	1.37
TH01	2.07	4.60	0.34	0.82	0.38	0.79
vWA	7.43	16.8	0.75	1.57	0.66	1.74
D21S11	8.51	12.3	0.72	1.42	1.07	1.90
D7S820	5.38	11.1	0.53	1.12	0.76	1.76
D5S818	6.53	11.1	0.55	1.12	1.00	1.88
TPOX	3.01	6.42	0.31	0.50	0.23	0.31
D8S1179	6.96	10.7	0.60	1.08	0.70	1.28
D12S391	9.68	17.9	1.01	2.40	0.81	2.38
D19S433	7.85	12.5	0.81	2.37	1.06	2.58
SE33	10.5	18.1	1.05	2.02	1.04	2.25
D22S1045	7.55	18.1	1.01	1.86	4.50	9.48
DYS391	6.79	8.71	0.77	1.64	0.73	2.02
FGA	7.21	12.4	0.81	1.86	0.77	2.21
DYS576	8.89	12.1	0.89	2.27	1.28	2.83
DYS570	8.96	12.9	0.97	1.62	1.07	1.82

	Half Backward n-2 bp	
	Mean %	Mean + 3SD %
D1S1656	1.68	2.97
SE33	4.46	5.53
FGA	0.47	0.97

	Half Forward n+2 bp	
	Mean %	Mean + 3SD %
SE33	1.26	2.25

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 7
REPORT WORDING

REPORT WORDING RULES

These are general wording guidelines for commonly occurring test results. Alterations must be approved by a supervisor. Many of the words in these guidelines that are in uppercase should be changed to reflect your results (e.g. PERSON, ITEM X, etc.). Words or phrases in [ ] are optional.

In this appendix, the word "subitem" refers to a part of a submitted item of evidence that is considered distinctly separate and would not be combined with other subitems for DNA testing (due to case circumstances, sample purpose, serological results, etc.), regardless of whether or not a sample is retained. (E.g., vaginal/cervical swabs and external genital swabs are subitems within a kit item; a belt and a shirt may be subitems within a whole item; two submitted swabs with different serology results are considered separate subitems.)

REPORT FORMATTING

1. Reports shall be typed into a Word document with specific formatting.

2. The Word document shall have no indents, tabs, special formatting, or font/size changes. The report shall be written in default Normal style.

3. **Original items** and their descriptions shall be bolded and underlined. Multiple original items shall be separated with a horizontal line of underscores.

   o If related, original items shall be addressed in additional reports such as additional standard comparisons, Y-STR and paternity requests (see example 10)

4. **Subitems** and their descriptions (if applicable) may be bolded (see examples 2,3 and 4).

   o Subitem headers and required formatting (bold, underlined, or italicized) are required when you have different tiers in the same report (e.g. in SAK's). Headers are not required for simple items, especially if nothing is retained or amplified (see example 9).

   o When a subitem is reported it shall be bolded and underlined if the original item is not present and was previously reported (as in additional submissions).

5. Sub-subitems may be grouped by results under underlined headings (see example 6).

6. *Sperm and Non-sperm fractions*, where DNA results are different, shall be separated under italicized headings (see item 001C in [example 1](#)).

7. *Sub-sub-subitems,* if needed, are listed under italicized headings.

8. Line breaks: At least one blank line shall separate all items, subitems, and other individual results. Multiple blank lines may be used to enhance clarity or readability.

9. Exclusions and comparisons without LRs (inclusions of unknown individuals) shall be listed with the contributor assumption before the bullets (see item 002A in [example 6](#)). The word excluded shall be bolded, except when *including* an individual (i.e., cannot be excluded).

10. Likelihood ratios:

    o Each likelihood ratio paragraph shall be preceded by a triangle text bullet: ▶

    o The word inclusion/exclusion/uninformative in the LR paragraph shall be bolded.

GENERAL REPORT RULES

1. Report wording may be altered with the approval of a Biology Unit Supervisor.

2. The appropriate appendix with appropriate definitions and explanations shall be attached to the end of each report. Reports in which the entire request has been withdrawn do not require an appendix.

3. Serology results shall be reported prior to any DNA results for each item.

4. No serology statement is needed if no serological testing is performed.

5. Item numbers shall include all leading zeroes.

6. Items shall be reported by item in numerical order.

7. Item descriptions in the results section:

    o The description of each item shall be similar to but need not be identical to the item description in the submission section.

    o The description shall not include the packaging.

    o The description shall include the number of swabs/samples in parentheses when it is not otherwise apparent.

8. If subitems created for DNA sampling are retained by the Laboratory, this shall be reported (see [examples](#)). When multiple swabs/samples within an item are being tested, the number of items tested shall be reported (see [examples](#)).

9. Retention statements generally will be in the opening paragraph for an item.

10. The significant contents of an item shall be listed in the results section.

11. At a minimum, the first and last name of any individuals being compared shall be used throughout the entire report. The item number of the standard shall not follow the name in the results unless individuals cannot otherwise be distinguished.

12. Individual items from another case should not be listed in the item descriptions or as separate items in the body of the report.

13. Similar results within an item may be grouped together.

14. Swabs utilized for DNA sampling, shall be reported as a "sample" of the item being tested or the sample itself. When multiple samples of an item are extracted separately, they are considered separate samples and the number of samples shall be reported.

15. All submitted samples in an item (excluding standards) not tested shall be described as not tested in the report using the appropriate wording.

16. When two or more samples are combined at any point in the analysis process, the report shall refer to the samples as "combined". The analyst's notes shall clearly describe which samples and at which stage of the process the samples were combined.

    o If all subitems or samples within the item (not collected by the analyst) are not combined into one extract, the report shall indicate how many subitems/samples were combined and how many were not tested.

17. If multiple swabs are collected by the analyst and treated as one sample, the use of "combined" is not required. In a sample with insufficient (male) DNA for amplification, where it is valuable to identify a male contributor from a female contributor (i.e., sexual assault cases), results shall be reported based on the Quantifiler® Trio Y target.

18. When differential extractions are employed, the terminology "sperm [cell] fraction" and "non-sperm [cell] fraction" shall be used in the report any time a portion of the sample is amplified or qualifies for amplification. The results shall be reported as single result not referring to the fractions in the following situations:

    o When both fractions of a differential extraction exhibit the same quantitation testing result and are not amplified, the results shall be reported as a single result referring to the item only and not the individual fractions.

    o If fractions of the same extract have different results regarding insufficient male DNA for autosomal analysis, insufficient male DNA for further analysis, and/or no male DNA, the results shall be reported as a single result using the appropriate insufficient male DNA wording that indicates the largest amount of male DNA present between the two fractions.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

DNA PROFILE COMPARISONS

1. When reporting a mixture, the assumed number of contributors shall be stated.

2. When a person or a group of people are visually excluded from a sample, they are not required to be mentioned at every sample from which they are visually excluded.

3. A unique unknown individual may be determined and reported if there are twelve or more complete autosomal loci with > 99% weighting in a single source sample or a single profile determined from a mixture.

   o A unique individual shall be identified in the report as male or female if an unambiguous determination can be made from Amelogenin and the Y-STR loci results.

   o A unique unknown individual can be included/excluded from any additional single source or mixed sample (including samples with fewer than 12 loci) at the analyst's discretion. Care should be taken to ensure that this conclusion is unambiguous as the comparison within STRmix™ will not take place until a standard is submitted.

4. For each report detailing a new evidentiary DNA profile, the appropriate CODIS statement shall be included. The CODIS statement shall be with the closing statements at the end of the report.

5. All positive associations shall utilize a STRmix™ deconvolution and have a statistical evaluation applied and reported. The only exceptions being the following:

   o The owner on an intimate sample or an elimination standard reasonably assumed to be present on an item. Report wording shall include a statement indicating that the assumption was made.

      ▪ A profile may be reported as being consistent with an assumed individual with no conclusions drawn on the remainder of the sample due to the inability to determine a number of contributors.

   o A profile from one fraction of a differential extraction that is consistent with a conditioned individual and a profile from the corresponding fraction.

      ▪ The profile from the corresponding fraction may be utilized if it is single source or a single source profile determined by conditioning.

STATISTICAL EVALUATIONS

1. When reporting out the Likelihood Ratio, it can be reported numerically or in word form, (i.e., 1,000,000 versus 1 million).

2. A reporting ceiling of 1 trillion will be utilized. When a calculated LR is more than 1 trillion times more likely for all four population groups the phrase "at least 1 trillion" shall be utilized in place of the calculated LR number.

    o   A verbal scale shall accompany the LR result in an effort to provide additional explanation of the weight of the evidence.

Likelihood Ratio	Support Level	Support for:
LR ≥ 1,000,000	Very strong	Inclusion
10,000 ≤ LR < 1,000,000	Strong	Inclusion
100 ≤ LR < 10,000	Moderate	Inclusion
2 ≤ LR < 100	Limited	Inclusion
1	Uninformative	Uninformative
2 ≤ (1/LR) < 100	Limited	Exclusion
100 ≤ (1/LR)	--	Exclusion

3. Analysts have the discretion to report unambiguous exclusions without using STRmix™ to calculate a LR.

4. DNA mixtures in which multiple reference profiles indicate inclusion (LR support for H1) shall have multiple LR propositions calculated. The reference profile LRs shall be run individually to indicate individual weight in the mixture. Additionally, LRs shall be calculated to indicate the weight of all included individuals combined together in the mixture.

    o   There may be scenarios when multiple individuals (possibly even more individuals than present in the mixture) have a LR supporting H1 when compared to an evidence profile in isolation; however, the LR could favor exclusion when the individuals are compared to the evidence profile in combination with each other. In this scenario, the evidence profile could not have come from that combination of individuals. Such groups are termed 'incompatible' as, combined, they are incompatible with the evidence profile. This scenario is not unexpected when related individuals are compared to minor or low level DNA profiles. When appropriate, an analyst can attempt to test different combinations to provide further information. However, it is not always possible to test or list all incompatible groups.

5. When multiple LR propositions are calculated, it is recommended that the analyst report the single proposition that most reasonably explains the evidence based on the case information available, if possible. Case circumstances will dictate which of these calculations are most appropriate for the report and multiple propositions may need to be reported.

    o   Reporting individual LRs is required when the LR favoring inclusion of one individual falls in a different level of the verbal scale than the other individual. However, if each individual LR falls in the same level of the verbal scale (i.e., all individuals indicate very strong support for H1), it is appropriate to only report the combined LR.

- o The combined LR shall be reported when the calculated LR of all individuals combined equals 0 (no support for the individuals making up the mixture together).

    - ▪ The appropriate inclusionary individual LRs shall be reported along with the additional report wording statement indicating the individuals cannot make up the mixture together.

6. If the analyst cannot clearly exclude an individual, STRmix™ may be used to assist in the comparison.   If STRmix™ is used to determine an exclusion, the appropriate exclusion statement shall be included in the report for that individual.

    - o LR values less than 1 favor the defense proposition (H2). LR values less than 1 will be evaluated for the verbal equivalent as 1/LR.

        - ▪ 1/LR values between 2 and 99 will be reported as limited support for exclusion and will include the hypotheses and calculated 1/LR value in the report as stated in the report wording guidelines.

        - ▪ 1/LR values above 99 shall use the general exclusion statement as stated in the report wording guidelines.

        - ▪ An LR value of zero indicates no scientific support for the prosecution proposition (H1) and shall use the general exclusion statement as stated in the report wording guidelines.

7. 99% 1-sided lower HPD LR values will be calculated for the four subpopulations (African American, Asian, Caucasian, and Hispanic).

    - o When all four LR values are in the support of H1, the lowest LR of the four calculated populations will be reported, rounded down to two significant figures.

        - ▪ If the lowest LR in support of H1 is less than 2, it shall be rounded down to 1.0 (uninformative).

    - o If any of the four LR values is in support of H2, the highest 1/LR value favoring H2 will be reported, rounded up to two significant figures.

        - ▪ If the highest LR in support of H2 is less than 2, it shall be rounded up to 2.0 (limited support for exclusion).

    - o If any of the four calculated populations have a LR of zero, the LR shall be reported as zero.

8. Additional LR calculations performed by request after the initial report has been released shall be reported in a Supplemental Certificate of Analysis.

9. Reports issued with a verbal equivalent statement prior to September 24, 2018 shall not be reissued unless new statistical comparisons are made. If new comparisons are made, the verbal equivalents shall be updated as appropriate and appropriate wording shall be added to the report to indicate the change.

10. Any statistical result for analysis reported prior to July 13, 2015 shall be recalculated with the original statistical method used and appropriate updated allele frequencies utilizing IndySTR v2.2_04-01-2016 if:

    o Additional evidence is submitted in the case;

    o The case is going to court; or

    o Upon request;

    o If the reported statistical results change, a new (additional evidence submitted) or amended (for court or upon request) Certificate of Analysis shall be issued with notification included in the report and the statistical worksheets uploaded under the associated request number. If the reported statistical results do not change, the new statistical worksheets shall be uploaded under the original request number as v2.

11. Previously calculated statistics and comparisons should utilize the appropriate report writing statements approved at the date the calculations were originally performed. Supervisor approval shall be obtained.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

SEROLOGY STATEMENTS
Citations shall refer to the Report Appendix to reflect the test(s) supporting the result.

NEGATIVE RESULT
No blood was detected.[1]
No seminal material was detected.[2]

POSITIVE CONFIRMATORY RESULT
Human blood was confirmed.[3] DNA analysis was performed on X samples.

Seminal material was confirmed.[4] DNA analysis was performed on X samples.

POSITIVE PRESUMPTIVE RESULT
Blood was indicated by presumptive testing.[1] [DNA analysis was performed on X samples.]
Seminal material was indicated by presumptive testing.[2] [DNA analysis was performed on X samples.]

NEGATIVE CONFIRMATORY RESULT
In addition to positive presumptive result.

Confirmatory testing was negative [for human blood/seminal material].[3]/[4]

SEXUAL ASSAULT KIT
Could apply to any item containing multiple subitems which need to be addressed.

Item 00X: Evidence Collection Kit – PERSON
Items 00XA through 00XX were present [or prepared from items] in the kit.

NO SEROLOGICAL TESTING PERFORMED
For cases where it is important to note a lack of staining to test; not for routine use.

No stains were observed for serological testing.

TRACE EVIDENCE
Potential trace evidence was observed.[6]

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

HAIR STATEMENTS

Hair demonstrating characteristics of human hair with the potential for nuclear DNA analysis was observed/tested as item XA.[5]

Human hair without roots, or other hairs may be considered trace evidence when not significant. The wording below is to be used when hair is of evidentiary value.

Hair demonstrating characteristics of human hair but without the potential for nuclear DNA analysis was observed.[5]

Hair demonstrating characteristics not consistent with human hair was observed.[5]

ITEMS OR SUBITEMS NOT EXAMINED
For subitems not examined, the statement is placed at the end of the item.

The ITEMDESCRIPTION, ITEMDESCRIPTION, and ITEMDESCRIPTION (items XA-XZ) were not examined.

AUTOSOMAL DNA STATEMENTS

EXCLUSION
Excluded visually or with an individual STRmix™ calculated LR=0 or 1/LR ≥100.
To be placed with the appropriate interpretation statement and any other inclusions, before the LR bullet points.

PERSON is **excluded** [as a contributor to the DNA profile/mixture].

IDENTICAL TWINS
To be placed at the beginning of the results section.

The DNA profiles obtained from TWIN1 and TWIN2 demonstrated the same DNA profile. This is typically associated with identical twins. The conclusions reflect the comparisons with the provided standards.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

SINGLE SOURCE STATEMENTS

The DNA profile [from ITEMDESCRIPTION] was interpreted as originating from a single individual.

► The DNA profile is at least 1 trillion/LR times more likely if it originated from PERSON than if it originated from an unknown, unrelated individual.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON is **included**.

UNKNOWN PROFILE
STRmix™ deconvolution performed, but no LR calculated.

The DNA profile [from ITEMDESCRIPTION] was interpreted as originating from a single unknown male/female/individual [(Unknown Male/Female/Individual #)].

CONSISTENT WITH ELIMINATION STANDARD
No deconvolution performed.

The DNA profile [from ITEMDESCRIPTION] was interpreted as originating from a single individual. Assuming PERSON as a contributor, no additional alleles were detected.

MIXTURE STATEMENTS

GENERAL MIXTURE STATEMENT
LR ≥ 2, result supports H1, inclusion of one person of interest.

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of N individuals.

► The DNA profile is at least 1 trillion/LR times more likely if it originated from PERSON and N-1 unknown individual[s] than if it originated from N unknown, unrelated individuals.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON is **included**.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

DESIGNATED UNKNOWN INDIVIDUAL IN A MIXTURE
Statement utilized any time a designated unknown is either determined at 12 or more loci OR not excluded from the mixture. Utilized at analyst discretion ('excluded' is not bolded).

Unknown Female/Male/Individual #1 cannot be excluded [as a contributor to the mixture].

MIXTURE WITH CONDITIONING
LR ≥ 2, result supports H1, inclusion of one person of interest.

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of two individuals. PERSON2 was assumed as a contributor.

► The DNA profile is at least 1 trillion/LR times more likely if it originated from PERSON2 and PERSON1 than if it originated from PERSON2 and an unknown, unrelated individual.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON1 is **included**.

