# Unit Technical Manual

## Forensic Biology

SAN DIEGO POLICE DEPARTMENT

June 9, 2025

Approved by: Adam Dutra

Forensic Biology

# Reagent Preparation (also refer to individual procedures)

Date: 06/29/2023 Approved by: ARD

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All preparations can be scaled up or down as needed, while maintaining the same proportions/concentrations of reagents.

1. ACETATE BUFFER, pH 5

   Add 5mL of glacial acetic acid to 10g of sodium acetate (anhydrous) in a suitable container. Bring volume to 500ml with $dH_2O$. Adjust pH to 5. Good for nine months (refrigerated).

2. AMMONIUM OXALATE (saturated, pH = 3.0)

   Make a saturated solution of ammonium oxalate by adding ammonium oxalate to 100mL of PCR grade water. Adjust to pH = 3.0 by adding HCL.

3. BLEACH, 10-20% (Sodium Hypochlorite)

   Dilute store bought bleach between 1/5 and 1/10 with water.

4. DTT (1M DTT, 10mM Sodium Acetate, pH = 5.2)

   Dissolve .77g dithiothreitol in 5mL autoclaved PCR grade water. Add 50µL 1M autoclaved sodium acetate pH= 5.2. Store at -20°C in 100-500µL aliquots. The aliquots are good for one year.

5. EDTA (0.5M, pH = 8.0)

   Dissolve 186.1g disodium ethylenediamine tetraacetate-$2H_2O$ to 800mL of distilled or PCR grade water. Stir vigorously and adjust the pH to 8.0 by adding NaOH pellets (approximately 20g). Adjust final volume to one liter with distilled or PCR grade water and autoclave.

6. HIDI Formamide (Applied Biosystems P/N 4311320)

   Dispense the formamide into 100-1000µL aliquots and store frozen. The frozen formamide can be used up to 1 year after being aliquoted.

   *Caution*: Formamide is a known teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a fume hood and wear chemical resistant gloves and safety glasses when handling.

7. HYDROGEN PEROXIDE 3%

   Prepare by 1/10 dilution of 30% stock $H_2O_2$ or use a commercial 3% solution.

8. LEUCOMALACHITE GREEN (LMG) SOLUTION

   0.1g LMG (p,p-benzylidene-bis-N,N-dimethylaniline)
   66mL glacial acetic acid
   33mL distilled or PCR grade water

   Store refrigerated over excess zinc. Caution: the reagent will evolve hydrogen gas continuously. Do not cap tightly!!

   Expiration: The reagent is usable as long as the LMG is in the clear (reduced) form. The color of the reagent should be evaluated before each use. A reagent which is lightly tinged green is still usable. A reagent that has become dark-green or opaque should be discarded.

9. NUCLEAR FAST RED STAIN

   Stain can be ordered pre-made from Seri or another manufacturer

   Dissolve 5.0g of aluminum sulfate in 100mL of hot distilled or PCR grade water and add 0.1g of Nuclear Fast Red (C.I. 60760). Stir, cool, and then filter. This solution is stable for many months stored at 8° C.

10. Ortho-DIANISIDINE WORKING SOLUTION

    Ortho-Dianisidine (fast blue salt BN) is added to acetate buffer until a yellow brown solution is produced. Prepare fresh or freeze into one-use aliquots.

11. PHENOLPHTHALIN STOCK SOLUTION

    To a single-neck round-bottom flask, add:

    10g phenolphthalein powder
    100g potassium hydroxide
    100g granular zinc (mesh size ≤30) or zinc shavings
    500mL distilled or PCR grade water

    Place the flask in a heating mantle and attach a reflux unit to the round-bottom flask, sealing the joint with vacuum grease. Adjust the mantle setting to gently boil the solution. Reflux until clear. Store refrigerated over excess zinc.

    Expiration: Stock solution is usable as long as it is in the clear (reduced) form. A pinkish solution has oxidized and should be re-refluxed or discarded.

    Caution: zinc dust/granular zinc with mesh size >30 (<500µm diameter) can be pyrophoric (spontaneously combust in air) and must be stored under an inert gas. Zinc dust or granular zinc with mesh size >30 can also react violently with water, releasing highly flammable hydrogen gas. Ensure zinc has particles with mesh size ≤30.

12. PHENOLPHTHALIN WORKING SOLUTION

    Add 4 parts of ethanol to one part phenolphthalin stock solution. The reagent will be cloudy immediately following preparation; let stand refrigerated overnight to clear. Store refrigerated over excess zinc.
    Expiration: The same precautions used for the stock solution should be applied. A pinkish solution should be discarded.

13. PICROINDIGOCARMINE STAIN

Stain can be ordered pre-made from Seri or another manufacturer

Caution: dry picric acid is an explosive similar to TNT. Crystals of picric acid can form on the bottles. Dry picric acid can explode with shock, friction, or heat. Handle with extreme caution!

To 100mL of saturated picric acid solution add 0.33g of Indigo Carmine (C.I. 73015) and stir overnight. Filter and store at 8°C. This solution is stable for many months under these conditions.

14. PROTEINASE K, 10 mg/mL

Dissolve 100mg of proteinase K in 10mL of autoclaved PCR grade water. Store at -20°C in 100-500µL aliquots. The aliquots are good for one year.

15. SODIUM ACETATE, pH 5.2

Dissolve 8.2g $C_2H_3NaO_2$ (sodium acetate) anhydrous in 80mL PCR grade water. Adjust to pH 5.2 by adding glacial acetic acid (approximately 2 mL). Adjust final volume to 100 mL. Autoclave. Store at room temperature.

16. SODIUM ALPHA-NAPHTHYL PHOSPHATE SOLUTION (saturated)

Sodium alpha-naphthyl phosphate (a calcium form can also be used) is prepared as a saturated solution in acetate buffer. Prepare fresh or freeze into one use aliquots.