UNKNOWN PROFILES ONLY
STRmix™ deconvolution performed, but no LR calculated. The sex and number of specific unknown individuals may be adjusted (or omitted) as appropriate.

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of two/three/four unknown individuals [, including at least one male].

UNKNOWN PROFILES ONLY WHEN VARNOC UTILIZED
The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of N or N+1 individuals [, including at least one male].

CONDITIONED PROFILE + UNKNOWN(S)
STRmix™ deconvolution performed, but no LR calculated. The sex and number of specific unknown individuals may be adjusted (or omitted) as appropriate.

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of two individuals. Assuming PERSON as a contributor, the mixture contains an unknown male/female/individual [(Unknown Male/Female/Individual #)].

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of three/four individuals [, including at least one male]. Assuming PERSON as a contributor, the mixture contains two/three unknown individuals.

CONSISTENT WITH MULTIPLE ELIMINATION STANDARDS
No deconvolution performed.

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of two individuals. Assuming PERSON1 and PERSON2 as contributors, no additional alleles were detected.

CONSISTENT WITH ELIMINATION STANDARD + DIFFERENTIAL FRACTION
In one differential fraction, may assume corresponding differential fraction. No deconvolution performed.

The DNA profile from the non-sperm/sperm fraction [of ITEMDESCRIPTION] was interpreted as a mixture of two individuals. Assuming PERSON and the corresponding sperm/non-sperm fraction as contributors, no additional alleles were detected.

LIMITED EXCLUSIONARY LR
STRmix™ LR calculated, 2≤1/LR<100, result supports H2.

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of N individuals.

► The DNA profile is LR times more likely if it originated from N unknown, unrelated individuals than if it originated from PERSON and N-1 unknown individual[s].
► This statistical analysis provides limited support for the proposition that PERSON is **excluded**.

INDIVIDUAL INCLUSIONS, BUT EXCLUDED TOGETHER
To be placed after the appropriate individual inclusionary statements.

Note:
Given the interpretation that the mixture originated from N individuals, there is support for the propositions that the DNA profile originated from PERSON1 or PERSON2 individually. However, there is no scientific support for the proposition that the DNA profile originated from PERSON1 and PERSON2 together. Refer to the independent inclusionary statistics reported above.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

INCONCLUSIVE LR
STRmix™ LR calculated, LR rounds to 1.0.

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of N individuals.

► A likelihood ratio of 1.0 was obtained. The DNA profile is equally as likely if it originated from PERSON and N-1 unknown individual[s] or if it originated from N unknown, unrelated individuals.
► This result is considered **uninformative**.

ALTERNATE PROPOSITIONS

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of two individuals. The probability of the evidence has been calculated by considering multiple propositions.

PERSON1 and PERSON2 were considered as contributors:
► The DNA profile is at least 1 trillion/LR times more likely if it originated from PERSON1 and PERSON2 than if it originated from two unknown, unrelated individuals.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that both PERSON1 and PERSON2 are **included**.

Assuming PERSON2 as a contributor, PERSON1 was considered [as a contributor]:
► The DNA profile is at least 1 trillion/LR times more likely if it originated from PERSON2 and PERSON1 than if it originated from PERSON2 and an unknown, unrelated individual.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON1 is **included**.

VARIABLE NUMBER OF CONTRIBUTORS
The appropriate mixture wording should be adjusted to accurately reflect results. One example below.

The DNA profile [from ITEMDESCRIPTION] was interpreted as a mixture of N or N+1 individuals.

► The DNA profile is at least 1 trillion/LR times more likely if it originated from PERSON and N unknown individual[s] than if it originated from N+1/N-1 unknown, unrelated individuals.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON is **included**.

INCONCLUSIVE SAMPLES

NO ALLELES DETECTED

ITEMDESCRIPTION failed to demonstrate a DNA profile.

INCONCLUSIVE NUMBER OF CONTRIBUTORS

The DNA profile [from ITEMDESCRIPTION] has limited data/is a mixture. No further conclusions can be drawn due to the uncertainty in the number of contributors.

CONSISTENT WITH ELIMINATION STANDARD + INCONCLUSIVE NOC
Consistent with elimination standard but no conclusion being drawn from additional alleles.

The DNA profile [from ITEMDESCRIPTION] is a mixture. Assuming PERSON as a contributor, no further conclusions can be drawn due to the uncertainty in the number of contributors.

>4 CONTRIBUTORS

No conclusion can be drawn [from ITEMDESCRIPTION] because the DNA profile is a mixture of more than four individuals.

FAILED CONTROL
Statement to be placed before listed results for an item. Not all possible combinations are given; consultation with the Technical Leader and/or Supervisor is required for situations not covered below.

During analysis [of ITEMDESCRIPTION], a positive/negative test control failed. Data was evaluated and determined to be suitable for interpretation.

During analysis [of ITEMDESCRIPTION], a positive/negative test control failed. Data was evaluated and determined to be unsuitable for interpretation; therefore, no conclusions can be drawn.

CONTAMINATION

When Database Search LR ≥ 10,000. Not all possible combinations are given; consultation with the Technical Leader and/or Supervisor is required.

The DNA profile [from ITEMDESCRIPTION] was interpreted as originating from a single identified individual from the Indiana State Police Laboratory Division Quality Assurance DNA Database. Assuming this individual as a contributor, no additional alleles were detected.

The DNA profile was interpreted as a mixture of N individuals. An identified individual from the Indiana State Police Laboratory Division Quality Assurance DNA Database was present and assumed as a contributor.

► The DNA profile is at least 1 trillion/LR times more likely if it originated from the Database individual and PERSON than if it originated from the Database individual and an unknown, unrelated individual.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON is **included**.

SAMPLES NOT AMPLIFIED / QUANT-OUTS

In a sample with insufficient (male) DNA for amplification, where it is valuable to identify a male contributor from a female contributor (i.e. sexual assault cases), the reported results shall be based on the Quantifiler® Trio Y target.

INSUFFICIENT HUMAN DNA

ITEMDESCRIPTION demonstrated an insufficient quantity of DNA for further analysis.

INSUFFICIENT MALE DNA for AUTOSOMAL ANALYSIS
Not eligible for autosomal, but male DNA is greater than 0.006 ng/µl

ITEMDESCRIPTION demonstrated an insufficient quantity of male DNA for autosomal STR analysis.

INSUFFICIENT MALE DNA
Not eligible for either autosomal or Y-STR analysis

ITEMDESCRIPTION demonstrated an insufficient quantity of male DNA for further analysis.

UNDETECTED MALE DNA

No male DNA was detected [on ITEMDESCRIPTION].

SAMPLE TRIAGED
Eligible for autosomal analysis, but not amplified

ITEMDESCRIPTION was/were extracted and quantified; however, no additional DNA analysis was performed [due to the development of DNA profiles on other items].

SAMPLE/SUBITEM NOT TESTED FOR DNA
May modify as shown if permission to consume is required.

No DNA analysis was performed. [If further DNA analysis is desired, permission to consume the sample is required. Please contact the reporting analyst.]

STANDARD ITEMS

Item X: DNA Standard – PERSON

STAIN CARD

A stain card was prepared [and returned to the original packaging].

PROFILE DEVELOPED/NOT DEVELOPED

The DNA profile [of PERSON] was [previously] developed.

No DNA profile [of PERSON] was developed [at this time].

SEXUAL ASSAULT KIT ORAL SWAB DEVELOPED AS STANDARD

If female victim:
Appropriate male quantitation statement. The DNA profile was developed as a standard for PERSON.

<u>If male victim</u>:
The DNA profile [from ITEMDESCRIPTION] was interpreted as originating from a single individual. This profile will be used as a DNA standard for PERSON.

PROFILE DEVELOPED BUT NOT INTERPRETED

The sample was processed but not interpreted due to a lack of evidence for comparison.

MIXED PROFILE OBTAINED
To be used only with Technical Leader consultation and approval.

The DNA profile was interpreted as a mixture of N individuals. A distinct female/male profile was determined to be used as the assumed standard for PERSON.

MULTIPLE STANDARDS

The standard was not examined due to the presence of multiple standards.

STANDARD COMPARED TO EVIDENCE IN OTHER CASE
For the case that contains the standard

The DNA profile of PERSON (item XXA) was compared to the evidence in Indiana State Police Laboratory Case XXX-XXXXX, Other Police Department Case XXXXX.

EVIDENCE COMPARED TO STANDARD FROM OTHER CASE
For the case that contains the evidence but not the standard. Placed at the beginning of the report.

Evidence was compared to the DNA profile of PERSON (item XA) developed in Indiana State Police Laboratory Case XXX-XXXXX, Other Police Department Case XXXXX.

CODIS STATEMENTS
All placed in the end section of the report.

[The] DNA profile[s] from [list item numbers] [was/were] entered into the Combined DNA Index System (CODIS) and will be searched on a routine basis. In the event of a database match, additional information will be provided separately.

PENDING STANDARD SUBMISSION

Upon submission of an appropriate DNA standard from PERSON, the DNA profile from item number will be re-evaluated for possible entry into the Combined DNA Index System (CODIS).

PENDING ADDITIONAL INFORMATION

Additional information is required regarding item number before it can be determined if any DNA profiles are eligible for the Combined DNA Index System (CODIS). Please contact the reporting analyst if a database search is desired.

NO CODIS ENTRIES
To be used when profiles are developed but none are suitable for entry.

No DNA profiles were entered into the Combined DNA Index System (CODIS).

REMOVAL FROM CODIS
To be used in supplemental reports when profiles are deleted from the database.

[The] DNA profile[s] from [list item numbers] [was/were] removed from the Combined DNA Index System (CODIS).

OTHER STATEMENTS
All wording in this section placed at the end of the report unless otherwise indicated.

ALTERNATE CALCULATIONS
To be used additional/alternate LRs were calculated but not included in report.

Alternate calculations have been performed for item(s) XA, XB and XC and are available upon request.

STATISTICS STATEMENT WHEN FREQUENCY REPORTED
To be placed prior to the appropriate SWGDAM statement.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

The data in this report was interpreted and evaluated prior to the implementation of Likelihood Ratios at the Indiana State Police Laboratory. The statistical estimates are presented in the form of a frequency.

POWERPLEX® FUSION 6C DATA WITH RMP/CPI

To be used when Fusion 6C data was reported with a frequency prior to November 1, 2017.

Statistical estimates were performed based on the recommended concepts endorsed by the Scientific Working Group on DNA Analysis Methods (1/12/17). Y-STR loci included with the PowerPlex® Fusion 6C System were used for interpretations but were not included in statistical estimates. Calculations for the Caucasian, African American, and Hispanic populations have been performed. The most common frequency has been reported unless all calculated frequencies exceed 1 in 8 trillion.

RE-EVALUATING DATA WITH NEW FBI ALLELE FREQUENCIES

Stats calculated prior to July 13, 2015 and re-calculated with updated allele frequencies.

The statistical calculations were adjusted to reflect the amended allele frequencies published by the FBI. *Moretti, T.R., Budowle, B. and Buckleton, J. S., Erratum, Journal of Forensic Sciences 2015; 60(4).*

RE-EVALUATING DATA WITH NEW INTERPRETATION GUIDELINES

Requires Supervisor's approval and shall be placed at the beginning of the results section.

In the DNA analysis detailed below, sample data was re-evaluated using methods implemented after the original report was issued. Accordingly, the following conclusions may differ from the original report.

UPDATING STATISTICS WITH VERBAL EQUIVALENT

Shall be placed at the beginning of the results section. Applies to reports issued with a verbal equivalent statement prior to September 24, 2018.

In the DNA analysis detailed below, sample conclusions were updated utilizing a verbal qualifying statement recommended by the Scientific Working Group on DNA Analysis Methods. Accordingly, the following conclusions may differ from the original report.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

RE-ANALYSIS OF SAMPLE FOR ADDITIONAL DATA
Requires Supervisor's approval and shall be placed at the beginning of the results section. Other situations may be listed as needed with supervisor approval.

Additional analysis of the ITEMDESCRIPTION was performed. The resulting profile was suitable/not suitable for familial searching/entry/updating in the Combined DNA Index System (CODIS). In the event of a database match, additional information will be provided separately.

REQUEST FOR STANDARDS

If additional comparisons are desired, please submit an appropriate DNA standard (such as an oral/buccal swab standard) from [victim/consensual partner/named suspect or] any [additional] suspect(s).

Y-STR/NGS ANALYSIS PENDING

When completed, results from [Y-STR/Next Generation Sequencing] analysis will be provided separately.

PATERNITY/KINSHIP ANALYSIS PENDING

When completed, Paternity/Kinship analysis of the DNA profiles determined for PERSON1, PERSON2, and PERSON3 will be provided separately.

SAMPLES SENT TO ANOTHER LABORATORY

Item(s) XA [were/was] sent to XYZ Laboratory, city, state for [additional] analysis as per the authority of John Smith, Police Department.

WITHDRAWAL STATEMENTS

The request for DNA analysis was withdrawn by John Smith, Police Department, on Date. The items will be returned to the submitting agency.

The request for DNA analysis was withdrawn. The evidence is not eligible for entry into the Combined DNA Index System (CODIS). Submission of appropriate DNA standard(s) (such as an oral/buccal swab standard) is required before analysis can be completed. The evidence will be returned to the submitting agency.

The request for DNA analysis was withdrawn in order to preserve the sampling area for Latent Print examination. The items will be returned to the submitting agency.

The request for laboratory examination was withdrawn per information found in MyCase, Cause Number: CAUSE NO, DATE. The items will be returned to the submitting agency. If analysis is needed, please re-submit the item(s) to the laboratory.

EXAMPLES

Example 1: Homicide with Kit

Item 001: Evidence Collection Kit – Jane Smith
Items 001A through 001K were present or prepared from items in the kit.

Item 001A: Blood standard
A stain card was prepared. The DNA profile was developed.

Item 001B: Vaginal wash
The vaginal wash demonstrated an insufficient quantity of male DNA for autosomal STR analysis.

Item 001C: Vaginal/cervical swabs (2)
Sperm fraction
The DNA profile from the combined swabs was interpreted as a mixture of two individuals. Jane Smith was assumed as a contributor.

► The DNA profile is at least 1 trillion times more likely if it originated from Jane Smith and John Doe than if it originated from Jane Smith and an unknown, unrelated individual.
► This statistical analysis provides very strong support for the proposition that John Doe is **included**.

Non-sperm fraction

The DNA profile from the combined swabs was interpreted as a mixture of two individuals. Assuming Jane Smith and the corresponding sperm fraction as contributors, no additional alleles were detected.

Item 001D: Neck swabs (2)

The DNA profile from the combined swabs was interpreted as a mixture of two individuals. Assuming Jane Smith as a contributor, the mixture contains an unknown male (Unknown Male #1).

Item 001E: External genital swab

The swab demonstrated an insufficient quantity of male DNA for autosomal STR analysis.

Item 001F: Anal swabs (6)

Four combined swabs demonstrated an insufficient quantity of male DNA for autosomal STR analysis. No DNA analysis was performed on two remaining swabs.

Item 001G: Underwear

Sperm fraction
The DNA profile from the sample was interpreted as originating from a single individual.

► The DNA profile is at least 1 trillion times more likely if it originated from John Doe than if it originated from an unknown, unrelated individual.
► This statistical analysis provides very strong support for the proposition that John Doe is **included**.

Non-sperm fraction
The sample demonstrated an insufficient quantity of male DNA for further analysis.

The pubic hair combings, head hair combings, foreign material collection, and underwear (items 001H through 001K) were not examined.

_____

Item 002: DNA standard – John Doe

The DNA profile was developed.

_____

Item 003: Knife

Human blood was confirmed.[3] DNA analysis was performed on two samples.

Item 003A: Knife blade
The DNA profile from the knife blade was interpreted as a mixture of two individuals. John Doe is **excluded** as a contributor. Unknown Male #1 cannot be excluded as a contributor.

► The DNA profile is 200 times more likely if it originated from Jane Smith and one unknown individual than if it originated from two unknown, unrelated individuals.
► This statistical analysis provides moderate support for the proposition that Jane Smith is **included**.

Item 003B: Knife handle
The DNA profile from the knife handle was interpreted as originating from a single unknown male (Unknown Male #1).
_____

Item 004: Shirt
Human blood was confirmed.[3] Seminal material was indicated by presumptive testing.[2] DNA analysis was performed on four samples.

Item 004A
Sperm Fraction
The DNA profile was interpreted as originating from a single unknown male (Unknown Male #2).

Non-Sperm Fraction
The DNA profile was interpreted as originating from a single individual. Assuming John Doe as a contributor, no additional alleles were detected.

Items 004B and 004C
The DNA profiles were each interpreted as originating from a single individual.

► Each DNA profile is at least 1 trillion times more likely if it originated from Jane Smith than if it originated from an unknown, unrelated individual.
► Each statistical analysis provides very strong support for the proposition that Jane Smith is **included**.

Item 004D
The sample demonstrated an insufficient quantity of DNA for further analysis.
_____

DNA profiles from items 001D and 004A were entered into the Combined DNA Index System (CODIS) and will be searched on a routine basis. In the event of a database match, additional information will be provided separately.

Example 2: Crime against person – single item multiple components
Use the kit format for a collection of different objects within one evidence item.

Item 005: Bag of trash
Items 005A-005D were present in the bag of trash.

Item 005A: Condom wrapper
The DNA profile was interpreted as originating from a single individual. Bob Roberts is **excluded** as a contributor to the DNA profile.

► The DNA profile is 17 times more likely if it originated from Jane Smith than if it originated from an unknown, unrelated individual.
► This statistical analysis provides limited support for the proposition that Jane Smith is **included**.

Item 005B: Piece of gum
Seminal material was indicated by presumptive testing.[2]

Sperm Fraction
The DNA profile was interpreted as originating from a single individual.

► The DNA profile is 17 times more likely if it originated from Bob Roberts than if it originated from an unknown, unrelated individual.
► This statistical analysis provides limited support for the proposition that Bob Roberts is **included**.

Non-Sperm Fraction
The DNA profile was interpreted as originating from a single individual.

► The DNA profile is at least one trillion times more likely if it originated from Jane Smith than if it originated from an unknown, unrelated individual.
► This statistical analysis provides very strong support for the proposition that Jane Smith is **included**.

_____

The banana peel and apple core (items 005C and 005D) were not examined.

Example 3: Crime against person – single item multiple components - clothing
Use the kit format for a collection of different objects within one evidence item.

Item 001: Bag of clothing
A shirt (item 001A), jeans (item 001B) and a pair of socks (item 001C) and a pair of shoes (item 001D) were present.

Item 001A: Shirt
Human blood was confirmed.[3] DNA analysis was performed on two samples.

Item 001A1
The DNA profile from sample was interpreted as originating from a single unknown male (Unknown Male #1).

Item 001A2
The DNA profile was interpreted as originating from a single individual. Assuming Jane Doe as a contributor, no additional alleles were detected.

Item 001B: Jeans
Seminal material was indicated by presumptive testing.[2]

Sperm Fraction
The DNA profile was interpreted as originating from a single unknown male (Unknown Male #2).

Non-Sperm Fraction
The DNA profile was interpreted as originating from a single individual. Assuming Jane Doe as a contributor, no additional alleles were detected.

Item 001C: Socks
Blood was indicated by presumptive testing on each sock.[1] DNA analysis was performed on three samples.

Item 001C1
Item 001C1a
The DNA profile from sample was interpreted as originating from a single unknown male (Unknown Male #1).

Item 001C1b

The sample demonstrated an insufficient quantity of DNA for further analysis.

Item 001C2
The DNA profile was interpreted as originating from a single individual. Assuming Jane Doe as a contributor, no additional alleles were detected.

Item 001D: Shoes
The DNA profile was interpreted as originating from a single individual. Assuming Jane Doe as a contributor, no additional alleles were detected.

_____

Item 002: DNA standard – Jane Doe
The DNA profile was developed.

_____

DNA profiles from items 001A1 and the sperm fraction of 001B were entered into the Combined DNA Index System (CODIS) and will be searched on a routine basis. In the event of a database match, additional information will be provided separately.

Example 4: Property crime with serological testing
Submitted swabs that are considered distinctly separate (due to case circumstances, sample purpose, serology results, etc.) get separate (subitem) designations. Item 001 in the following example contains swabs that are distinct because of serological results and may have different DNA treatment. Item 002 contains swabs that have no reason to be considered separately.

Item 001: Swabs of window (2)

Item 001A
Blood was indicated by presumptive testing.[1] Confirmatory testing was negative.[3]

The DNA profile was interpreted as originating from a single unknown female (unknown female #1).

Item 001B
No blood was detected on the remaining swab.[1] No DNA analysis was performed.

_____

Item 002: Swabs of floor (2)
Blood was indicated by presumptive testing on the two swabs.[1] Human blood was confirmed on one swab.[3] No confirmatory testing was performed on the remaining swab.

The DNA profile from the combined swabs was interpreted as originating from a single unknown male (unknown male #1).

_____

Item 003: Buccal swab standard – Robert Jones
The DNA profile was developed.

Example 5: Property crime – no serological testing

Item 001: Crowbar
A sample of the crowbar demonstrated an insufficient quantity of DNA for further analysis.

_____

Item 002: Swabs of dresser drawer (2)
The DNA profile from the combined swabs was interpreted as originating from a single individual. Assuming Sally Smith as a contributor, no additional alleles were detected.

_____

Item 003: DNA standard – Sally Smith
The DNA profile was developed.

Example 6: Crime against person – many areas on one item

Item 001: Victim's Blanket
Human blood was confirmed.[3] DNA analysis was performed on six samples.

Items 001A and 001B
Each sample demonstrated an insufficient quantity of DNA for further analysis.

Items 001C and 001D
Each DNA profile was interpreted as originating from a single individual. Assuming Mary Doe as a contributor, no additional alleles were detected.

Items 001E and 001F
The DNA profiles were each interpreted as mixtures of two individuals. Mary Doe and Bob Doe are **excluded** as contributors.

► Each DNA profile is at least 1 trillion times more likely if it originated from John Doe and an unknown individual than if it originated from two unknown, unrelated individuals.

► Each statistical analysis provides very strong support for the proposition that John Doe is **included**.

_____

Item 002: Shirt
Human blood was confirmed.[3]

The DNA profile was interpreted as a mixture of two individuals. The probability of the evidence has been calculated by considering multiple propositions. Bob Doe is **excluded** as a contributor.

Mary Doe was considered as a contributor.
► The DNA profile is 40,000 times more likely if it originated from Mary Doe and one unknown individual than if it originated from two unknown, unrelated individuals.
► This statistical analysis provides strong support for the proposition that Mary Doe is **included**.

John Doe was considered as a contributor.
► The DNA profile is 8,500 times more likely if it originated from John Doe and one unknown individual than if it originated from two unknown, unrelated individuals.
► This statistical analysis provides moderate support for the proposition that John Doe is **included**.

Note:
Given the interpretation that the mixture originated from two individuals, there is support for the propositions that the DNA profile originated from Mary Doe or John Doe individually. However, there is no scientific support for the proposition that the DNA profile originated from Mary Doe and John Doe together. Refer to the independent inclusionary statistics reported above.

Example 7: Possession of firearm (swabs submitted)

Item 001: Swabs of Firearm (3)

Items 001A1 and 001A3
The DNA profiles from the swabs were each interpreted as originating from a single unknown male (Unknown Male #1).

Item 001A2
The swab demonstrated an insufficient quantity of DNA for further analysis.

Example 8: Possession of firearm (firearm submitted)

Item 001: Firearm
Three samples were tested.

Items 001A1 and 001A3
The DNA profiles from two samples were each interpreted as originating from a single unknown male (Unknown Male #1).

Item 001A2
The swab demonstrated an insufficient quantity of DNA for further analysis.

Example 9: Simple items (swabs distinguished by description, headers not required)

Item 001: Swabs from Chevy (2)
The swabs each demonstrated an insufficient quantity of DNA for further analysis.

_____

Item 002: Swabs from Ford (2)
The DNA profile from the steering wheel swab was interpreted as originating from a single unknown male (Unknown Male #1).

The swab of the gear shift demonstrated an insufficient quantity of DNA for further analysis.

_____

Example 10: Additional reports without subitems in which the original parent item is related in LIMS (new comparisons, Y-STR, paternity testing, etc)

Item 001: Evidence Collection Kit – Jane Smith

Item 001A: DNA standard – Jane Smith
The DNA profile was previously developed.

_____

The Appendix below will be added to all Biology and Y-STR Requests in Justice Trax after the analyst's signature, formatted as a stand-alone page.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

Biology Report Appendix

The following includes supplemental information on biological analysis performed at the Indiana State Police Laboratory and definitions of some commonly used terms. Not all terms referenced in the appendix may apply to the testing in the associated case report. Please contact the reporting analyst with specific questions regarding the DNA analysis in this case.

Serological testing: Citations in the report refer to the test(s) listed below.
Presumptive testing indicates the possible presence of the substance in question.
1. Blood: Kastle-Meyer/phenolphthalein chemical test
2. Seminal material: Acid Phosphatase chemical test
Confirmatory testing identifies the substance in question.
3. Human blood: HemDirect™ immunological test + Kastle-Meyer/phenolphthalein test
4. Seminal material: microscopic sperm search + Acid Phosphatase test
Other examinations:
5. A macroscopic and microscopic examination of a potential hair is performed to determine if it is consistent with human hair and if root material may be present.
6. Trace evidence may include but is not limited to paint, glass, hair, and fibers.

I. The terms **"sperm fraction"** and **"non-sperm fraction"** refer to the two portions of the extract generated during a differential extraction procedure. The presence of DNA in the "sperm fraction" does not confirm the presence of seminal material and may or may not indicate the type of cells from which the DNA originated.

II. **Insufficient DNA:** Following extraction and quantitation, DNA profiles are not developed from samples which do not yield enough DNA based on the laboratory's established threshold.

III. **Insufficient Male DNA:** In cases where male DNA is of interest, autosomal DNA profiles are not developed from samples which did not yield enough male DNA based on the laboratory's established threshold. These samples may still be eligible for Y-STR analysis. Male DNA quantitation at low levels can be unreliable and does not confirm the presence of male DNA.

IV. An **unknown individual** may be given a designation (e.g., "Unknown Male #1") to distinguish between multiple unknown profiles within a case or to indicate multiple samples consistent with the same unknown profile.

V. A **mixture** is a sample that contains DNA from more than one person. Mixtures with limited or low quality data and mixtures of more than four contributors are not interpretable by the laboratory's current methods.

VI. An individual is **assumed** to be a contributor when their contribution to a DNA sample is reasonably expected based upon the context of the sample and the presence of their DNA is not in question. This may include samples recovered from the individual's own body or from an item they regularly use. Using the DNA profile of the assumed individual allows for a more accurate interpretation and representative statistic for the portion of the profile that is in question.

VII. Statistics are presented in the form of a **likelihood ratio** (LR), which is a comparison between two competing hypotheses to explain the evidence profile. If all parties agree that the DNA profile originated from the same individual(s), the LR would equal 1. An LR of 0 is an exclusion. Calculations for the Caucasian, African American, Hispanic, and Asian populations are performed. The lowest LR is reported unless all calculated LRs exceed 1 trillion. The LR is based upon the case information provided. If additional information should be considered, please contact the reporting analyst.

VIII. In some DNA mixtures, false inclusions of other closely related **family members** (such as parents, offspring, and siblings) can occur due to the elevated sharing of genetic information between relatives. When first order relatives share many alleles with a person of interest, an adventitious likelihood ratio can give strong support for inclusion. The rate of adventitious support with related individuals increases with increasing mixture complexity. If a case involves potential related individuals, the best course of action is to submit known DNA standards for direct comparison and statistical evaluation.

IX. The statistical calculations are reported with a **verbal qualifying statement** to express the strength of statistical support. These statements follow the verbal scale recommended by the Scientific Working Group on DNA Analysis Methods (7/12/18). The scale reflects stronger degrees of support as the likelihood ratio increases. Reported exclusion(s) may include statistical calculations to support the verbal qualifying statement.

Likelihood Ratio	Support Level	Support for:
LR ≥ 1,000,000	Very strong	Inclusion
10,000 ≤ LR < 1,000,000	Strong	Inclusion
100 ≤ LR < 10,000	Moderate	Inclusion
2 ≤ LR < 100	Limited	Inclusion
1	Uninformative	Uninformative

2 ≤ (1/LR) < 100	Limited	Exclusion
100 ≤ (1/LR)	--	Exclusion

X. **Evidence disposition:** Unless otherwise noted, all portions of the evidence not consumed during analysis will be returned to the submitting agency. Retained items will remain at the Indiana State Police Laboratory. The remainder of the evidence or the original packaging will be returned.

XI. **DNA chemistry:** Unless otherwise noted, DNA profiles are developed using Polymerase Chain Reaction (PCR) with the PowerPlex® Fusion 6C System by Promega Corporation. Y-STR loci may be used for interpretations but are not included in statistical estimates.

Relationship Testing Wording

INCONCLUSIVE

The DNA profile [from ITEMDESCRIPTION] has [limited data/is a mixture]. [1]/[2]/[3] No further conclusions can be drawn due to insufficient quality of the data.

DNA STANDARD RESULT STATEMENTS
Citations shall refer to the Report Appendix to reflect the technology and amplification kit used to generate the profile.

MIXTURE - PRODUCTS OF CONCEPTION

The DNA profile was interpreted as a mixture of two individuals. Assuming BIOMOTHER as a contributor, a distinct male/female profile was determined to be used as the standard for the product of conception. [1]/[2]/[3]

MIXTURE - SECONDARY STANDARDS

The DNA profile was interpreted as a mixture of two/three individuals. A distinct male/female profile was determined to be used as the secondary standard for PERSON. [1]/[2]/[3]

TRASH PULL – PROFILE TO BE USED FOR STATISTICAL CALCULATIONS

The DNA profile [from ITEMDESCRIPTION] was interpreted as [a mixture / originating from a single individual]. A [distinct] male/female profile was determined from the sample and was purported to belong to PERSON. [1]/[2]/[3]

MULTIPLE EVIDENTIARY OR STANDARD SAMPLES
For varied mixture results

DNA profiles were developed for # additional samples of ITEMDESCRIPTION; however, these profiles were not used for statistical purposes. [1]/[2]/[3]

PARENTAGE RESULTS

ALLEGED INDIVIDUAL CANNOT BE EXCLUDED

STANDARD TRIO AND SINGLE PARENTAGE

PERSON1 and CHILD were evaluated as biological father and child by considering the following propositions:

► H1: [PERSON2 is the mother and] PERSON1 is the father of CHILD.
► H2: [PERSON2 is the mother and] A[a]n unknown, unrelated man is the father of CHILD.

►The genetic results are CPI times more likely if PERSON1 is the father of CHILD than if an unknown, unrelated man is the father of CHILD.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON1 and CHILD are related as biological father and child.

INCESTUOUS PARENTAGE

PERSON1 and CHILD were evaluated as biological father and child by considering the following propositions:

► H1: PERSON2 is the mother of CHILD; PERSON1 is the father of PERSON2 and CHILD.
► H2: PERSON2 is the mother of CHILD; PERSON 1 is the father of PERSON2; and an unknown, unrelated man is the father of CHILD.

►The genetic results are CPI times more likely if PERSON1 is the father of CHILD than if an unknown, unrelated man is the father of CHILD.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON1 and CHILD are related as biological father and child.

REVERSE PARENTAGE

PERSON1 and PERSON2 were evaluated as the biological parents of CHILD by considering the following propositions:

► H1: PERSON1 and PERSON2 are the parents of CHILD.
► H2: Two unknown, unrelated individuals are the parents of CHILD.

►The genetic results are CPI times more likely if PERSON1 and PERSON2 are the parents of CHILD than if two unknown, unrelated individuals are the parents of CHILD.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON1 and PERSON2 are the biological parents of CHILD.

ALLEGED INDIVIDUAL CAN BE VISUALLY EXCLUDED

Given the lack of genetic markers that must be contributed to the child by the biological father in the genetic results, PERSON can be excluded as the biological father of CHILD.

OTHER RELATIONSHIPS

SIBSHIP (LR SUPPORT FOR RELATIONSHIP)

CHILD1 and CHILD2 were evaluated as full siblings by considering the following propositions:

► H1: CHILD1 and CHILD2 are related as full siblings.
► H2: CHILD1 and CHILD2 are unrelated.

►The genetic results are CPI times more likely if CHILD1 and CHILD2 are related as full siblings than if CHILD1 and CHILD2 are unrelated.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that CHILD1 and CHILD2 are related as full siblings.

PEDIGREE OPTION 1

PERSON1, PERSON2, and PERSON3 were evaluated for relatedness by considering the following propositions:

► H1: PERSON1 is the X of PERSON2 and [and the Y of] PERSON3.
► H2: PERSON1 is the X of PERSON2; PERSON3 is unrelated.

►The genetic results are CPI times more likely if PERSON1, PERSON2, and PERSON3 are related as alleged (H1) than if PERSON3 is unrelated to PERSON1 and PERSON2 (H2).
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that includes the alleged relationship (H1).

PEDIGREE OPTION 2

PERSON1, PERSON2, and PERSON3 were evaluated for relatedness by considering the following propositions:

► H1: PERSON1 is the X of PERSON2 and [and the Y of] PERSON3.
► H2: PERSON1 is the X of PERSON2; PERSON3 is unrelated.

►The genetic results are CPI times more likely if PERSON1, PERSON2, and PERSON3 are related as X [and Y] than if PERSON3 is unrelated to PERSON1 and PERSON2.
► This statistical analysis provides limited/moderate/strong/very strong support for the proposition that PERSON3 is a X [and Y] of PERSON1 and PERSON2.

ADDITIONAL STATEMENT FOR LIMITED SUPPORT FOR H1

The statistical results for RELATIONSHIP are in a range where adventitious support with unrelated individuals can occur. These results should be considered in the context of other information known in the case when making determinations of the identity of the individual. Additional genetic testing is recommended if further information is needed. Please contact the reporting analyst if desired.