Method: **Use of the Crime-lite® AUTO Alternate Light Source**

Date: 01/31/2024 Approved by: ARD

MATERIALS:

1. Crime-lite® AUTO
2. Makita® Rechargeable battery
3. Mains Power Adaptor (cable with 2-prong electrical plug)

PROCEDURES:

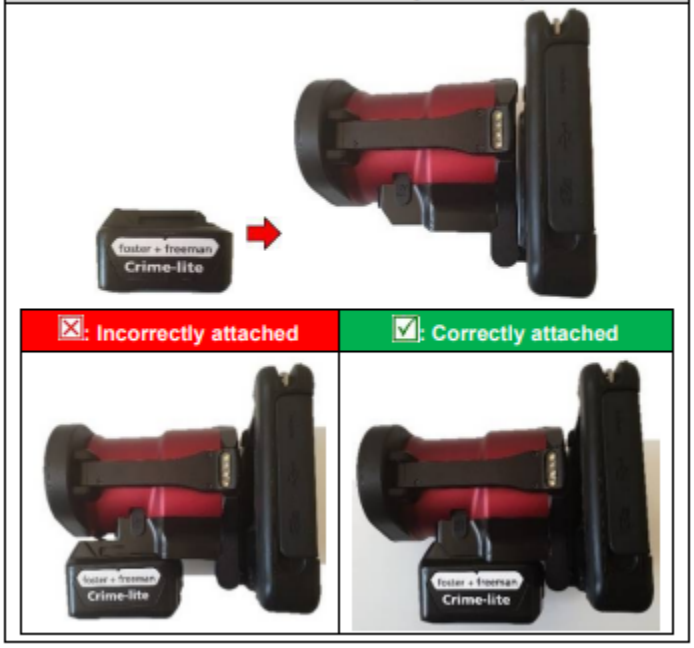
Note: The Crime-lite® AUTO is a portable high intensity light source, which if used inappropriately has the potential to be a hazard to the eyes and skin. Users should use the Crime-lite® AUTO with the awareness that both their own eyes and skin, and those in close proximity should be protected at all times. Exposure time needs to be considered.

1. Connect a power source, either the Mains Power Adapter or the Makita® Rechargeable battery for portable use. If using the rechargeable battery, ensure it has been charged. It is recommended to have the extra, rechargeable batteries available in the kit charging on deck while one rechargeable battery is in current use.

**Rechargeable battery**

Engage the battery with the mounting on the underside of the *Crime-lite*®AUTO main unit and slide it into position. The battery will click into place when it is correctly attached.

*Crime-lite*®AUTO: Attaching the battery

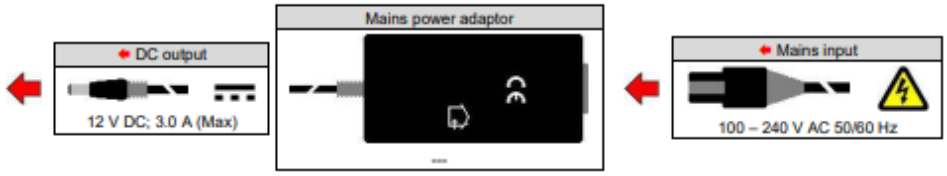



The equipment is now ready for use.

If the battery has a low charge or unavailable, attach the Mains Adaptor to the Crime-lite® AUTO and the nearest outlet.

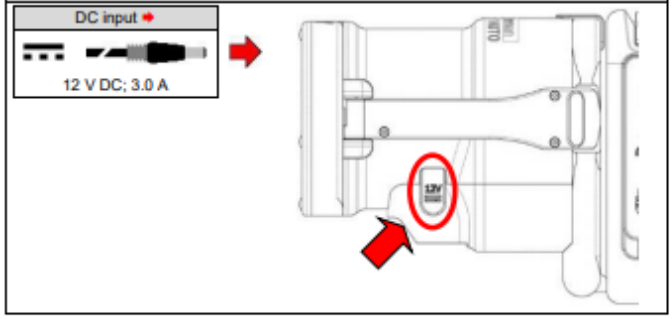
**Mains power adaptor**

Appearance and specification may vary




⚠ Do not operate the equipment with an incompatible mains electricity supply.

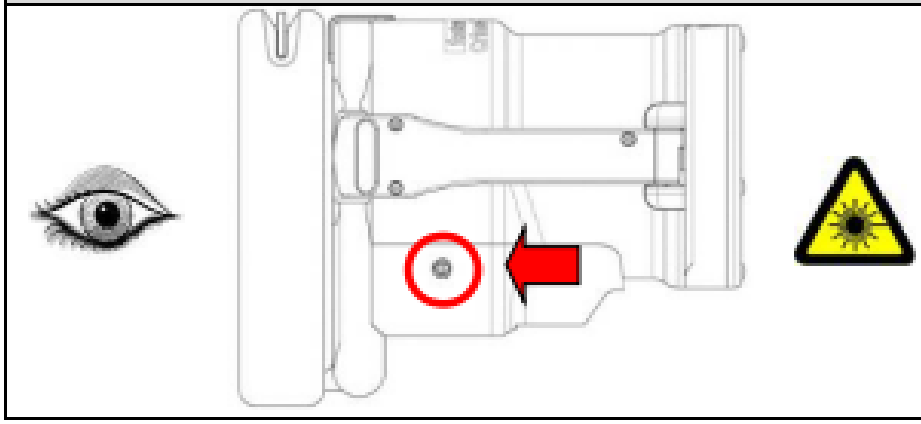
*Crime-lite*®AUTO: Main unit (DC power)



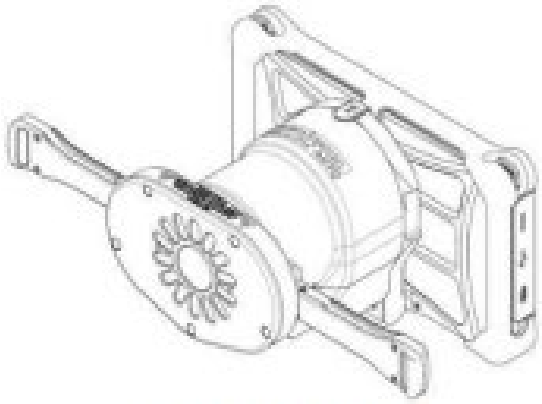

Remove the protective cover from the input socket. Interconnect the equipment with the cables provided. The equipment is now ready for use.

2. Power on the Crime-lite® AUTO and remove the magnetic lens cap.

*Crime-lite*®AUTO: On/Off control (Main unit)



3. Swing open the wide-angle illumination arms of the Crime-lite® AUTO



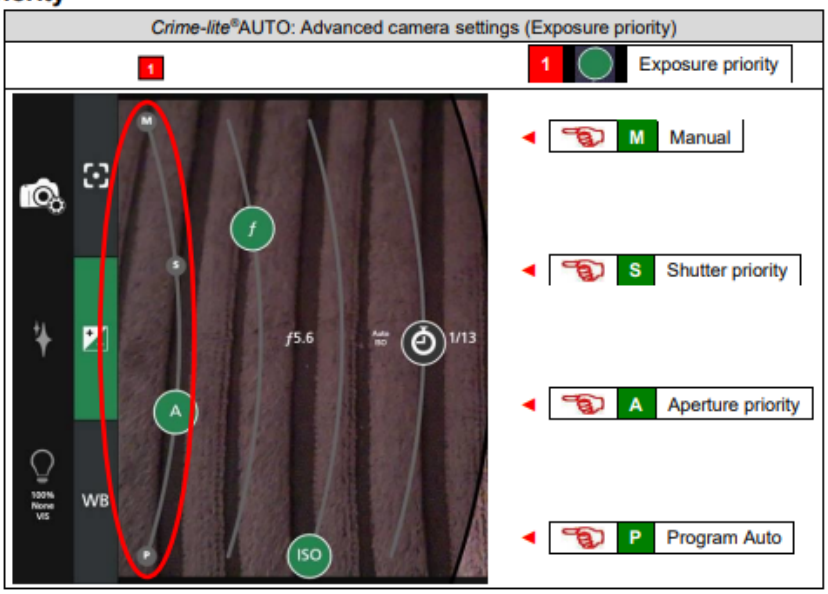
Extended arms

4. On the home screen, click the light bulb button in the lower left-hand corner to select the appropriate light source. Blue light should be selected for the observation of potential body fluids and IR should be selected for the observation of potential bloodstains. The appropriate filter will be automatically selected.

   Note: If you are having difficulty focusing on the item being examined, ensure the camera settings are all set to Auto. To adjust the camera settings, click the camera settings button in the top left-hand corner. The top tab [+] changes the focus. Set the focus to A or AL for auto focus. The middle tab +/- changes the exposure priority. Set the exposure to P for auto

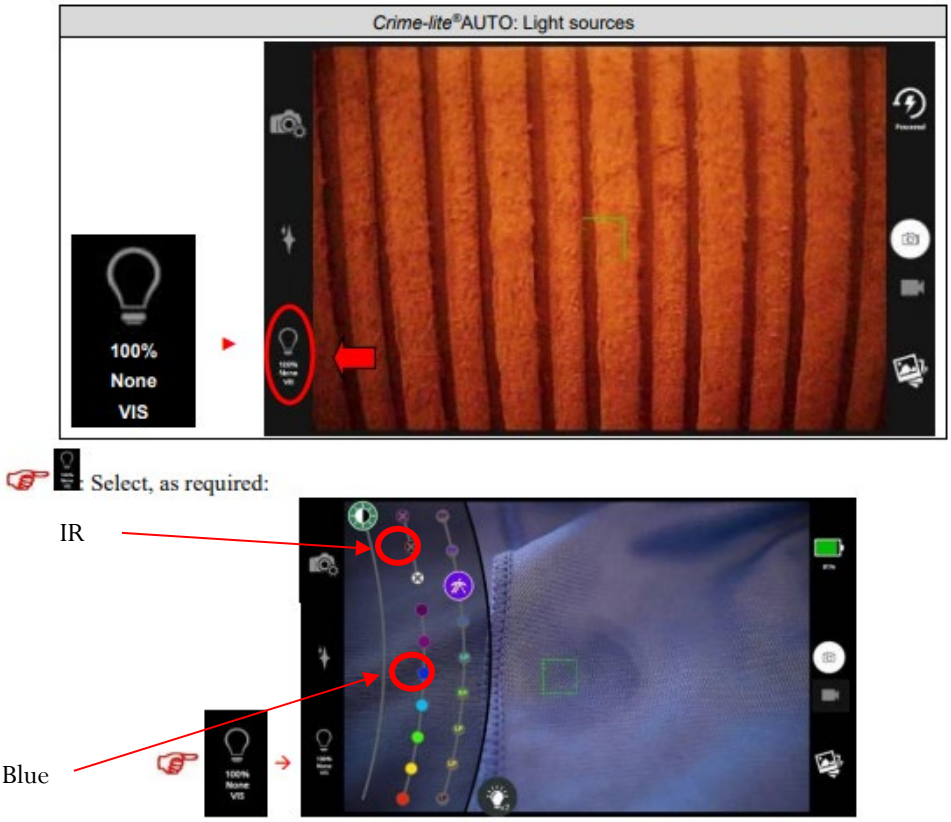

[A] [AL] [M]: Select, as required.

**Exposure priority**

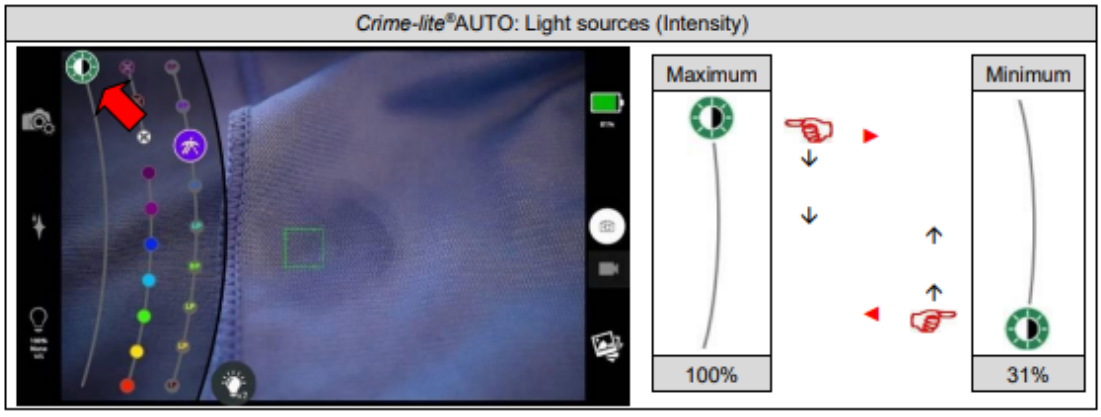



[M] [S] [A] [P]: Select, as required.

**Light sources**




Note: The intensity of the light can be adjusted by the sliding scale depicted below:

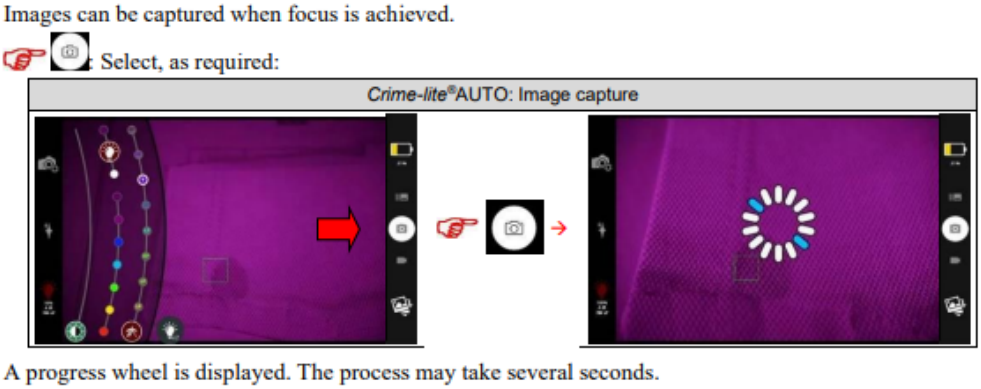



5. Conduct a quality control check of the Crime-Lite® AUTO on known biological stains prior to examining evidence and record the results in the

case notes.