UNINFORMATIVE RESULTS

CHILD1 and CHILD2 were evaluated as full siblings by considering the following propositions:

► H1: CHILD1 and CHILD2 are related as full siblings.
► H2: CHILD1 and CHILD2 are unrelated.

►The genetic results are equally as likely if CHILD1 and CHILD2 are related as full siblings or if CHILD1 and CHILD2 are unrelated.
► This result is considered uninformative.

LR SUPPORT FOR LIMITED EXCLUSION

CHILD1 and CHILD2 were evaluated as full siblings by considering the following propositions:

► H1: CHILD1 and CHILD2 are related as full siblings.
► H2: CHILD1 and CHILD2 are unrelated.

► The genetic results are CPI times more likely if CHILD1 and CHILD2 are unrelated than if CHILD1 and CHILD2 are related as full siblings.
► This statistical analysis provides limited support for the proposition that CHILD1 and CHILD2 are unrelated.

The statistical results for full siblings are in a range where the alleged relationship is not supported despite there being a potential biological relationship. These results should be considered in the context of other information known in the case when making determinations of the identity of the individual. Additional genetic testing is recommended if further information is needed. Please contact the reporting analyst if desired.

ALLEGED INDIVIDUAL IS EXCLUDED BY CPI (1/CPI≥100)

CHILD1 and CHILD2 were evaluated as full siblings by considering the following propositions:

► H1: CHILD1 and CHILD2 are related as full siblings.
► H2: CHILD1 and CHILD2 are unrelated.

► The genetic results are CPI times more likely if CHILD1 and CHILD2 are unrelated than if CHILD1 and CHILD2 are related as full siblings.
► This statistical analysis supports the proposition that CHILD1 and CHILD2 are unrelated individuals.

ALLEGED INDIVIDUAL IS EXCLUDED BY FULL Y-STR ANALYSIS

Given the inconsistencies between the Y-STR genetic markers, CHILD1 can be excluded as the biological full sibling of CHILD2.

ALLEGED INDIVIDUAL IS INCLUDED BY FULL Y-STR ANALYSIS (NO INCONSISTENCIES)

The Y-STR profile from PERSON 1 was compared to the Y-STR profile developed from PERSON 2. No mismatches were observed out of # evaluated Y-STR loci. Based on this comparison, it is likely that the donors of the Y-STR profiles are paternally related. These results are based only on the Y-STR data developed at the time of reporting and do not supersede any additional genetic testing that may be performed. They should be evaluated in context with the autosomal kinship statistical results reported above.

STATEMENTS LOCATED AT THE END OF THE REPORT

OBSERVED MUTATION or NULL ALLELE

Incorporating a null allele into kinship calculations requires Technical Leader permission.

A single genetic inconsistency between CHILD and ALLEGED FATHER was observed at LOCUS. This has been incorporated into the calculation as a [mutation/null allele].

OBSERVED MUTATION AT A Y-STR LOCUS WITH FULL Y-STR ANALYSIS

A single genetic inconsistency between the decedent and JOHN DOE was observed at LOCUS. This has been incorporated into the calculation as a mutation. The remaining 22 Y-STR loci were not evaluated statistically; however, these Y-STR results support the proposition that John DOE and the decedent are paternally related.

piSNP and aiSNP STATEMENT

Analysis of phenotype informative and ancestry informative SNPs has been completed and will be provided separately.

BODY IDENTIFICATION WITH CODIS ENTRY

If a positive identification is made, notify the reporting analyst such that associated family reference standards may be removed from CODIS as required.

NGS STATEMENT FOR ACCREDITATION PROCESS

The Indiana State Police Laboratory approved Next-Generation Sequencing with the ForenSeq™ DNA Signature Prep Kit, Primer Set B for casework use on July 1, 2023. The Laboratory is currently in the process of adding this type of analysis to its ISO/IEC 17025 Forensic Testing Scope of Accreditation by ANSI National Accreditation Board (ANAB).

The Appendix below will be added to all Paternity and Missing Person Requests in Justice Trax after the analyst's signature, formatted as a stand-alone page.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

Relationship Comparison Report Appendix
The following includes supplemental information on relationship comparisons performed at the Indiana State Police Laboratory and definitions of some commonly used terms. Not all terms referenced in the appendix may apply to the testing in the associated case report. Please contact the reporting analyst with specific questions regarding the DNA analysis in this case.

DNA Kit Chemistry and Technology: Citations in the report refer to the test(s) listed below.
1. PowerPlex® Fusion 6C: DNA profiles are developed using Polymerase Chain Reaction (PCR) and capillary electrophoresis with the PowerPlex® Fusion 6C System by Promega Corporation. Y-STR loci may be used for interpretations but are not included in statistical estimates.
2. ForenSeq™ DNA Signature Prep, Primer Set B: DNA profiles are developed using Polymerase Chain Reaction (PCR) and Next-Generation Sequencing (NGS) with the ForenSeq™ DNA Signature Prep Kit, Primer Set B. Y-STR and identity informative SNP loci may be used for interpretations but may not be included in statistical estimates. X-STR loci may be used for interpretations but are not included in statistical estimates. Phenotype informative SNP loci and ancestry informative SNP loci may be used for investigative purposes only.
3. PowerPlex® Y23: Y-STR profiles were developed using Polymerase Chain Reaction (PCR) and capillary electrophoresis with the PowerPlex® Y23 System by Promega Corporation. Y-STR loci may be used for interpretations but are not included in statistical estimates for relationship comparisons unless a mutation is suspected.

I. **Insufficient DNA:** Following extraction and quantitation, DNA profiles are not developed from samples which do not yield enough DNA based on the laboratory's established threshold.

II. An **unknown individual** may be given a designation (e.g., "Unknown Male #1") to distinguish between multiple unknown profiles within a case or to indicate multiple samples consistent with the same unknown profile.

III. A **mixture** is a sample that contains DNA from more than one person. Mixtures with limited or low quality data and mixtures of more than four contributors are not interpretable by the laboratory's current methods.

IV. An individual is **assumed** to be a contributor when their contribution to a DNA sample is reasonably expected based upon the context of the sample and the presence of their DNA is not in question. This may include samples recovered from the individual's own body or from an item they regularly use. Using the DNA profile of the assumed individual allows for a more accurate interpretation and representative statistic for the portion of the profile that is in question.

V. Statistics are presented in the form of a **likelihood ratio** (LR), which is a comparison between two competing hypotheses to explain the profiles. If all parties agree that the alleged relationship is true, the LR would equal 1. An LR of 0 indicates no support for the relationship. Calculations are only reflective of the relationship(s) tested. If an alternate relationship should be considered, please contact the reporting analyst.

VI. Calculations are performed utilizing the population data associated with the racial information provided for the alleged individual and is documented in the case record. Population data from the Caucasian, African American, Hispanic, and Asian populations are available for calculation. If an individual is multi-racial or if racial information is unknown the Combined population data is utilized. If additional information should be considered for this case, please contact the reporting analyst.

VII. Closely related **family members** (such as parents, offspring, and siblings) share genetic information. If a case involves a related individual as an additional person of interest, the best course of action is to submit known DNA standards for direct comparison and statistical evaluation.

VIII. The statistical calculations are reported with a **verbal qualifying statement** to express the strength of statistical support. These statements follow the verbal scale recommended by the Scientific Working Group on DNA Analysis Methods (7/12/18). The scale reflects stronger degrees of support as the likelihood ratio increases.

Likelihood Ratio	Support Level	Support for:
LR ≥ 1,000,000	Very strong	Alleged Relationship
10,000 ≤ LR < 1,000,000	Strong	Alleged Relationship
100 ≤ LR < 10,000	Moderate	Alleged Relationship
2 ≤ LR < 100	Limited	Alleged Relationship
1	Uninformative	Uninformative
2 ≤ (1/LR) < 100	Limited	Unrelated
100 ≤ (1/LR)	--	Unrelated

IX. **Evidence disposition:** Unless otherwise noted, all portions of the evidence not consumed during analysis will be returned to the submitting agency. Retained items will remain at the Indiana State Police Laboratory. The remainder of the evidence or the original packaging will be returned.

RELATIONSHIP TESTING EXAMPLES

Example 1: Standard paternity trio

Item 001: DNA standard - John Smith
The DNA profile was developed.[1]

_____

Item 002: Product of conception
The DNA profile was interpreted as a mixture of two individuals. Assuming Sally Smith as a contributor, a distinct male profile was determined to be used as the standard for the product of conception.[1]

_____

Item 003: DNA standard - Sally Smith
The DNA profile was developed.[1]

_____

John Smith and the product of conception were evaluated as biological father and child by considering the following propositions:

► H1: Sally Smith is the mother and John Smith is the father of the product of conception.
► H2: Sally Smith is the mother and an unknown, unrelated man is the father of the product of conception.

►The genetic results are 1,000,000 times more likely if John Smith is the father of the product of conception than if an unknown, unrelated man is the father of the product of conception.
► This statistical analysis provides very strong support for the proposition that John Smith and the product of conception are related as biological father and child.

Example 2: Direct comparison of remains to secondary standard

Item 001: DNA standard - Decedent
The DNA profile was interpreted as originating from a single individual.[1]

► The DNA profile is at least 1 trillion times more likely if it originated from Ron Smith than if it originated from an unknown, unrelated individual.

► This statistical analysis provides very strong support for the proposition that Ron Smith is **included**.

_____

Item 002: Toothbrush (secondary standard) - Ron Smith
The DNA profile was interpreted as a mixture of two individuals. A distinct male profile was determined to be used as the secondary standard for Ron Smith.[1]

Example 3: Sibship with limited support for exclusion

Item 001: DNA standard – John Smith
The DNA profile was developed.[1]

_____

Item 002: DNA standard – Sally Smith
The DNA profile was developed.[1]

_____

John Smith and Sally Smith were evaluated as full siblings by considering the following propositions:

► H1: John Smith and Sally Smith are related as full siblings.
► H2: John Smith and Sally Smith are unrelated.

► The genetic results are 10 times more likely if John Smith and Sally Smith are unrelated than if John Smith and Sally Smith are related as full siblings.
► This statistical analysis provides limited support for the proposition that John Smith and Sally Smith are unrelated.

The statistical results for sibship are in a range where the alleged relationship is not supported despite there being a potential biological relationship. These results should be considered in the context of other information known in the case when making determinations of the identity of the individual. Additional genetic testing is recommended if further information is needed. Please contact the reporting analyst if desired.

Example 4: Pedigree, same nuclear family

Item 001: DNA standard – John Smith
The DNA profile was developed.[1]

_____

Item 002: DNA standard – Sally Smith

The DNA profile was developed.[1]

Item 003: DNA standard – Jane Smith

The DNA profile was developed.[1]

John Smith, Sally Smith, and Jane Smith were evaluated for relatedness by considering the following propositions:

► H1: John Smith is the father of Sally Smith and Jane Smith; Sally Smith is the full sibling of Jane Smith.
► H2: John Smith is the father of Sally Smith; Jane Smith is unrelated.

►The genetic results are 1,000,000 times more likely if John Smith, Sally Smith, and Jane Smith are related as alleged (H1) than if Jane Smith is unrelated to John Smith and Sally Smith (H2).
► This statistical analysis provides very strong support for the proposition that includes the alleged relationship (H1).

Example 5: Pedigree, extended family

Item 001: DNA standard – John Smith

The DNA profile was developed.[1]

Item 002: DNA standard – Sally Smith

The DNA profile was developed.[1]

Item 003: DNA standard – Jane Smith

The DNA profile was developed.[1]

John Smith, Sally Smith, and Jane Smith were evaluated for relatedness by considering the following propositions:

► H1: John Smith is the grandfather of Sally Smith and Jane Smith; Sally Smith is the cousin of Jane Smith.
► H2: John Smith is the grandfather of Sally Smith; Jane Smith is unrelated.

►The genetic results are 99 times more likely if John Smith is the grandfather of Sally Smith and Jane Smith, and Sally Smith is the cousin of Jane Smith, than if Jane Smith is unrelated to John Smith and Sally Smith.

► This statistical analysis provides limited support for the proposition that John Smith is the grandfather of Sally Smith, and Jane Smith and Sally Smith is the cousin of Jane Smith.

The statistical results for the familial relationships are in a range where adventitious support with unrelated individuals can occur. These results should be considered in the context of other information known in the case when making determinations of the identity of the individual. Additional genetic testing is recommended if further information is needed. Please contact the reporting analyst if desired.

Example 6: Trash pull, single parentage

Item 001: DNA standard – Sally Smith
The DNA profile was developed.[1]

Item 002: Water bottle
The DNA profile [from the water bottle] was interpreted as originating from a single individual. A male profile was determined from the sample and was purported to belong to Ron Smith.[1]

The contributor of the DNA profile from item 002A and Sally Smith were evaluated as biological father and child by considering the following propositions:

► H1: The contributor of the DNA profile from item 002A is the father of Sally Smith.
► H2: An unknown, unrelated man is the father of Sally Smith.

►The genetic results are 1,000,000 times more likely if the contributor of the DNA profile from item 002A is the father of Sally Smith than if an unknown, unrelated man is the father of Sally Smith.
► This statistical analysis provides very strong support for the proposition that the contributor of the DNA profile from item 002A and Sally Smith are related as biological father and child.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
MEMO FOR Y-STR TESTING TO SUPPORT INVESTIGATIVE GENETIC GENEALOGY:

Investigator Name
Agency
Address
City, IN Zip

Date
Agency Case Number:
ISP Laboratory Case Number:

This notice is to inform you of potential investigative information. Y-STR testing has been performed to aid in the genealogical investigation for the above referenced case.

This information is for investigative purposes only. No statistical analysis has been performed to give statistical weight to any potential familial relationships or the frequency of the Y-STR profile(s) in the population and is therefore not appropriate to support criminal prosecution. It is recommended that standards of any persons of interest be submitted for direct comparison to the profile in question.

The genetic results are based only on the data developed at the time of reporting and do not supersede any additional genetic testing that may be performed. Male individuals in the same paternal line are expected to share the same Y-STR profile. Differences in Y-STR profiles may indicate distantly related male individuals due to mutations or it may indicate a non-relationship. As the differences accumulate, this indicates more distant relationships or provides more support for a non-relationship. A variety of factors, including the number of Y-STR loci analyzed and mutation rate estimates, currently impede the ability to make specific conclusions on suspected familial relationships. Additional kinship analysis may provide more information.

DNA results for these items have been described in ISP issued Certificates of Analysis XXX. The Y-STR profile from *sample description* (item #) was compared to the Y-STR profile developed from *John Doe* (item #). # mismatches were observed out of # evaluated Y-STR loci. Based on this comparison, it is likely that the donors of the Y-STR profiles are *paternally related/probably paternally related/possibly paternally related/not related by a recent common paternal ancestor*. These results should be utilized with caution.

Analyst
Biology Section
Indiana State Police Laboratory

Year/Lab/Memo#

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

MEMO FOR ANCESTRY AND PHENOTYPE ESTIMATIONS:

Investigator Name
Agency
Address
City, IN Zip

Date
Agency Case Number:
ISP Laboratory Case Number:

This notice is to inform you of potential investigative information. Analysis of phenotype (pi) and ancestry informative (ai) SNP markers has been performed to aid in the identification of the unidentified human remains for the above referenced case. This information is for investigative purposes only.

The genetic results are based only on the data developed at the time of reporting and do not supersede any additional genetic testing that may be performed. Conclusions based on genetic prediction models may not account for age-related changes, human factors that affect physical appearance (i.e., dying, bleaching, highlighting), and/or differences in subjective descriptions. Therefore, genetic modeling of phenotypic traits may not correspond with the physical characteristics of an individual. These results should be utilized with caution.

The following results supplement DNA information for item XXX described in the ISP issued Certificate of Analysis XXX, dated X. The following phenotype estimations were yielded from *sample description*.

Hair Color			
Brown	Red	Black	Blond

Eye Color		
Brown	Intermediate	Blue

The genetic analysis suggests that the unidentified human remains are consistent with an individual of *European/East Asian/African/Ad Mixed American* ancestry. [This individual may possess *Brown/Blue/Intermediate* eye color and *Brown/Red/Black/Blond* natural hair color. (>80%)/A range of phenotype results was observed; therefore, specific hair and eye color characteristics could not be determined/Additional descriptions with supervisor approval.]

Phenotypic hair and eye color traits are an estimate and should not be used as the sole basis for an inclusion or exclusion of an individual. To confirm an identification, submit appropriate family reference DNA standards. Please contact the reporting analyst for further discussion regarding the information provided in this report.

> Analyst
> Biology Section
> Indiana State Police Laboratory
> Analyst work phone number
> Analyst work email

Year/Lab/Memo#

Y-STR Report Wording

EXCLUSION

PERSON is **excluded** [as a contributor to the Y-STR profile].

SINGLE SOURCE STATEMENTS

UNKNOWN PROFILE
Standard is excluded or no standard is present

The Y-STR profile [from ITEMDESCRIPTION] is consistent with an unknown male or male lineage.

CONSISTENT STATEMENT
Profile obtained from sample; no differences from standard

The Y-STR profile [from ITEMDESCRIPTION] was interpreted as originating from a single male or male lineage.

► The [Y-STR] profile is LR times more likely if it originated from PERSON or any of his male paternal relatives than if it originated from an unknown, unrelated male individual.
► This statistical analysis provides limited/moderate support for the proposition that PERSON is **included**..

CONSISTENT STATEMENT + ADDITIONAL ALLELE
Profile obtained from sample; no differences from standard with additional allele

The Y-STR profile [from ITEMDESCRIPTION] was interpreted as originating from a single male or male lineage. However, one additional allele was detected from which no conclusion can be drawn.

► The [Y-STR] profile is LR times more likely if it originated from PERSON or any of his male paternal relatives than if it originated from an unknown, unrelated male individual.
► This statistical analysis provides limited/moderate support for the proposition that PERSON is **included**.

CONSISTENT WITH MALE VICTIM

The Y-STR profile [from ITEMDESCRIPTION] was interpreted as originating from a single male or male lineage. Assuming PERSON as a contributor, no additional [Y-STR] alleles were detected.

MIXTURE STATEMENTS

INDISTINGUISHABLE MIXTURE

The Y-STR profile is a mixture in which a major profile could not be determined. The results do not qualify for statistical calculations; therefore, no further conclusions can be drawn.

MAJOR/MINOR COMPONENTS

The Y-STR profile [from ITEMDESCRIPTION] demonstrated the presence of a mixture with a major profile assumed to be a single male or male lineage. No conclusion can be drawn regarding the additional [Y-STR] alleles..

► The major [Y-STR] profile is LR times more likely if it originated from PERSON or any of his male paternal relatives than if it originated from an unknown, unrelated male individual.
► This statistical analysis provides limited/moderate support for the proposition that PERSON is **included**.

MAJOR/MINOR COMPONENTS, NO STANDARD:

The Y-STR profile [from ITEMDESCRIPTION] demonstrated the presence of a mixture with a major profile assumed to be a single unknown male or male lineage. No conclusion can be drawn regarding the additional [Y-STR] alleles.

MAJOR CONSISTENT WITH MALE VICTIM

The Y-STR profile [from ITEMDESCRIPTION] demonstrated the presence of a mixture with a major profile assumed to be a single male or male lineage. Assuming PERSON as a contributor, no conclusion can be drawn regarding the additional [Y-STR] alleles.

INCONCLUSIVE/NOT EXAMINED

LIMITED DATA

The Y-STR profile has limited data; therefore, no further conclusions can be drawn.

NO PROFILE

ITEMDESCRIPTION failed to demonstrate a Y-STR profile.

FAILED CONTROL

No conclusion can be drawn regarding ITEMDESCRIPTION because the/a test control[s] did not meet required parameters.

SAMPLE NOT EXAMINED

No Y-STR analysis was performed on # additional samples at this time.

SAMPLE NOT SUITABLE

Upon evaluation, no samples were determined to be suitable for Y-STR analysis.

STANDARDS

STANDARD PROFILE DEVELOPED

The (autosomal and) Y-STR profile [of PERSON] was(were) developed.

STANDARD NOT EXAMINED

No Y-STR analysis was performed [on the DNA standard of PERSON] due to a lack of evidence for comparison.

CONTAMINATION FROM LABORATORY PERSONNEL
Not all possible situations are given, consultation with the Technical Leader and/or Supervisor is recommended

SINGLE SOURCE

No Y-STR profile of apparent value was identified on ITEMDESCRIPTION. The Y-STR profile of a laboratory analyst/non-laboratory person was identified.

MIXTURE

The Y-STR profile obtained from ITEMDESCRIPTION demonstrated the presence of a mixture with a major profile. The major [Y-STR] profile is consistent with the Y-STR profile of a laboratory analyst/non-laboratory person.

REQUIRED STATEMENTS (PLACED AFTER RESULTS):

PROFILES GENERATED USING POWERPLEX® Y23

In the DNA analysis detailed above, Y-STR profiles were developed using Polymerase Chain Reaction (PCR) with the PowerPlex® Y23 System by Promega Corporation.

OR

NO SAMPLES AMPLIFIED

Extraction and quantification were performed in the Y-STR analysis of the sample(s) detailed above.

REQUESTS FOR STANDARDS

If [additional] Y-STR comparisons are desired, please submit an appropriate male DNA standard (such as an oral/buccal standard) from any suspects.

WORDING OF STATISTICAL ANALYSIS

Statistical estimates were performed based on the recommended concepts endorsed by the Scientific Working Group on DNA Analysis Methods (3/2/22). Y-STR results for item[s] XA have been searched using the Y Chromosome Haplotype Reference Database (https://yhrd.org), Release R# on date. Calculations for the Caucasian, African American, Hispanic, and Asian populations have been performed and a likelihood ratio has been reported for the combined populations.

CODIS STATEMENT

Placed at the end of any report detailing new profiles

No Y-STR profiles were entered into the Combined DNA Index System (CODIS).

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 8
DEFINITIONS

1. 99% 1-Sided Lower HPD – A confidence interval is applied to the point estimate to account for uncertainty. The interval gives a range of where the true allele probabilities lie. The lower end of the HPD is reported to be the most conservative to the person of interest.
2. Acid Phosphatase – a chemical component of semen; also found in other body fluids at significantly lower concentrations.
3. Allele – the alternative form of a gene.
4. Allele Frequency – the proportion of a particular allele found in a population.
5. Allelic Ladder – a set of DNA fragments of the commonly known alleles for each locus. By comparing the samples to the allelic ladder, the correct allele designation may be assigned.
6. Ambiguous Zygosity – a locus where a single allele is present in which it is unclear if the locus is homozygous or if the sister allele is dropped out or masked.
7. Amelogenin – the marker for determining the gender of the individual contributor to a DNA profile.
8. Amplification – using the PCR process to create many copies of a specific DNA sequence(s). An increase in the number of copies of a specific DNA fragment.
9. Analyst Discretion - the use of individual judgment, based on an analyst's training and experience to determine the optimum modes of analysis for an item of evidence.
10. Artifact – non-allelic product of the amplification process, an anomaly of the detection process, or a by-product of primer synthesis. A data peak that does not represent a true allele.
11. Assumed reference – A standard from an individual whose DNA is reasonably expected based upon the context of the sample and the presence of their DNA is not in question. This may include samples recovered from the individual's own body or from an item they regularly use. It may also include a consensual partner of an individual.
12. Base Pairs – paired nucleotides which make up the DNA molecule. Two complementary nucleotides joined by a hydrogen bond.
13. Blood – the fluid that circulates through the body, containing red and white blood cells carrying oxygen and nutrients to cells throughout the body; and carrying away waste and carbon dioxide.
14. Burn-in – The initial 10,000 accepts during the MCMC process that allow the chains to converge on a "good space". These iterations are discarded.
15. Capillary Electrophoresis – a method to separate DNA fragments based on size using electrical current. The DNA sample is placed in a thin tube (capillary) containing polymer and subjected to high voltage current allowing the DNA fragments to migrate through the tube.
16. Chromosome – the structure on which genes are naturally arranged and how DNA is organized.
17. Combined DNA Index System (CODIS) – refers to the DNA database and its software. It is composed of National (NDIS), State (SDIS), and Local (LDIS) components. It contains DNA profiles from offenders, crime scenes and includes a missing person database.
18. Conditioning – A likelihood ratio in which an individual is included in both H_1 and H_2.
19. Contamination – the process of making a sample impure or unusable.
20. Deconvolution – Process to determine the potential contributor genotypes to a mixed DNA profile.

21. Deduced – the inference of an unknown contributor's DNA profile after taking into consideration the contribution of an assumed contributor's DNA profile in an intimate sample based on quantitative peak height information.

22. Degradation – the breaking down of the DNA molecule into smaller fragments.

23. Denaturation – the separation of the double stranded DNA molecule into two single strands.

24. Deoxyribonucleic Acid (DNA) - the genetic material present in the nucleus of most cells. Also located in cell mitochondria.

25. DNA Sequence - a specific order of base pairs.

26. Electropherogram – the visual representation of the DNA fragments contained in each sample; generated by the analysis software of the Capillary Electrophoresis Instrument.

27. Electrophoresis – the method to separate molecules based on their size by placing them in a medium and applying an electrical current. The molecules will travel through the medium at different rates, the smaller molecules traveling through the medium more quickly than the larger ones.

28. Enzyme – a protein which acts as a catalyst, speeding up a specific chemical reaction without being changed or consumed in the process.

29. Epithelial Cells – skin cells and other surface cells such as from the mouth (buccal cells) or the vaginal cavity.

30. Factor of N! – Likelihood ratio which compares the person of interest to the total mixture at the sub-source level, independent of contributor order.

31. Gelman-Rubin – A value which informs the user whether the MCMC chains have converged.

32. Gene – the basic functional unit of heredity. Most genes determine the structure and function of proteins.

33. Genotype – the specific genetic sequence of a person's DNA.

34. Genotype Probability Distribution – A summary of the genotype combinations accepted at each locus and the percentage of accepts per genotype.

35. H_1/H_p – Interchangable terms for the most likely proposition assigned to the prosecution.

36. H_2/H_d – Interchangable terms for the most likely proposition assigned to the defense.

37. Hair – a slender outgrowth from the skin of mammals.

38. Heme – the iron containing complex of the hemoglobin molecule.

39. Hemin – the oxidized heme molecule.

40. Hemoglobin - the iron containing protein in blood.

41. Heterozygous – having different alleles at a particular locus.

42. Homozygous – having two of the same allele at a particular locus.

43. Internal Lane Standard (ILS) – a set of DNA fragments of known length(s). The ILS is simultaneously injected with all DNA samples during electrophoresis. This allows accurate measurement of the length of each allele in a DNA sample.

44. Intimate samples – biological sample that is obtained directly from an individual's body.

45. Iteration – A proposed genotype combination and mass parameters that are either accepted or rejected during the MCMC process.

46. Likelihood Ratio – A ratio of the probability of the evidence given two different propositions (H_1 and H_2).

47. Locus (loci) – the physical location of a gene on a chromosome.

48. LUS – Longest uninterrupted sequence for an allele variation.

49. Marker – a gene of known location and phenotype used as a point of reference.

50. Mass Parameters – Collection of parameters (degradation rate per contributor, amplification efficiencies per locus, replicate amplification strength, template per contributor, and kit efficiency), that are used to determine expected peak heights during deconvolution. Collectively referred to as DARTB.

51. Maximum Likelihood Estimate (MLE) LR – A likelihood ratio calculated when a variable number of contributors deconvolution was utilized. The MLE assigns an LR using the most probable number of contributors under H_1 and H_2. This means the number of contributors in the numerator and denominator may be the same or they may be different. The number of contributors will depend on which number of contributor analysis has the highest likelihood given the proposition for each.

52. MCMC (Markov Chain Monte Carlo) – A mathematical method that uses a process of random re-sampling to give the best explanation of observed data.

53. Meaningful profile – a profile that is developed from an item or location where the individual's profile would not reasonably be expected. A person's profile may be expected on an item or sample from their own body, their own clothing, or their property such as their house or vehicle. All other samples will generally be considered meaningful.

54. Minimum allele frequency - a conservative frequency correction for alleles observed less than 5 times in the STR population frequency tables.

55. Mitochondria - a part of a cell used in energy production. Contains circular DNA inherited from the mother only.

56. Modified Random Match Probability - an estimate based on the assumption of the number of contributors within a sample, of the probability of selecting an unrelated individual at random that would be included as a potential contributor to the mixed profile.

57. Nucleus - the part of a cell that contains the double stranded DNA inherited from both parents.

58. Obligate allele – an allele in a mixed DNA typing result that is a) foreign to an assumed contributor, or b) based on quantitative peak height information, determined to be shared with the assumed contributor.

59. Partial profile – DNA profile for which typing results were not obtained for all tested loci.

60. Phenotype – the expressed genotype.

61. Polymerase – an enzyme that initiates the duplication of a DNA molecule.

62. Polymerase Chain Reaction (PCR) – a process for amplifying (copying) the DNA molecule.

63. Polymorphism – the presence of more than one possible allele set for a specific gene.

64. Population – a stable group of random individuals, chosen for genetic analysis.

65. Primer – a short nucleotide fragment of known sequence used to locate its complementary sequence on the DNA molecule for the initiation of PCR. Primers target the specific loci to be amplified.

66. Proficiency Testing – a test to evaluate the competency of an analyst in a specific procedure.

67. Property crime – a category of crime that includes, among other crimes, burglary, theft, arson, drug offenses, vandalism, and criminal mischief. Property crimes do not involve force or threat of force.

68. Proposition – A proposed explanation of the data. Interchangeable with "hypothesis".

69. Post Burn-in – The iterations after burn-in from which the deconvolution is determined.

70. Random Match Probability (RMP) - the probability of obtaining a match between two distinct and unrelated individuals.

71. Replicate – A reamplification of the same extract.

72. Saliva – digestive fluid from the mouth.

73. Semen/Seminal Fluid – the male ejaculate. In a dried state may be referred to as seminal material. Semen normally contains spermatozoa.

74. Short Tandem Repeat (STR) – small sections of DNA that contain short segments (2, 3, 4 or more base pairs) which repeat several times. The number of repeat units may vary between individuals. STRs are located between specific genes and are considered non-functional.
75. Source attribution - a RMP threshold at which a degree of confidence can be assured that an unknown sample can be attributed to a known reference sample.
76. Spermatozoa – the male reproductive cell, sperm cell.
77. Stochastic effect – peak imbalance observed in a locus and/or allele drop-out due to random, disproportionate amplification of alleles in low quality/quantity template samples.
78. STRmix™ - A fully continuous probabilistic genotyping software which combines biological modeling with mathematical processes to deconvolute DNA profiles and then compare them to reference standards and provide statistical weight in the form of a likelihood ratio.
79. Stratified Likelihood Ratio – A single LR that samples across all populations.
80. Stutter – an artifact that occurs as a by-product of the PCR process. It is observed as a minor peak typically observed one repeat unit smaller than a primary STR allele caused by strand slippage during amplification. Stutter has been observed and documented in other positions.
81. Substrate – the material on which a biological sample is deposited such as at a crime scene.
82. SWGDAM – Scientific Working Group on DNA Analysis Methods.
83. Theta - a value to adjust for population substructure in homozygous loci.
84. Unified Likelihood Ratio – LR that takes into account that the unknown contributors are made up of both relatives and unrelated people.
85. Validation – a study to assess whether a particular procedure (or instrument) can obtain a desired result reliably and reproducibly. The study includes looking at the conditions necessary to obtain those results and the limitations of the procedure/instrument. All DNA analysis methods shall be validated prior to implementation.
86. Verbal Scale – A verbal representation of the relative strength of the likelihood ratio results.
87. Work Product – the material that is generated as a function of analysis, which may include extracts and amplified product, in tubes or plates, and any aliquots thereof.
88. X chromosome – a sex chromosome, present twice in female cells and once in male cells.
89. Y chromosome – a sex chromosome, present once in male cells (and absent in female cells), paired with the X chromosome.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 9
DNA SERVER INSTRUCTIONS

The DNA servers will be used for storage and archiving of data and photos. Real Time PCR files, 3500 run folders, GeneMapper™ ID-X projects, genotypes table .txt files, STRmix™ files, photos, and NGS files shall be saved to the server. Any worksheets, statistic sheets, typed notes pages, or workbooks shall NOT be saved to the server. The completed case record in LIMS will be the official, tracked copy. We want to avoid duplication of items between LIMS and the server.

On the server, each analyst will have a folder to organize and store their data files. Other analysts will have access to the items in the folder for purposes of review, but they shall not change, delete, or add items within the folder. Within the analyst's folder there will be one folder for each laboratory case number and request. Each folder will contain all associated data for that request. Any projects or run folders that contain batched data will be saved individually within each of the associated case folders. The following nomenclature should be used:

Analyst's Folder (created by Lab IT)

- Laboratory case and request folder – "case number_request number" – ex. "10I1234_1" – the request number corresponds to the laboratory request number assigned in LIMS.
    - RT-PCR projects: "1st case on the plate_date_plate#" – ex. "10I1234_01Jan11_01"
    - 3500 run folder: "1st case on the plate_date_plateinjection#" – ex. "10I1234_01Jan11_01" – plate injection number indicates how many times that plate preparation has been put in the instrument for injection; if the same plate preparation is run on a different date, the original date should still be used from when the plate was prepared. If a second preparation of a plate is made on the same date as the first, it shall be designated with a "-2" after the date. Ex. "10I1234_01Jan11-2_01"
    - GeneMapper™ ID-X project name: must contain the date the 3500 plate was prepared, so the project matches the run folder. If there were multiple injections of the plate or multiple preparations of the plate on the same date, a number shall be added to clarify which run folder the data was pulled from. - ex. "01Jan11", "10I1234_01Jan11", "Controls_01Jan11" or "10I1234_01Jan11 (02)". The exported genotypes table .txt file will maintain the same name as the project with the automatic extension "Genotypes Table" added during export.
    - STRmix™ results folders: The deconvolution results folder should include "case number_item number_Decon number". The LR from previous results folder should include "case number_item number_Decon number-LRPrev number".   If additional characters are added to the name of the either the deconvolution results folder or the LR from previous results folder, those characters shall be added after the specified naming convention.
    - DBLR™ analysis name: At a minimum, the name shall include the case number, followed by an underscore and the DBLR run number, increasing sequentially (e.g., 23L1234_DBLR1, 23L1234_DBLR2). If additional characters are added to the "Analysis Name" designation, those characters shall be added after the specified naming convention.
    - Photos: create a folder per item photographed – folder name "case_item#" ex. "10I1234_item1". The individual photo file names shall, at a minimum, have the laboratory case number and the Forensic Scientist's or Forensic Technician's initials, name, or permanent employee (PE) number.