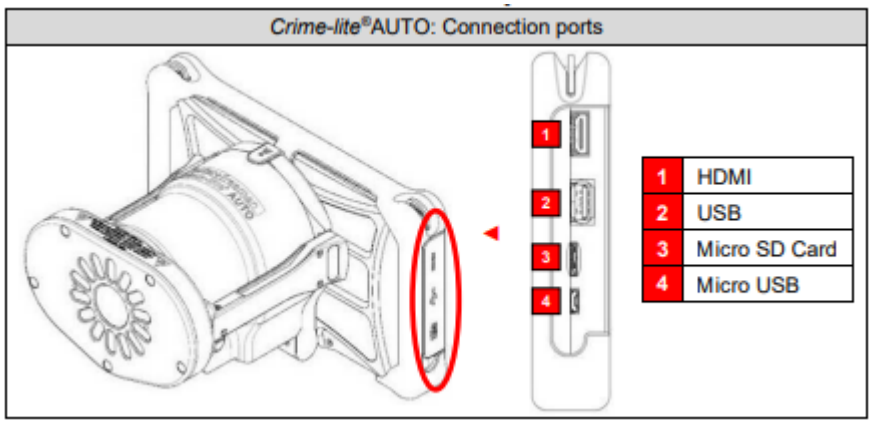
6. Systematically pass the light over the entire item to be examined. A fluorescent area with the Blue light may indicate the presence of a body fluid stain. Note the area for further testing. When the IR source is used, the image is automatically gray-scaled. A dark spot may indicate the presence of a bloodstain. Note the area for further testing.

7. If desired, capture an image of the stains you saw. Tap on the screen directly to focus the camera on the desired location. Click the camera button on the right side, center of the screen.




Note: When not actively using the Crime-lite® AUTO, put it into Sleep mode. To put into sleep mode, click the power button and select "Sleep" from the touch screen options.

8. Images can be exported by inserting a flash drive into the Crime-lite® AUTO or by removing the Micro SD card and uploading the images via the card reader.




If exporting images via the flash drive, from the home screen, select the stack of photos button in the bottom right hand corner, press and hold on any image to display the selection tools, check the images you would like to export (or select "check all images"), and then click the export button (the export button looks like a square with an arrow pointing to the right).

9. Once the examination is complete, turn off the power.

10. Secure the Crime-lite® AUTO by either placing it on a stable surface or in its carrying case (for long term storage) and removing the Mains Adaptor or Rechargeable Battery.

## Charging the Makita® rechargeable battery:

There are 3 total *Makita*® Rechargeable batteries provided in the Crime-lite® AUTO kit: 2 BL1021B batteries and 1 BL1041B battery. These *Makita*® Rechargeable battery are lithium-ion 10.8-12V, DC that supplies a Crime-lite® AUTO sufficient power for up to 60-120 minutes at a fully charged capacity. The 2 BL1021B provide power for 60 min and the 1 BL1041B provides power for up to 120 min.


1. Insert the battery cartridge into the charger until it stops adjusting to the guide of charger.

2. When the battery cartridge is inserted, the charging light color will change from green to red and charging will begin. The charging light will remain steadily during charging.

3. One red charging light indicates charged condition in 0 - 80% and one red and one green light indicates 80 – 99%.

4. With finish of charge, the charging lights will change from one red and one green light to one green light only.

5. The charging time is approximately 30 minutes for the *Makita*® BL1021B Rechargeable batteries and 60 minutes for the *Makita*® BL1041B Rechargeable battery.

6. After charging, unplug the charger from the power source. To remove the battery from the charger, press and release the retaining catch and slide the battery away from the charger.

REFERENCES:

1. Foster and Freeman- Crime-lite® AUTO User Manual (February 2022)

Method: **Microscopic Screening of Evidence for the Presence of Semen**

Date: 04/02/1998 Revision Date: 06/29/2023 Approved by: ARD

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Note: This procedure has been specifically written for Analysts involved in screening evidence for the presence of semen but not involved in DNA testing. Depending on the scenario and previous testing, one or more sections may not be necessary.

Presumptive testing:

1. Remove a very small portion of the putative semen stain and perform a test for the presence of acid phosphatase activity.

Water wash microscopy:

1. Remove approximately 1/4 of a swab or a suitable portion of the substrate and place in 1.5mL tube. Add 1mL of PCR grade water and incubate for 30 minutes at room temperature. Vortex several times during that 30 minutes. A reagent blank tube should be created at this point which will undergo all of the following manipulations and will be used as a control to monitor potential contamination, if the extracts prepared will be used for DNA testing.

2. Using a pipette tip (or wooden stick) remove as much liquid from the substrate as possible, transfer it to a second microcentrifuge tube, and retain.

3. Microcentrifuge the sample extract at maximum speed for 3-5 minutes.

4. The supernatant may be used for serological tests for blood, semen, saliva. [Consult the specific procedure for details]. Remove an appropriate amount of the supernatant for any desired assays.

5. Carefully pipette off all but 30-100µL of the supernatant being careful not to disturb the pellet. The supernatant can be discarded. At least 30µL of supernatant should be left behind to ensure the cell pellet is not disturbed.

6. Resuspend the cell pellet in the remaining supernatant. Pipette 3-10µL of the sample on to the well of a slide (different amounts may be used). Place the slide in an incubator or on a heat block to dry.

7. Stain the slide with Xmas Tree Stain (refer to the Identification of Cellular Material using Xmas Tree Staining procedure).

8. If only screening is being performed and enough sperm are present in a sample, no further work on that sample may be necessary and that sample (along with the reagent blank) can be referred for DNA testing. In general, at least 100 sperm cells in the sample are needed for successful DNA typing. If no sperm are observed in a sample, then proceed with the following steps.

Digest microscopy:

1. Add back the substrate to what remains of the water extract and add 500-750µL of digest buffer and 20-25µL of proteinase K to the tube. Incubate the sample for at least one hour at 56°C.

2. Retain as much liquid from the substrate as possible and discard the substrate.

3. Microcentrifuge the sample at maximum speed for 3-5 minutes.

4. Carefully pipette off all but 30-100µL of the supernatant being careful not to disturb the pellet. The supernatant can be discarded. At least 30µL of supernatant should be left behind to ensure the cell pellet is not disturbed.

5. Add 1mL of water to the cell pellet and resuspend by lightly vortexing.

6. Microcentrifuge the sample at maximum speed for 3-5 minutes.

7. Carefully pipette off all but 30-100µL of the supernatant being careful not to disturb the pellet. The supernatant can be discarded. At least 30µL of the supernatant should be left behind to ensure the cell pellet is not disturbed.