- ForenSeq™ DNA Signature Prep results: The batch ID shall include the batch number, year, (ie., 001-2023).

Note: RT-PCR and 3500 Plates generated by the automation team may substitute Batch ID # for Lab Case #. RT-PCR plate names generated by the automation team shall also include the automation analyst initials and PE number.

Note: For RT-PCR projects and 3500 run folders – only the first case on the plate should be included in the name. Do not write more than one case number and do not write "et al." after the name.

Note: ForenSeq™ DNA Signature Prep batch data is maintained on the IRL DNA Data drive.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 10
RELATIONSHIP COMPARISON STATISTICAL REFERENCE SHEET

Formulas for Paternity Index (PI) and Random Man Not Excluded (RMNE) *(Table below adapted from the AABB, Standards for Relationship Testing Laboratories, 9th Edition, Appendix 7 and 8)*

Mother	Child	Alleged Father	PI	RMNE	PE
BD	AB	AC	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
BC	AB	AC	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
BC	AB	AB	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
BC	AB	A	$1/a$	$1-(1-a)^2$	$(1-a)^2$
B	AB	AC	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
B	AB	AB	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
B	AB	A	$1/a$	$1-(1-a)^2$	$(1-a)^2$
AB	AB	AC	$1/[2(a+b)]$	$1-(1-a-b)^2$	$(1-a-b)^2$
AB	AB	AB	$1/(a+b)$	$1-(1-a-b)^2$	$(1-a-b)^2$
AB	AB	A	$1/(a+b)$	$1-(1-a-b)^2$	$(1-a-b)^2$
AB	A	AC	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
AB	A	AB	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
AB	A	A	$1/a$	$1-(1-a)^2$	$(1-a)^2$
A	A	AB	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
A	A	A	$1/a$	$1-(1-a)^2$	$(1-a)^2$
	AB	AC	$1/4a$	$1-(1-a-b)^2$	$(1-a-b)^2$
	AB	AB	$(a+b)/4ab$	$1-(1-a-b)^2$	$(1-a-b)^2$
	AB	A	$1/2a$	$1-(1-a-b)^2$	$(1-a-b)^2$
	A	AC	$1/2a$	$1-(1-a)^2$	$(1-a)^2$
	A	A	$1/a$	$1-(1-a)^2$	$(1-a)^2$

Paternity Index for Null allele in standard trio comparisons =

$$\frac{cs}{(2cs + c^2) \times (a + s)}$$

    Known parent = AB, Child = AA or A*S*, Alleged parent = CC or C*S*
    a = known parent shared allele frequency
    c = alleged parent unshared allele frequency
    s = silent allele frequency = 0.001

Adapted from ISFG 2022 Kinship Analysis Workshop and California Criminalistics Institute Kinship Analysis Course

Combined Paternity Index (CPI) = PI$_1$ x PI$_2$ x PI$_3$…

Probability of Paternity (W) = [CPI x Pr]/[CPI x Pr + (1-PR)]
    Pr = Prior Probability

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

Formulas for Reverse Paternity
(Table below adapted from the AABB, Guidelines for Mass Fatality DNA Identification Operations, 2010, Appendix B)

Mother	Possible Child	Father	Formula
A	A	AB	$1/2a^2$
A	AB	AB	$1/4ab$
A	AB	BC	$1/4ab$
AB	A	AB	$1/4a^2$
AB	A	AC	$1/4a^2$
BC	AB	AB	$1/8ab$
BC	AB	AC	$1/8ab$
BD	AB	AC	$1/8ab$
A	A	A	$1/a^2$
AB	A	A	$1/2a^2$
B	AB	A	$1/2ab$
BC	AB	A	$1/4ab$
AB	AB	AC	$1/8ab$
AB	AB	A	$1/4ab$
AB	AB	AB	$1/4ab$

Formulas for Sibship
(Table below adapted from the AABB, Guidelines for Mass Fatality DNA Identification Operations, 2010, Appendix B)

Sibling	Possible Full Sibling	Formula
AB	AB	$(1+a+b+2ab)/8ab$
A	A	$(1+a)^2/(2a)^2$
A	AB	$(1+a)/4a$
AB	AC	$(1+2a)/8a$
AB	CD	$\frac{1}{4}$

Half Sibling	Possible Half Sibling	Formula
AB	AB	$(a+b+4ab)/8ab$
A	A	$(1+a)/2a$
A	AB	$(1+2a)/4a$
AB	AC	$(1+4a)/8a$
AB	CD	$\frac{1}{2}$

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

Mutation Rates

Mutation rates for CSF1PO, D13S317, D16S539, D18S51, D19S433, D21S11, D2S1338, D3S1358, D5S818, D7S820, D8S1179, FGA, Penta D, Penta E, TH01, TPOX, and vWA reported in AABB, Standards for Relationship Testing Laboratories, 10th Edition, Appendix 6.

Average paternal and maternal mutation rates calculated from tetranucleotide markers listed in AABB, Standards for Relationship Testing Laboratories, 10th Edition, Appendix 6 for D1S1656, D2S441, D10S1248, D12S391, D22S1045, D17S1301, D20S482, D4S2408, D6S1043, D9S1122.

Mutation rates for SE33 reported in AABB Annual Report 2003.

The average mutation rate used for iiSNP loci is set to 1 x 10^{-8}, the default parameter set in DBLR™ and supported by Nachman, M.W., Crowell, S. (2000) Estimate of the Mutation Rate per Nucleotide in Humans. Genetics. 156: 297-304.

The mutation factor for a single discordant Y-STR locus contained in the kit shall be incorporated as a haplotype Relationship Index and incorporated manually into the CRI by multiplying by 0.001 for all Y-STR loci except DYS570, DYS576, DYS612, and DYF387S1. This mutation rate value is supported by Ballantyne, K.N., et. al., (2012) A New Future of Forensic Y-chromosome Analysis: Rapidly Mutating Y-STRs for Differentiating Male Relatives and Paternal Lineages. Forensic Science International: Genetics. 6: 208-218. DYS570, DYS576, DYS612, and DYF387S1 are identified as rapidly mutating Y-STR loci. The mutation factor for a single discordant Y-STR locus defined as rapidly mutating will be incorporated as a haplotype Relationship Index and incorporated manually into the CRI by multiplying by 0.01. This rapidly mutation Y-STR definition and mutation rate value is supported by Boattini, A., et. al., (2016) Mutation Rates and Discriminating Power for 13 Rapidly-Mutating Y-STRs between Related and Unrelated Individuals. PLoS ONE.

LOCUS	PATERNAL	MATERNAL
CSF1PO	0.002021	0.000283
D10S1248	0.001692	0.000409
D12S391	0.001692	0.000409
D13S317	0.001743	0.000436
D16S539	0.001127	0.000481
D18S51	0.002530	0.000748
D19S433	0.000745	0.000596
D1S1656	0.001692	0.000409
D21S11	0.001709	0.001295
D22S1045	0.001692	0.000409
D2S1338	0.001526	0.000245
D2S441	0.001692	0.000409
D3S1358	0.001691	0.000211
D5S818	0.001742	0.000300
D7S820	0.001348	0.000073
D8S1179	0.002031	0.000333
FGA	0.003713	0.000522
Penta D	0.000259	<0.000253
Penta E	0.000260	<0.000253
SE33	0.006400	<0.003000
TH01	0.000070	0.000043
TPOX	0.000130	0.000081
vWA	0.003258	0.000494

D17S1301	0.001692	0.000409
D20S482	0.001692	0.000409
D4S2408	0.001692	0.000409
D6S1043	0.001692	0.000409
D9S1122	0.001692	0.000409

NIST 1036 Revised U.S. Population Dataset (July 2017)
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., Valone, P.M. (2017) Corringendum to 'U.S. Population Data for 29 Autosomal STR Loci'. Forensic Sci. Int. Genet. 31: e36-e40.

NIST 1036 Expanded U.S. Population Dataset
Gettings, K.B., Borsuk, L.A., Steffen, C.R., Kiesler, K.M., Vallone, P.M. (2018). Sequence-based U.S. population data for 27 autosomal STR loci. Forensic Sci. Int. Genet. 37: 106-115.

SNP Allele Frequencies
Churchill, J.D., Novroski, N.M.M., King, J.L., Seah L.H., & Budowle, B. (2017). Population and performance analyses of four major populations with Illumina's FGx Forensic Genomics System. Forensic Sci. Int. Genet. 30: 81-92.

Linkage Map for use in DBLR™
Matise, T.C. *et al.* (2007). A second-generation combined linkage physical map of the human genome. Genome Res**.** 17:1783-6.

Nato, A.Q., Buyske, S., and Matise, T.C. The Rutgers map: A third-generation combined linkage-physical map of the human genome. *Human Genetics Institute of New Jersey Second Research Day.* Life Sciences Building, Rutgers University, Piscataway, NJ, USA. (Manuscript in Process)

Phillips, C., Ballard, D., Gill, P., Syndercombe Court, D., Carracedo, Á. & Lareu, M.V. (2012). The recombination landscape around forensic STRs: Accurate measurement of genetic distances between syntenic STR pairs using HapMap high density SNP data. Forensic Sci. Int. Genet. 6. 354–365.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

APPENDIX 11

Applied Biosystems® 3500 Genetic Analyzer - Data Collection Software version 2.0 - Instrument

(only necessary the first time PowerPlex® Y23 samples are run or if the parameters change)

See [Appendix 3](#) for security, user roles, and user account settings.

1.0 Create an Instrument Protocol

    1.1 Navigate to the **Library** and select "Instrument Protocols".

    1.2 Select "Create".

    1.3 Select "HID" for the Application Type.

    1.4 Select "36" for the Capillary Length.

    1.5 Select "POP4" for the Polymer.

    1.6 Select the appropriate Dye Set: "Promega G5 WEN".

    1.7 Select "HID36_POP4" for the Run Module.

    1.8 Enter the desired Injection Time and Injection Voltage.

    1.9 Name the protocol with the kit name and the injection voltage and time (e.g., "PPY23_1.2kV15s").

    1.10 Ensure settings match the figure below.

    1.11 Mark "Locked" and click "Save".

Instrument Protocol:

2.0 Create a Size Standard

2.1 Navigate to the **Library** and select "Size Standard".

2.2 Select "Create".

2.3 Assign the appropriate name: "WEN ILS 500 Y23".

2.4 Select "Orange" for the Dye Color.

2.5 Type the following into the left column: 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500. Click "Add Size(s)".

2.6 Mark "Locked" and click "Save".

Size Standard:

Edit Size Standard WEN ILS 500 Y23

Setup a Size Standard

* Size Standard   WEN ILS 500 Y23   ☐ Locked

Description:

* Dye Color: Orange ▼

Enter sizes in the field below separated by a comma, space, or return then click the "Add Size(s)>>" button to add them to the current size standard definition.

Enter new Size Standard definition: (e.g. 11.0, 34.2, 55)

* Current Size Standard definition: Delete Selected Sizes

60.0
65.0
80.0
100.0
120.0
140.0
160.0
180.0
200.0
225.0
250.0
275.0
300.0
325.0
350.0
375.0
400.0
425.0
450.0
475.0
500.0

Add Size(s) >>

Close   Save

3.0 Create a QC Protocol

3.1 Navigate to the **Library** and select "QC Protocols".

3.2 Select "Create".

3.3 Assign the appropriate name: "WEN_ILS_500_Y23".

3.4 Change settings to match the figure below.

3.5 Mark "Locked" and click "Save".

QC Protocol:

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

4.0 Create an Assay

4.1 Navigate to the **Library** and Select "Assays".

4.2 Select "Create".

4.3 Assign a descriptive assay name (e.g., "PPY23_1.2kV15s").

4.4 Select "HID" for the Application Type.

4.5 Select the appropriate QC Protocol: "WEN_ILS_500_Y23".

4.6 Select the appropriate instrument protocol.

4.7 Mark "Locked" and click "Save".

Assay:

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

5.0 Create a Naming Convention

5.1 Navigate to the **Library** and Select "File Name Convention".

5.2 Select "Create".

5.3 Assign a descriptive name (e.g., PPY23_FNC).

5.4 Select desired attributes and click "Add".

5.5 Select desired delimiters and click "Add".

5.6 Leave file location as the default.

5.7 Look at the "Preview of File Name" field for accuracy.

5.8 Mark "Locked" and click "Save".

File Name Convention:

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

6.0 Create a Results Group

6.1 Navigate to the **Library** and Select "Results Group".

6.2 Select "Create".

6.3 Assign a descriptive assay name (e.g., . PPY_ResultsGroup).

6.4 Select the desired attributes and click "Add".

6.5 Select the desired delimiters, if there are multiple delimiters click the "Add between attributes" box and click "Add".

    6.5.1 The order of the attributes and delimiters can be changed using the "Move Up" and "Move Down" buttons.

6.6 Ensure that "Store reinjection sample file with original sample files (same level)" is selected.

6.7 Leave file location as the default.

6.8 Click so the "Include a Result Group Name folder" box is selected.

6.9 Look at the "Preview of "Results Group Name" field for accuracy.

6.10 Mark "Locked" and click "Save".

Results Group:

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 12

GeneMapper™ ID-X Version 1.6 Software - PowerPlex® Y23 Software Settings

1.0 Importing Panel and Bin Files for use with WEN ILS 500

1.1 Open the GeneMapper™ ID-X Version 1.6 software.

1.2 Select **Tools → Panel Manager**.

1.3 Highlight the "Panel Manager" icon in the navigation pane.

1.4 Select **File → Import Panels**.

1.5 Navigate to the saved panel, bin, and stutter files. Select "PowerPlexY23_Panels_IDX_v2.0" then click "Import".

1.6 Select the "ISP Casework Security Group". Click "OK".

1.7 In the navigation pane, highlight the "PowerPlexY23_Panels_IDX_v2.0" folder.

1.8 Select **File → Import Bin Set**.

1.9 Select "PowerPlexY23_Bins_IDX_v2.0" then click "Import".

1.10 In the navigation pane, highlight the "PowerPlexY23_Panels_IDX_v2.0" folder.

1.11 Select **File → Import Marker Stutter**. A warning box will appear asking to overwrite the current values. Select "Yes".

1.12 Select "PowerPlexY23_Stutter_IDX_v2.0" then click "Import". This will import the Promega Marker Stutter file that has been modified to include the Indiana State Police PowerPlex® Y23 stutter percentages for filtering out stutter as determined by the internal validation studies.

1.13 In the Panel Manager window, select "Apply", then "OK".

2.0 Creating a Casework Analysis Method

2.1 Select **Tools → GeneMapper Manager**.

2.2 Select the "Analysis Methods" tab.

2.3 Select "New" and a new analysis method dialog box will open.

2.4 Enter the name: "PowerPlex Y23 WEN".

2.5 Select the "ISP Casework Security Group".

2.6 Select the "Allele" tab. In the "Bin Set" drop-down menu select "PowerPlexY23_Bins_IDX_v2.0". Ensure that the "Use marker-specific stutter ratio and distance if available" box is checked. The table values may be left as the factory defaults.

The Allele Tab:

2.7 Select the "Peak Detector" tab. Change the settings to match the figure below.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

The Peak Detector Tab:

2.8 The Peak Amplitude Threshold (analytical threshold) values were determined during the internal validation by calculating the noise level of each instrument and determining the cut-off in which the majority of the noise peaks would be below.

2.9 Select the "Peak Quality" tab. Change the settings to match the image below.

The Peak Quality Tab:

2.10 Leave the "SQ and GQ Settings" tab set to the factory defaults.

2.11 Select "Save".

3.0 Creating a Size Standard

3.1 Select **Tools → GeneMapper Manager.**

3.2 Select the "Size Standard" tab and click "New"

3.3 Create the "WEN ILS 500" size standard (see figure below for exact settings and sizes).

3.4 Select "OK".

Size Standard Editor

Edit

Size Standard Description

Name:	WEN ILS 500
Security Group:	ISP Casework Security Group ▼
Description:	
Size Standard Dye:	Orange ▼

Size Standard Table

Insert   Delete

	Size in Basepairs
1	60.0
2	65.0
3	80.0
4	100.0
5	120.0
6	140.0
7	160.0
8	180.0
9	200.0
10	225.0
11	250.0
12	275.0
13	300.0
14	325.0
15	350.0
16	375.0
17	400.0
18	425.0
19	450.0
20	475.0
21	500.0

OK   Cancel   Help

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

4.0 Creating a Table Setting

4.1 Select **Tools → GeneMapper Manager**.

4.2 Select the "Table Setting" tab and click "New".

4.3 Under the "General" tab, name the Table Setting "PPY23" and select the "ISP Casework Security Group".

4.4 Under the "Samples" tab, ensure that check marks are located next to only the following: Status, Sample File, Sample Name, Sample Type, Analysis Method, Panel, Size Standard, Sizing Quality Overridden, Sample File Not Found, Sample Off-Scale, and Sizing Quality. Leave the font "Arial" and the size "11". Sort by "Sample Type" and then by "Sample Name". Select the "Ascending" option for both, and leave the final sort option to "None".