8. Resuspend the cell pellet in the remaining supernatant. Pipette the remainder (or portion) of the sample on to the well of the slide. Place the

slide in the incubator or on a heat block to dry.

18. Stain the slide with Xmas Tree Stain (refer to the Identification of Cellular Material using Xmas Tree Staining procedure).

Note: The following scale can be used for documentation of microscopic examinations:

0 = No cells observed
1* = One cell observed
1+ = Cells observed in a few fields of view
2+ = Cells observed in most fields of view
3+ = Numerous cells observed in most fields of view
4+ = Too many cells to count

19. If sufficient sperm are present in a sample, no further work on that sample may be necessary and that sample can be referred for DNA testing. If no sperm were present for a sample even after the second round of microscopy, it may be necessary to prepare an extract of a second sample from the evidence.

20. If warranted, a small portion can be extracted for p30 analysis. Refer to the Detection of Seminal Protein p30 Using the Abacus p30 Diagnostic Card procedure.

REMARKS:

The systematic approach to the analysis of semen provides the analyst with the opportunity to assay for body fluids, to perform microscopy on cells, and to perform DNA testing from a single extract of a potentially limited sized evidence sample. It represents a superior approach for creating individual extracts for each class of testing.

The systematic approach to the analysis of semen is carried out by creating a single water extract from a substrate that may contain semen and other body fluids. Following centrifugation of the water extract, the supernatant can be used to test for the presence of blood, semen, and saliva. The cellular portion of

the extract, after it has been resuspended in the remaining supernatant, can be analyzed microscopically for the presence of sperm and other cells. Depending on the results of these initial tests, the remaining sample can be used for DNA testing.

The reagent blank will be subjected to all reagents used in the extraction process and all manipulations carried out on the evidence samples. The reagent blank is necessary to ensure that any contaminant introduced during the extraction process is detected. The use of a reagent blank is mandatory if the samples are to be tested using the extremely sensitive polymerase chain reaction (PCR) DNA tests. If the extracted materials are to be used for DNA analysis, the PCR grade water and microcentrifuge tubes used in the "Systematic Approach to the Analysis of Semen" must have been autoclaved.

REFERENCE:

Blake, Sensabaugh, Bashinski, "A Systematic Approach to the Analysis of Semen Evidence." CAC Meeting, November, 1980

Method: **Two-Step Acid Phosphatase Screening Test for Semen**

Date: 09/13/2001 Revision Date: 06/29/2023 Approved by: ARD

REAGENTS:

1. SODIUM ALPHA-NAPHTHYL PHOSPHATE SOLUTION (saturated)
2. Ortho-DIANISIDINE WORKING SOLUTION
3. ACETATE BUFFER, pH 5

MATERIALS:

1. Spray apparatus
2. Disposable pipettes

PROCEDURES:

A quality control check of the reagents against a known semen sample and a negative of reagents only before use. The results of the quality control test must be recorded in the case notes.

CUTTING METHOD

1. Place a small portion of the sample in a small test tube or on filter paper.
2. Add 1-5 drops of alpha-naphthyl phosphate.
3. Add 1-5 drops of ortho-dianisidine and observe for any color change. Positive tests show a red-purple precipitate.

SWAB METHOD

Wet a cotton swab with $dH_2O$ then rub the sample and proceed as above with step 2 on the swab.

MAPPING

1. Wet a piece of filter paper with $dH_2O$. Press against the suspected semen stain

sample for a few seconds.
2. Drip or spray alpha-naphthyl phosphate onto the paper.
3. Drip or spray ortho-dianisidine onto the paper. Positive tests show a red-purple color on the paper.

INTERPRETATION:

Acid phosphatase is an enzyme found in high concentrations in semen, but is also found in other body fluids and is produced by other organisms such as bacteria, yeast, fungi, and plants. Acid phosphatase can be found in concentrated vaginal discharge or secretions.

Appropriate results from the negative and positive control must be obtained with the reagents prior to their use on unknowns.

No color change should be observed after adding alpha-naphthyl phosphate. If a color occurs prior to adding ortho-dianisidine, the results are inconclusive.

The color change observed after adding ortho-dianisidine should occur within 45 seconds to be considered a positive reaction. Color change reactions observed after 45 seconds are considered negative.

When mapping an item, it is important to remember that as seminal fluid is deposited onto a fabric, the concentration of spermatozoa will usually be highest in the middle of the stain with the greatest concentration of acid phosphatase usually along the outer edges.

REFERENCES:

1. Blake, Sensabaugh, Bashinski. A Systematic Approach to the analysis of Semen Evidence. CAC Meeting, 6 November 1980
2. Gaensslen, R.E. Sourcebook in Forensic Serology, Immunology, and Biochemistry, 1983
3. Saferstein, Baechtel, "The Identification and Individualization of Semen Stains", Forensic Science Handbook, Vol. 2,1988
4. Metropolitan Lab. Biology Methods Manual, 1978

**Method: One-Step SERI AP Spot Test Detection of Acid Phosphatase**

Date: 01/11/2001 Revision Date: 06/29/2023 Approved by: ARD

REAGENTS:

1. 0.26g SERI AP Spot Test (SERI Catalog #R558)
2. 10mL distilled or PCR grade water

Different volumes of the reagent may be prepared. The ratio of reagent to water should not be changed

MATERIALS/EQUIPTMENT:

1. Whatman Filter Paper and/or cotton swabs
2. Disposable pipettes

PROCEDURE:

A quality control check of the reagent against a known semen sample and a negative of reagents only before use. The results of the quality control test must be recorded in the case notes.

1. Dissolve the AP Spot Test reagent in water and test the reagent against known positive and negative semen standards before use.

2. Moisten a piece of Whatman filter paper or a cotton swab and vigorously press or rub it against your questioned sample. Alternatively, a small cutting from the questioned sample may be taken and placed on a piece of filter paper.

3. Drip the AP reagent onto the filter paper, swab, or cutting on filter paper.

INTERPRETATION:

Acid phosphatase is an enzyme found in high concentrations in semen, but is also found in other body fluids and is produced by other organisms such as bacteria and yeast. Acid phosphatase can be found in concentrated vaginal discharge or secretions.

Appropriate results from the negative and positive control must be obtained with the reagents prior to their use on unknowns.

The color change observed after adding ortho-dianisidine should occur within 45 seconds to be considered a positive reaction. Color change reactions observed after 45 seconds are considered negative.

REMARKS:

Acid phosphatase is an enzyme found in high concentrations in semen, but is also found in other body fluids and is produced by other organisms such as bacteria, yeast, fungi, and plants. Acid phosphatase can be found in concentrated vaginal discharge or secretions.