4.5 Under the "Genotypes" tab, ensure that check marks are located next to only the following: Sample Name, Marker, Allele, and Height. Sort by "Sample Name", then by "Marker", and then by "None". Select the "Ascending" option for all three. Change the "Show number of alleles" to 8 and check "Keep Allele, Size, Height, Area, Data Point, Mutation and Comment together". (If the option to "Keep Allele, Size, Height…" is not visible in the window, expand the window size until the option appears.) Leave the font "Arial" and the size "11".

4.6 Select "OK".

5.0 Creating a Plot Setting – Samples, Controls, and Ladders

5.1 Select **Tools → GeneMapper Manager**.

5.2 Select the "Plot Settings" tab and click "New".

5.3 Under the "General" tab, name the Plot Setting "PPY23" and select the "ISP Casework Security Group".

5.4 Under the "Sample Header" tab, ensure that check marks are located next to the following: Sample File, Sample Name, Panel, Sizing Quality Overridden, Sample Off-Scale and Sizing Quality.

5.5 Under the "Genotype Header" tab, ensure that check marks are located next to the following: Sample File, Sample Name, Panel, Marker, Sample Off-Scale, Out of Bin Allele, Peak Height Ratio, Control Concordance, and Genotype Quality.

5.6 Under the "Sizing Table" tab, ensure that check marks are located next to the following: Dye/Sample Peak, Sample File Name, Marker, Allele, Size, Height, Area, and Data Point. Leave the font "Arial" and the size "11".

5.7 Select the "Labels" tab. Change the settings to match the following figure. Alternatively, if the allele edit comment" function is being used, "Label 4" for "Assigned Allele", "Custom Allele", and "Artifact" can be changed to "AE Reason for Change".

Labels Tab:

5.8 Under the "Display Settings" tab, change the display settings so that they match the following figure and select "OK".

Display Settings Tab:

6.0 GeneMapper™ ID-X Maintenance

6.1 The number of audit records on the GeneMapper™ ID-X database should be routinely checked. Audit records should be backed-up, saved to the DNA server, and then deleted from the GeneMapper™ ID-X database monthly or if the number of records exceeds 40,000. If the number of audit records exceeds 60,000, the performance of the software may be affected.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

6.2 The amount of database space in the GeneMapper™ ID-X software should be routinely checked. If the occupied space exceeds 80%, additional disk space should be allocated.

POWERPLEX® Y23 STUTTER TABLE

Stutter Guidelines for PowerPlex® Y23						
	reverse (e.g., n-4)		double reverse (e.g., n-8)		forward (e.g., n+4)	
	Mean	Mean + 3SD	Mean	Mean + 3SD	Mean	Mean + 3SD
DYS576	9.82%	12.57%	0.74%	1.78%	1.39%	4.00%
DYS389 I	5.51%	7.14%	0.57%	1.50%	0.44%	1.12%
DYS448	2.17%	2.55%	n/a	n/a	n/a	n/a
DYS389 II	11.32%	14.80%	1.23%	2.30%	1.05%	2.44%
DYS19	5.71%	8.48%	n/a	n/a	0.66%	1.55%
DYS391	7.00%	9.74%	0.71%	2.19%	0.28%	1.02%
DYS481	17.78%	25.93%	3.05%	5.04%	2.52%	5.12%
DYS549	6.61%	9.66%	0.99%	1.69%	1.11%	2.02%
DYS533	6.68%	9.05%	0.69%	1.68%	0.99%	2.19%
DYS438	3.03%	5.10%	n/a	n/a	0.66%	1.61%
DYS437	4.94%	7.96%	0.69%	0.98%	0.69%	1.75%
DYS570	9.69%	12.25%	0.69%	1.30%	0.90%	1.88%
DYS635	6.27%	10.67%	0.69%	1.74%	0.48%	1.26%
DYS390	8.55%	12.80%	1.01%	2.67%	0.44%	1.14%
DYS439	6.06%	9.19%	0.51%	1.02%	0.87%	2.31%
DYS392	9.64%	14.09%	1.09%	2.23%	5.93%	12.11%
DYS643	2.33%	5.41%	n/a	n/a	0.50%	1.31%
DYS393	9.78%	13.05%	1.03%	1.97%	1.44%	2.71%
DYS458	9.73%	12.71%	0.84%	1.31%	0.80%	1.88%
DYS385	7.89%	14.14%	1.09%	2.22%	0.82%	1.58%
DYS456	10.71%	15.71%	1.37%	2.93%	1.85%	2.68%
YGATAH4	7.41%	10.05%	1.09%	2.10%	1.44%	2.25%

	n-2				n+2	
	Mean	Mean + 3SD			Mean	Mean + 3SD
DYS19	7.76%	9.09%		DYS19	2.81%	4.36%
DYS391	0.50%	1.05%				

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 14
OUTSOURCED SAMPLE ANALYSIS

Samples may be sent to an external laboratory for analysis at the request and authorization of the investigating agency, prosecutor or by court order. Payment arrangements for additional analysis shall be agreed upon by the appropriate entity prior to sending the samples. Authorization to send the samples shall be documented in the case record.

If possible, only a portion of the evidence should be sent with the remainder retained by the laboratory or returned to the contributing agency. A subitem (or sub-subitem) of the evidence shall be generated for this purpose for tracking in LIMS. Items and/or subitems sent for analysis shall be tracked in the Chain of Custody, including extracts. Even though extracts are defined as work product, they shall be tracked in the Chain of Custody if being sent outside of the laboratory for the purpose of testing. The Chain of Custody shall document "Carrier Delivery" and the laboratory name of where the evidence is being sent. Copies of the shipping paperwork and paperwork filled out for the laboratory doing the testing shall be documented in the case record.

A Certificate of Analysis to the original submitting agency shall be generated to document the sample was sent out utilizing the wording specified in the report wording guidelines.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

APPENDIX 15
Electronic Case Record Generation and Review

1. Electronic Generation of Case Record

1.1. Analysts may electronically generate case notes using software (e.g., Microsoft Word), provided that the notes are clear, legible, and may be easily printed (if a paper record is being generated) or incorporated into PDF file format (if an electronic copy is being generated).

1.1.1. If an official worksheet exists for the examination of a particular evidence workflow (e.g., the sexual assault kit worksheets), it shall be used (an analyst may not create their own). If a table is included in an analyst's electronic case notes to record serology results and reagent lot numbers, only the approved table shall be used.

1.1.1.1. Official worksheets and approved tables are available on SharePoint.

1.2. Results of serological testing shall be recorded on the official stain identification worksheets or in the analyst's generated case notes.

1.2.1. All required controls, lot numbers, etc. shall be documented.

2. Electronic Technical and Administrative Review

2.1. At the reporting case analyst's discretion, a case record may be created in or converted to an electronic (PDF) format prior to technical/administrative review.

2.1.1. Exceptions shall be approved by a Unit Supervisor and documented in the case record.

2.2. The electronic copy of the case record becomes the official, tracked copy, and is the copy utilized for technical and administrative review.

2.2.1. All data sheets, notes, and other generated information which are normally kept in the paper case record shall be included in the electronic file.

2.2.2. Every page shall include analyst's initials and case number.

2.2.2.1. These may be inserted by software (e.g., typed) or hand-written.

2.2.3. Scanned documents from an original paper case record may be merged with electronically generated files to create the electronic copy.

2.2.4. When the case has been administratively reviewed and uploaded into LIMS, any remaining paper copies should be destroyed.

2.2.4.1. Paper copies should be retained at least 1 week prior to destruction in order to allow for a LIMS server backup.

2.2.4.2. Paper copies should be destroyed within 2 months of administrative review.

2.3. The analyst may make alterations to their electronic case record using software (e.g., Adobe), including but not limited to merging files, inserting/rearranging pages, adding text, strikethroughs, highlighting, and drawing lines.

2.3.1. All alterations (except those with no data gain/loss, such as rearrangement) shall be initialed and dated.

2.4. If a page of the electronic copy needs to be added, recreated, or removed, a watermark or other notation shall be placed on the original page stating the reason (e.g. "not used" or "page added") with the case analyst's initials and date. When data is rejected, the reason shall be listed. New pages should be inserted before or after original pages when appropriate or as best organizes the data.

2.4.1. Examples: additional deconvolution worksheets generated after first review, or removal of a CODIS sheet for a profile deemed ineligible

2.5. Completed case records ready for technical review shall be placed into a folder on the reporting analyst's personal data drive, in a subfolder marked "Electronic Reviews".

2.5.1. Example: \ DNAData \ IRL \ JDoe \ Electronic Reviews.

2.5.2. The case report printed to PDF from LIMS should be saved in the same folder as a separate electronic document. The case analyst shall verify the most up to date copy of the report is ready present for technical review.

2.5.3. This folder shall be separate from the case folder and should include only case records for technical and administrative reviews.

2.5.4. The analyst shall also upload the un-reviewed electronic copy to LIMS prior to technical review. This will preserve the record prior to changes.

2.5.5. If changes are made to the electronic case record by the analyst after a technical reviewer has been assigned, the casework analyst shall notify the technical reviewer of such changes.

2.6. Electronic records shall be retained as indicated in Appendix 9 unless otherwise indicated below.

2.6.1. The original case record PDF file shall be named as follows:
DNA_CASE#_REQUEST#_NOTES_ PE#_V0

2.6.2. The final, technically reviewed case record PDF file shall be named as follows:
DNA_CASE#_REQUEST#_NOTES_ PE#_V1

2.7. If the case analyst is converting their case record to electronic format, they shall notify the appropriate supervisor/manager when their electronic case record is ready for technical review.

2.7.1. If someone other than the case analyst has converted the case record to electronic format, they shall notify the case analyst and supervisor/manager (as appropriate). The individual who scanned the case record shall place it in the case analyst's data drive as indicated above. The case analyst shall then upload the electronic record to LIMS as indicated above.

2.8. The assigned technical reviewer shall use the electronic copy of the case record in the case analyst's personal folder for review.

2.8.1. The technical reviewer shall save a copy of the technical review worksheet to the case analyst's personal folder to document the review.

2.8.2. The technical reviewer shall check to ensure that the V0 copy is uploaded to LIMS prior to review.

2.8.3. The technical reviewer should save comments to the analyst's electronic copy.

2.8.4. The technical reviewer's electronic alterations to the case record should be limited to drawings with the drawing tool and/or comment boxes (i.e., "sticky notes") within the comment tool in Adobe.

2.8.4.1. All temporary alterations/comments shall be deleted by the technical reviewer before the casefile is finalized.

2.8.4.2. Technical review notation made using the comment tool may be removed by printing the PDF file as a PDF file; there is an option in the print menu that allows keeping/omitting such markups and shall be selected as appropriate.

2.8.5. A technical reviewer may opt to print the case record for their own review, at their discretion, but any generated paper copies shall be destroyed upon completion of review.

2.9. When technical review is complete, the case analyst shall add the completed technical review worksheet as the first page of the PDF case notes, save the electronic copy as the final copy,

named as outlined above and upload it to LIMS. The case analyst shall notify the appropriate supervisor/individual that it is ready for administrative review.

2.10.   If an additional request for lab exam is generated as part of the case exam (e.g., a Y-STR request), the case analyst is responsible for creating a print copy and forwarding that request to the case manager following administrative review.

    2.10.1. A copy of additional request form(s) should be retained in the original case notes.

3. CODIS Upload and Review

3.1. When a DNA profile is being submitted to CODIS for upload, the corresponding CMF file(s) shall be generated from the current version of STRmix and saved in the appropriate CMF server file.

    3.1.1.   Paper submissions may be accepted in place of CMF files, pending the Local CODIS Administrator's approval.

3.2. When ready for CODIS upload, the technical reviewer shall send an email to all appropriate CODIS administrators which indicates the case number, and case analyst associated with the upload. Multiple cases may be sent in one email.

3.3. The CODIS administrators will access the case record and CODIS sheet via the case analyst's data review folder.

    3.3.1.   A PDF file of the Specimen Detail Sheet (SDS) will be placed in the case analyst's data review folder. The SDS will be labeled with the case number and "spec detail". This becomes the official, tracked copy of the SDS.

    3.3.2.   Paper submissions will be returned as a paper copy.

3.4. A CODIS administrator will notify the case analyst via email when the upload is completed.

3.5. The case analyst shall merge any electronic copies of the SDS with the electronic copy of the case record during review.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 16
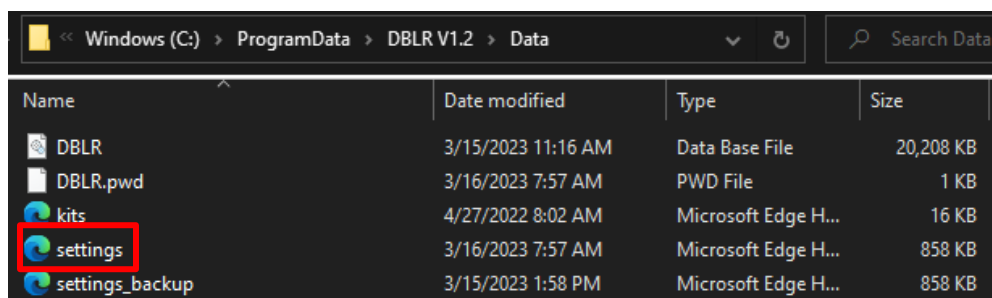# DBLR™ SOFTWARE SETTINGS

1. **DBLR™ Installation and Set-up**

   1.1.   Install the current version of DBLR™ (v1.2.24).

   1.2.   Install ISP settings.

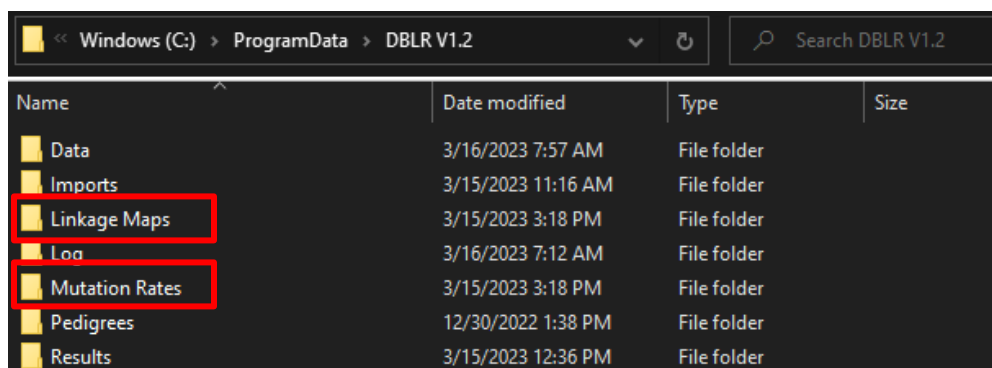   1.2.1.   Navigate to BiologyQA→Validations→DBLR→DBLR install files.

       1.2.2.   Open C:\ProgramData\DBLR V1.2\Data. Copy and replace the default settings file with the settings file contained on the Biology QA drive.

       1.2.3.   Back out to C:\ProgramData\DBLR V1.2. Copy and replace the Linkage Maps folder and Mutation Rates folder with the same folders contained on the Biology QA drive.

           1.2.3.1.   Optionally, for analysts trained to perform relationship comparisons, previously developed pedigrees may be imported in the Pedigrees folder.