In the presence of acid phosphatase, the SERI AP Spot Test reagent contains all the components necessary to carry out the following reaction: liberation of naphthol from sodium alpha-naphthyl phosphate by the enzyme and the formation of a purple azo dye by the coupling of naphthol with buffered fast blue B.

The reconstituted reagent will remain stable and sensitive for one day's use at room temperature.

REFERENCE:

Serological Research Institute. Laboratory Protocol, September 29, 1989

Method: **Identification of Cellular Material Using Xmas Tree Staining**

Date: 01/20/2000 Revision Date: 06/29/2023 Approved by: ARD

REAGENTS:

1. NUCLEAR FAST RED STAIN
2. PICROINDIGOCARMINE STAIN

PROCEDURE:

1. Extract the sample according to the procedure for Differential DNA Extraction or Screening for the Presence of Semen and fix the sample(s) to the slide.

2. Cover sample well(s) on the slide(s) with a drop of Nuclear Fast Red stain (Seri Stain A) and leave for approximately 10 minutes.

3. Wash away Nuclear Fast Red stain with distilled or PCR grade water dispensed from a wash bottle.

4. Add one drop of Picroindigocarmine stain (Seri Stain B) to the sample well(s) without drying the slide. Rotate the dye on the slide by hand for 15-30 seconds. Wash the stain from the slide with EtOH dispensed from a wash bottle. Dry the slide and mount a cover slip with Permount.

5. Examine microscopically. The slide can be examined by phase contrast before or after staining with Xmas Tree stain.

REMARKS:

Nuclear material is stained red by the Nuclear Fast Red dye. Sperm heads are usually well differentiated with the acrosome staining less densely than the distal region of the head. Epithelial membranes and sperm tails are stained green by the Picroindigocarmine. Nuclei inside epithelial cells appear red to purple. Yeast cells also stain red, however, the stain is uniform throughout the

cells and extends into polyp-like structures which are occasionally observed with yeast cells. Under phase contrast, the sperm heads will appear bright white with a dark acrosome.

The following scale can be used for documentation of microscopic examinations:

0 = No cells observed
1* = One cell observed
1+ = Cells observed in a few fields of view
2+ = Cells observed in most fields of view
3+ = Numerous cells observed in most fields of view
4+ = Too many cells to count

REFERENCES:

Gaensslen, R., Sourcebook in Forensic Serology, Immunology and Biochemistry. US
Government Printing Office, 1983.

## Method: p30 Detection Using the Abacus p30 Diagnostic Card

Date: 03/31/2000 Revision Date: 07/31/2024 Approved by: ARD

REAGENT:

Sterile PCR grade Water.

MATERIALS/ EQUIPMENT:

1. Abacus p30 Diagnostic Test Card Kit. The kit includes the test cards and plastic droppers. Abacus Diagnostics catalog #308322 (25 tests/kit).
2. Microcentrifuge (capable of 10,000-15,000 x g).

PROCEDURE:

1. Place a cutting* of the stain or swab into a sterile 1.5mL tube. Add 350µL PCR grade water to saturate the material. Extracts of positive control samples (neat semen and a 1/20 dilution of semen) and a negative control (reagents only) should also be run with the unknowns.

* If a high amount of semen is expected, add 75µL or enough PCR grade water to just saturate the material, leaving a small amount of excess water. A 1/10 dilution of the extract will be generated prior to its addition to the test card.

Note: The positive control samples can be pre-made stains of neat semen and a 1/20 semen dilution. Alternatively, the 1/20 dilution may be made from the extract of the neat semen stain.

2. Incubate the samples for 45 minutes at room temperature. Vortex or agitate the samples three or four times during the incubation.

3. Remove the substrate or swab and place it into a spin basket. Centrifuge the spin basket for 5 minutes at maximum speed to collect residual extract from the substrate.

4. Remove the spin basket containing the substrate and retain the substrate, if necessary. If a separate spin tube was used to remove the liquid from the substrate, combine the liquid from the original tube with the liquid from the spin tube and centrifuge the combined liquid for 5 minutes at maximum speed. The tube will contain a supernatant with water-soluble proteins such as the p30 protein and the cell pellet. The supernatant will be used for the Abacus p30 Diagnostic Test and the cell pellet can be microscopically examined for the presence of cellular material. Do not disturb the cell pellet at the bottom of the tube.

5. If a high amount of semen is expected, create a 1/10 dilution of the supernatant, otherwise skip to Step 6. To generate the 1:10 dilution, add 20µL of the supernatant to a sterile 1.5mL tube containing 200µL of water.

6. Load 80µL of the supernatant from Step 4 (or the dilution from step 5) onto the sample loading "S" area of the test card.

7. Immediately start timing the reaction. The test card result should be read at ten minutes. Record the results as positive or negative on the Abacus p30 Test Worksheet. Document the results with a digital camera and add the image to the Abacus p30 Test Worksheet. Once the image is printed there is no need to keep a permanent data file of the image.

Note: Under no circumstances should results obtained after 10 minutes be recorded.

8. If a positive result for the p30 protein is obtained for a given sample, it may be appropriate to also test a substrate control for that sample. For positive results from a questioned stain it could prove useful to test additional serial dilutions of the sample to provide information on how concentrated the stain is.

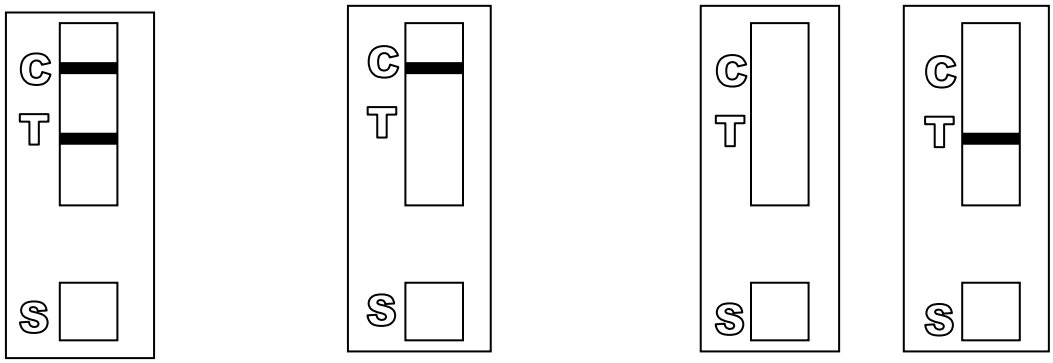
INTERPRETATION:

A positive test result for the presence of the protein p30 is indicated by the presence of pink bands at both the control line "C" and the test line "T" on the test card. The presence of a pink band at the control line but the absence of a pink band at the test line indicates a negative result. A pink band will always

appear at the control "C" area. The band in the "C" area is an internal control of the test strip. If a pink band does not appear at the control "C" line of the test card the test is invalid.