       1.2.4.   Confirm the file transfers.

           1.2.4.1.   Open the Linkage Maps folder on your computer and check for **ForenSeq DNA Sig Autosomal STR iSNP_v3** files.

           1.2.4.2.   Open the Mutation Rates folder on your computer and check for **ISP maternal_v2** and **ISP paternal_v2 files**.
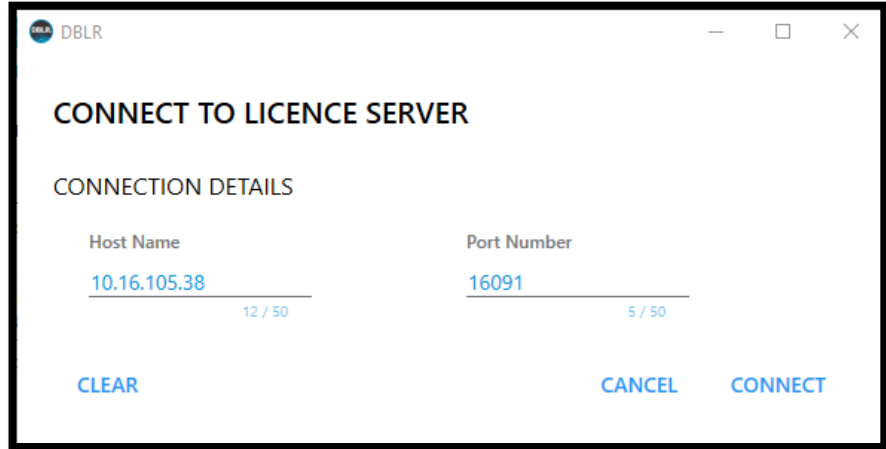
   1.3.   Open DBLR to connect to the floating license server:

       1.3.1.   A warning window may open that indicates "The fingerprint of settings.xml does no match…". Select "OK".

1.3.2.   Enter Host Name: 10.16.105.38

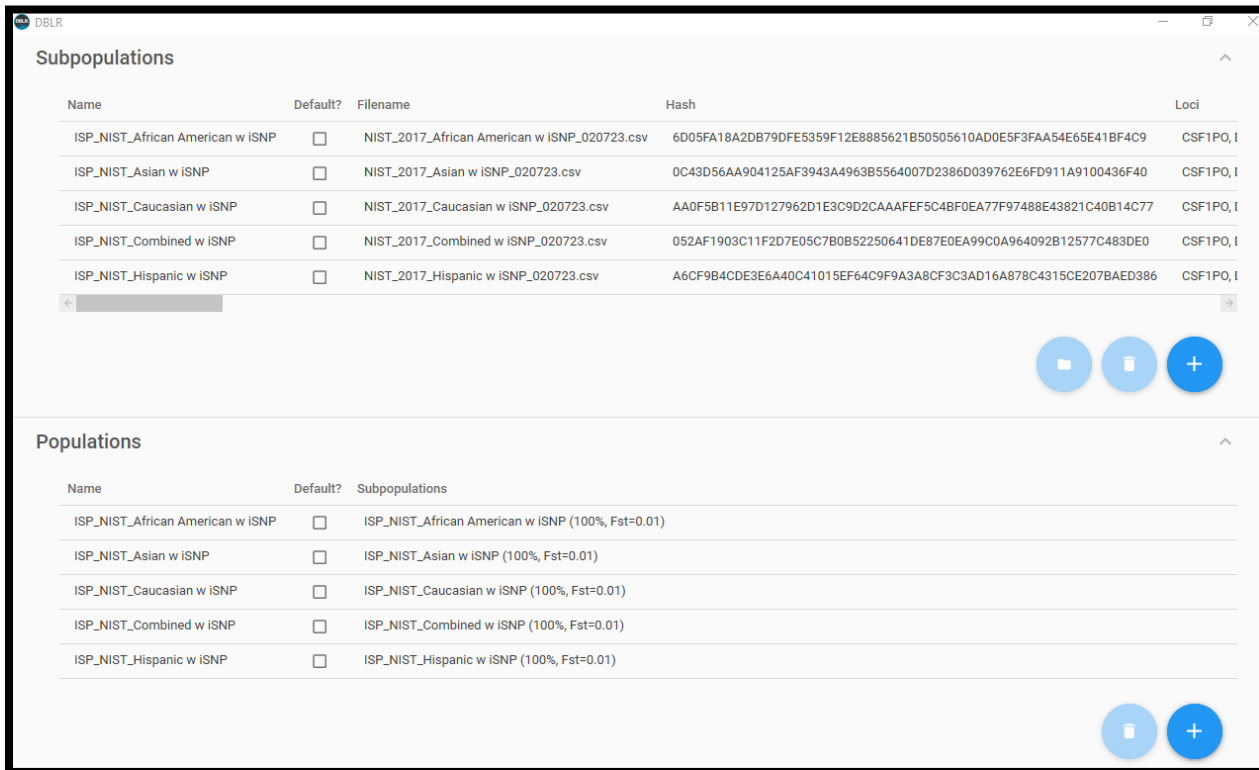1.3.3.   Confirm Port Number: 16091

1.3.4.   Select "Connect".

1.3.5.   Review and agree to Software License Agreement.

1.4.   Confirm the settings update.

1.4.1.   Select the "Settings" tab in the main menu. Open the drop-down menus to confirm the images below.

1.4.1.1.   When finished, close the settings window by selecting "Save changes".

DBLR

Subpopulations   ⌄

Populations   ⌄

Search Strategies   ⌃

Name	Default?	Relationship (H1)	Relationship (H2)	Population	Use Log10(LR) Threshold?	Log10(LR) Threshold

(▶) (🗑) (+)

Loci   ⌃

Name	Non-Autosomal?	Do not count towards minimum # loci for database upload?
AMEL	☑	☑
DYS391	☑	☑
DYS570	☑	☑
DYS576	☑	☑
Yindel	☑	☑

(🗑) (+)

Special allele labels   ⌃

Label	Is blank observation?
0	☑

(🗑) (+)

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 17
Verogen's Universal Analysis Software v1.3

1 Setting Up User Profile

1.1 Open the UAS.

1.2 Select "Create One" next to the section that states "Need an account?"

1.3 Enter the new user's ISP email address and create password.

1.4 Click the blue bar with a check mark.

1.5 The new user must be approved by an administrator.

    1.5.1 Log into an administrator account.

    1.5.2 Select the administrator's email address at the top of the screen.

    1.5.3 Select "Maintenance" from the menu.

    1.5.4 Select "User Management".

    1.5.5 Approve the new user.

    1.5.6 Users can also be changed from standard to administrator as well as enabled or disabled on this screen.

2 Creating an Analysis Method

2.1 Log into an administrator account.

2.2 Select the administrator's email address at the top of the screen.

2.3 Select "Maintenance" from the menu.

2.4 Select "Analysis Values".

    2.4.1 The ISP default analysis method is "SigPrep_ISP_v1"

        2.4.1.1 Analytical threshold is 1.5% for most loci, 5% for DYS389II, and 3.3% for DYS448 and DYS635.

        2.4.1.2 Interpretation threshold is 4.5% for most loci, 15% for DYS389II, and 10% for DYS448 and DYS635.

        2.4.1.3 See Appendix 18 for stutter value table.

2.5 Select "New Template".

2.6 Enter a name for the new template and select "Save".

2.7 Ensure the correct primer mix (Mix A or Mix B) is selected.

2.8 Click the check boxes by the loci the need to be edited.

2.9 Edit the necessary information (Analytical Threshold, Interpretation Threshold, or Stutter Filter).

2.10 To edit the Intralocus Balance flag percentage, select the "Edit" button in the gray banner containing that information.

2.11 Edit the appropriate Intralocus Balance value and select "Save".

3 Enable Sample History

3.1   Log into an administrator account.

3.2   Select the administrator's email address at the top of the screen.

3.3   Select "Maintenance" from the menu.

3.4   Select "Define Content"

3.5   Sample History is at the top of the page.

3.6   Ensure "Enable Sample History" is toggled on (to the right).

4   Data Management

4.1   Log into an administrator account.

4.2   Select the administrator's email address at the top of the screen.

4.3   Select "Maintenance" from the menu.

4.4   Select "Export Data".

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
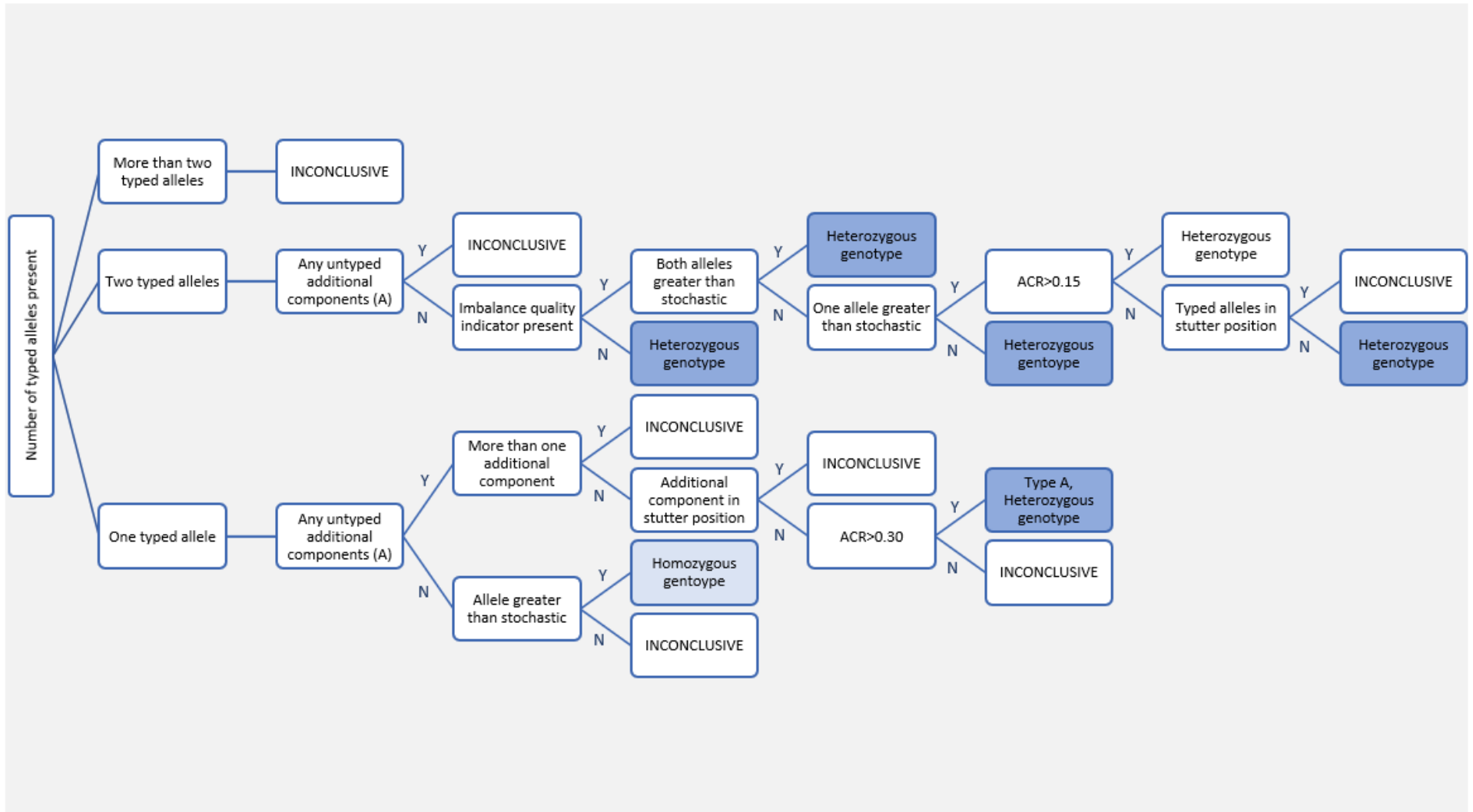# TEST METHODS
APPENDIX 18
ForenSeq™ DNA Signature Prep Stutter Filters

Stutter filters utilized by the Universal Analysis Software have been internally determined and validated through methods outlined in the Verogen MiSeq FGx® and ForenSeq™ DNA Signature Prep Kit Primer Set B validation. The back stutter filter values listed in the table below are the mean + 3SD of the average observed stutter for each locus rounded to the nearest whole percent. The forward and double back stutter are listed as the square of the corresponding back stutter.

	Back Stutter	Forward & Double Back Stutter			Back Stutter	Forward & Double Back Stutter
D1S1656	23.00%	5.29%		DXS7132	21.00%	4.41%
TPOX	7.00%	0.49%		DXS10074	12.00%	1.44%
D2S441	5.00%	0.25%		DXS10103	17.00%	2.89%
D2S1338	23.00%	5.29%		HPRTB	6.00%	0.36%
D3S1358	13.00%	1.69%		DXS7423	7.00%	0.49%
D4S2408	6.00%	0.36%		DYS505	10.00%	1.00%
FGA	25.00%	6.25%		DYS570	20.00%	4.00%
D5S818	10.00%	1.00%		DYS576	14.00%	1.96%
CSF1PO	7.00%	0.49%		DYS522	9.00%	0.81%
D6S1043	15.00%	2.25%		DYS481	44.00%	19.36%
D7S820	11.00%	1.21%		DYS19	13.00%	1.69%
D8S1179	23.00%	5.29%		DYS391	15.00%	2.25%
D9S1122	14.00%	1.96%		DYS635	10.00%	1.00%
D10S1248	17.00%	2.89%		DYS437	11.00%	1.21%
TH01	11.00%	1.21%		DYS439	8.00%	0.64%
vWA	21.00%	4.41%		DYS389I	17.00%	2.89%
D12S391	30.00%	9.00%		DYS389II	19.00%	3.61%
D13S317	7.00%	0.49%		DYS438	3.00%	0.09%
PentaE	9.00%	0.81%		DYS612	40.00%	16.00%
D16S539	20.00%	4.00%		DYS390	12.00%	1.44%
D17S1301	20.00%	4.00%		DYS643	7.00%	0.49%
D18S51	25.00%	6.25%		DYS533	9.00%	0.81%
D19S433	13.00%	1.69%		Y-GATA-H4	15.00%	2.25%
D20S482	17.00%	2.89%		DYS385a-b	24.00%	5.76%
D21S11	12.00%	1.44%		DYS460	12.00%	1.44%
PentaD	5.00%	0.25%		DYS549	7.00%	0.49%
D22S1045	15.00%	2.25%		DYS392	23.00%	5.29%
DXS10135	20.00%	4.00%		DYS448	4.00%	0.16%
DXS8378	8.00%	0.64%		DYF387S1	20.00%	4.00%

APPENDIX 19
ForenSeq™ DNA Signature Prep Interpretation Guide (STRs)

ForenSeq™ DNA Signature Prep Interpretation Guide (SNPs)
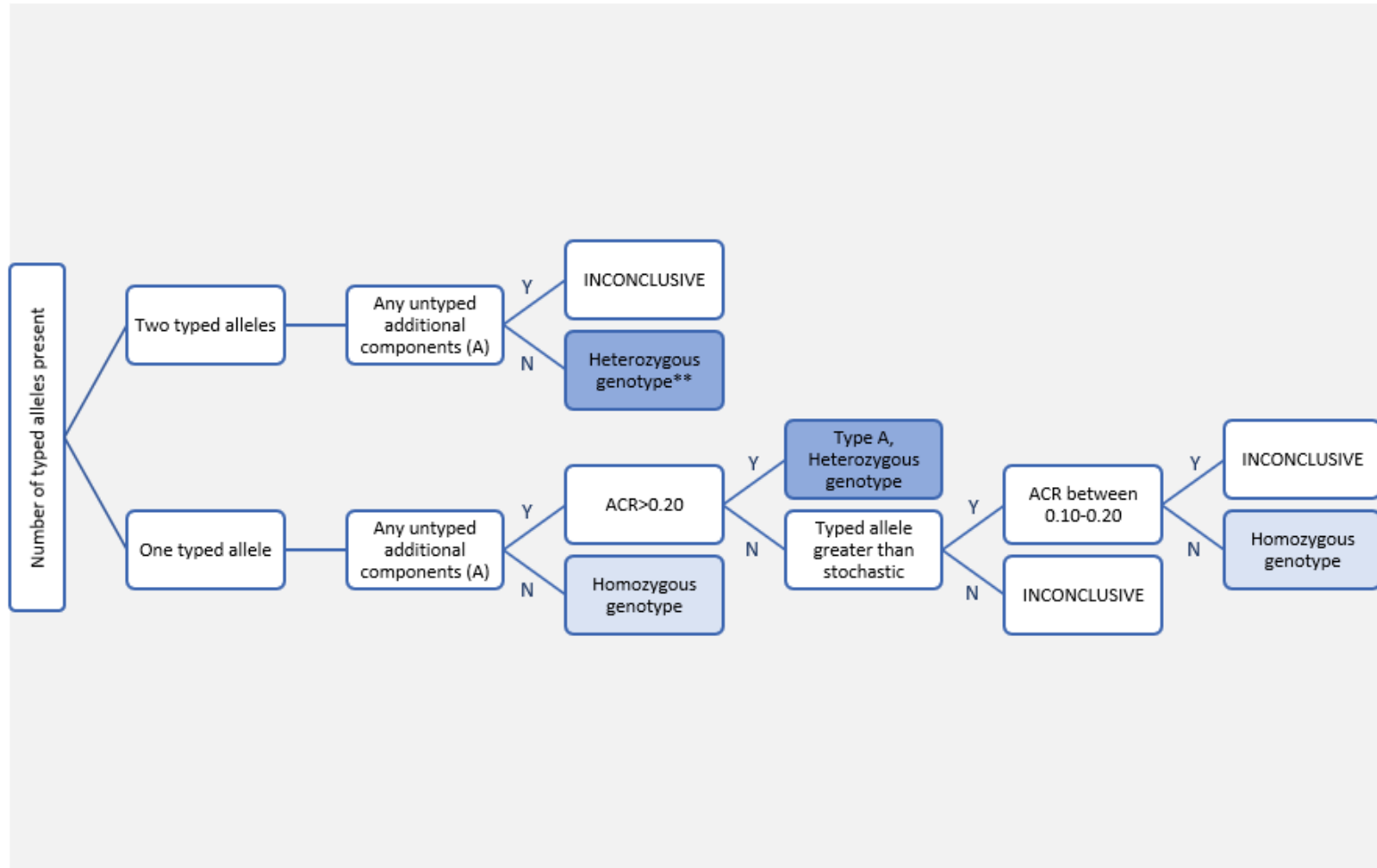
**If ACR is <0.10, the locus may be called inconclusive at the analyst's discretion.