For a test series to be interpreted the positive and negative controls must yield the expected results.

Legend: "S" = Sample loading area "T" = Test line "C" = Control line




**POSITIVE** **NEGATIVE** **INVALID**

REMARKS:

Validation work on the Abacus p30 Test Card performed at the San Diego Police Department indicated that male urine can produce positive test results. The p30 protein is used as a diagnostic marker for prostate cancer where it is significantly over expressed. In instances of prostate cancer, the p30 protein can be found in very high levels in both urine and in blood.

*Analysts should be cautious of inferring that semen is indicated from samples that do not exhibit some other indication (e.g., AP positive result) when testing is indicative of other body fluids (e.g., blood) being present in the sample.*

REFERENCES:

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11. Stowell LI et al. An enzyme-linked immunabsorbent assay (ELISA) for p30. Forensic Science Int. v50(1), p125-38, 1991.
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Method: **Leucomalachite Green (LMG) Presumptive Test for Blood**

Date: 04/01/1999 Revision Date: 06/29/2023 Approved by: ARD

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REAGENTS:

1. LEUCOMALACHITE GREEN (LMG) SOLUTION
2. HYDROGEN PEROXIDE 3%

MATERIALS:

Cotton swabs or filter paper (test substrates)

PROCEDURE:

1. Test the reagents against positive and negative blood standards before using. Record these results in your notes.

2. Moisten a cotton swab or filter paper (the test substrate) with distilled or PCR grade water. Gently press or rub the stain with the substrate.

   Alternatively, a portion of the stained material can be cut and used directly as the test substrate.

3. Add 1 or 2 drops LMG solution to the test substrate. Observe briefly for any color change.

4. Add 1 or 2 drops of 3% hydrogen peroxide. Observe for any color change.

INTERPRETATION:

A swiftly developing (within 5 seconds) blue-green color after step 4 is a positive test and presumptively indicates the presence of blood. When evaluating the color change in this reaction, the appearance of the stain, the amount of material being tested, and the condition of the stain should all be taken into consideration.

Lack of a color change before 30 seconds indicates a negative reaction and the absence of blood in detectable quantities.

Any change to a blue-green color after step 3 may indicate the presence of an oxidizer and should not be interpreted as a positive. A color change at this step should be deemed inconclusive.

The test depends on the oxidation of LMG from a colorless to a colored form. This reaction is catalyzed in the presence of heme and its derivatives. The reaction can also take place in the presence of other catalysts, such as peroxidases, or inorganic oxidizers. Therefore, the test cannot be considered completely specific for blood.

REFERENCES:

1. Burdett, P.E. "Presumptive Tests for Blood - A Comparative Survey", CRE Report No. 201, October 1976
2. Cox, M. "A Study of the Sensitivity and Specificity of Four Presumptive Tests for Blood", J Forensic Sci. Vol. 36, no. 5 (Sept. 1991), pp. 1503 - 1511
3. Gaensslen, R.E. Sourcebook in Forensic Serology, Immunology, and Biochemistry, section 6.5
4. Grodsky et al., "Simplified Preliminary Blood Testing - An Improved Technique and Comparison of Methods", J. Criminal Law, Criminology, and Police Science Vol. 42, (1951), pp. 95-104.

Method: **Phenolphthalin Presumptive Test for Blood (Kastle-Meyer Test)**

Date: 04/01/1999 Revision Date: 06/29/2023 Approved by: ARD

REAGENTS:

1. PHENOLPHTHALIN STOCK SOLUTION
2. PHENOLPHTHALIN WORKING SOLUTION
3. HYDROGEN PEROXIDE 3%

MATERIALS:

Cotton swabs or filter paper (test substrates)

PROCEDURE:

1. Test reagents against positive and negative blood standards before using. Record these results in your notes.

2. Moisten a cotton swab or filter paper (the test substrate) with distilled or PCR grade water.

   Gently press or rub the stain with the substrate. Alternatively, a portion of the stained material can be cut and used directly as the test substrate.

3. Add 1 or 2 drops phenolphthalin working solution to the test substrate. Observe briefly (up to 30 seconds) for any color change.

4. Add 1 or 2 drops of 3% hydrogen peroxide. Observe for any color change.

INTERPRETATION:

A swiftly developing (within 5 seconds) pink to magenta color after step 4 is a positive test and presumptively indicates the presence of blood. When evaluating the color change in this reaction, the appearance of the stain, the amount of material being tested, and the condition of the stain should all be taken into consideration.

Lack of a color change before 30 seconds indicates a negative reaction and the absence of blood in detectable quantities.

Bloodstains may change to a greenish color after step 3. Any change to a pink color at this stage may indicate the presence of an oxidizer and should not be interpreted as a positive.

The test depends on the oxidation of clear phenolphthalin to colored phenolphthalein. This reaction is catalyzed in the presence of heme and its derivatives. The reaction can also take place in the presence of other catalysts, such as peroxidases, or inorganic oxidizers. Therefore, the test cannot be considered completely specific for blood.

REFERENCES:

1. Cox, M. "A Study of the Sensitivity and Specificity of Four Presumptive Tests for Blood", J Forensic Sci. Vol. 36, no. 5 (Sept. 1991), pp. 1503 - 1511
2. Gaensslen, R.E. Sourcebook in Forensic Serology, Immunology, and Biochemistry, section 6.3
3. Grodsky et al., "Simplified Preliminary Blood Testing - An Improved Technique and Comparison of Methods", J. Criminal Law, Criminology, and Police Science Vol. 42, (1951), pp. 95-104.

**Method: Abacus HemaTrace Detection of Blood**

Date: 12/08/1999 Revision Date: 07/31/2024 Approved by: ARD

REAGENTS:

Kit supplied extraction buffer.

MATERIALS/ EQUIPMENT:

Abacus HemaTrace Test Card Kit. The kit includes test cards, plastic droppers, and extraction buffer. Abacus Diagnostics catalog #708424 (25 tests/box).

PROCEDURE:

1. Place a cutting of the stain or swab into a sterile 1.5mL tube. Add 100µL of kit extraction buffer. A solution of 5% ammonia may also be used to extract a stain that is difficult to remove from the substrate. Extracts should also be prepared from a known human blood stain (positive control) and a reagent blank (negative control).

Note: If stains remain in extraction buffer or 5% ammonia in a refrigerator for extraction, they must be warmed to room temperature before proceeding with step 2.

2. Extract the stain (cutting or swab) for sufficient time to allow for the stain to solubilize in the extraction buffer. A minimum of 15 minutes of extraction time is recommended. For stains older than 5 years, extraction time should be at least 30 minutes. Extraction time will depend on the age and concentration of the stain.

3. If the substrate is large and/or if the supernatant from steps 1 and 2 is straw yellow or clear, transfer the substrate to a spin basket and centrifuge the spin basket for 5 minutes at maximum speed to collect residual extract from the substrate. Recombine the liquid collected from the spin basket with the original tube containing the extract from step 2, if necessary.

4. Add enough of the supernatant created in steps 1-3 to 80µL of kit extraction buffer to create a light straw yellow colored solution.

Note: If the supernatant created in steps 1-3 is clear or light straw yellow, dilution in additional kit extraction buffer is not necessary. Alternative volumes of the kit extraction buffer may also be used to obtain the light straw yellow color.

If the supernatant is clear or nearly clear, a negative result may result from samples containing a small amount of blood, because the extract is too dilute. Interpretation of HemaTrace results from samples with clear extracts should be performed with caution.

5. Load 80µL of the extraction solution onto the test card (area S).

6. Immediately start timing the reaction. The test card result(s) should be read at ten minutes. Record the results as positive or negative on the Abacus HemaTrace Test Worksheet. Document the results with a digital camera and add the image to the Abacus HemaTrace Test Worksheet. Once the image is printed there is no need to keep a permanent data file of the image.

Note: Under no circumstances should results obtained after 10 minutes be recorded.

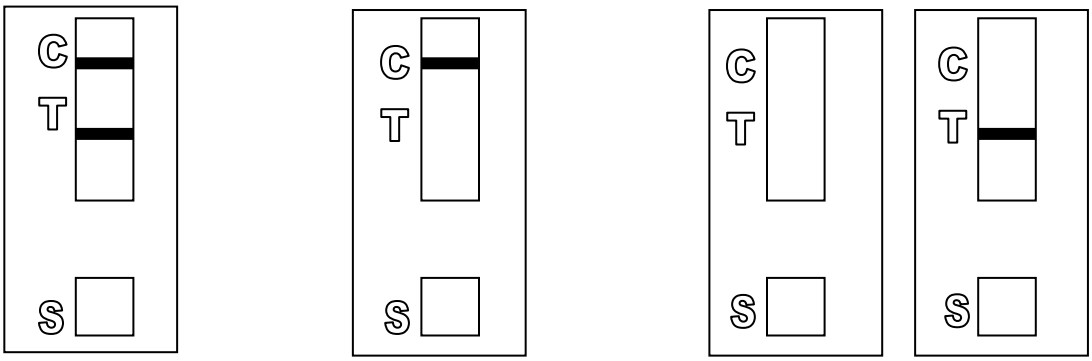
If a positive result for hemoglobin is obtained for a given sample, it may be appropriate to also test a substrate control for that sample.

INTERPRETATION:

A positive test result for the presence of human blood is indicated by the presence of pink bands at both the control line "C" and the test line "T" on the test card. The presence of a pink band at the control line but the absence of a pink band at the test line indicates a negative result. A pink band will always appear at the control "C" area. The band in the "C" area is an internal control of the test strip. If a pink band does not appear at the control "C" line of the test card the test is invalid.

For a test series to be interpreted the positive and negative controls must yield the expected results.

Legend: "S" = Sample loading area "T" = Test line "C" = Control line




**POSITIVE** **NEGATIVE** **INVALID**

REMARKS:

Validation work on the Abacus HemaTrace Test Card performed at the San Diego Police Department indicates that the HemaTrace card is specific for hemoglobin and will produce positive results with hemoglobin from certain higher primates and ferrets. The HemaTrace test can also produce positive results from other human body fluids that may contain hemoglobin at low levels. *Analysts should be cautious of inferring that blood is indicated from samples that do not exhibit some other indication (e.g., blood-like color) of blood being present.*

Validation of the HemaTrace Test Card by the Abacus Company indicates that treatment with Luminol, Coomasie Blue, Ninhydrin, bleach, or detergent did not interfere with detecting hemoglobin. Hemoglobin, when mixed with soil, plant material, leather, or washed jeans also yielded a positive result.

REFERENCES:

1. OneStep ABAcard HemaTrace for the Forensic Identification of Human Blood (kit insert), Abacus Diagnostics, 1999.
2. Swander, C.J., Stites, J.G. Evaluation of the ABAcard HemaTrace for the Forensic Identification of Blood, MAFS 1998 Annual Meeting.

3. Kristaly, A., Smith, D.A.S. Validation of the One-step ABAcard HemaTrace for the Rapid Forensic Identification of Blood, 1999.
4. Culliford, B., The Examination and Typing of Bloodstains in the Crime Laboratory, US Department of Justice, Washington D.C., 1964.
5. Spear, T.F., Binkley, S.A. The Hemeselect Test; a Simple and Sensitive Forensic Species Test, Journal of Forensic Science Society, V34 (1), p 41-46, 1994.
6. Fernando, S.A., Wilson, G.S. Studies of the Hook Effect in the One-Step Sandwich Immunoassay. Journal of Immunology Methods, V151 (1-2), p 47-66, 1992.
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METHOD: **Phadebas Amylase Test**

Date: 05/01/2012 Revision Date: 06/29/2023 Approved by: ARD

REAGENTS:

1. PHADEBAS WORKING SOLUTION
   0.02g crushed Phadebas tablet
   550µL PCR grade water
   This recipe is for 5 sample tests, but can be scaled up as needed.

STANDARDS AND CONTROLS:

1. Human salivary α-amylase standard, Sigma-Aldrich #A1031 (500 µL of 1/100,000 dilution of stock standard is required for the test)
   [The stock standard was reconstituted to ~2mg/mL with PCR grade water and is stored frozen. 1/100 dilutions of this stock have been prepared for use when analysts run this test. These dilutions have an expiration date of 6 months from the date prepared.]

2. Neat saliva. This is generally prepared fresh on a swab (of which a ~1/4 swab is used) or on filter paper (of which an ~5x5mm cutting is used) for each test.

3. Blank (PCR grade water)

4. Substrate control (if appropriate)

PROCEDURE:

1. Clear 1.5mL microcentrifuge tubes should be used for this test. For unknowns, add 500 µL of PCR grade water to an ~5x5 mm cutting or an ~1/4 swab. The frozen amylase standard aliquot should be diluted 1/1000 prior to use (suggested dilution is 5µL in 5mL). For the controls, 500 µL of the amylase standard dilution should be used for the test, 500µL PCR grade water should be used as a negative, and 500µL of PCR grade water should be added to the neat saliva sample as the positive control.

2. Add 100µL of the Phadebas working solution to each sample and vortex.

3. Incubate the samples at 37°C for 30 minutes.

4. Vortex, then centrifuge the samples at maximum speed for 2 minutes.

INTERPRETATION:

Positive: The presence of α-amylase is indicated if the blue color in the supernatant is darker than that of the 1/100,000 human salivary α-amylase standard.

NOTE: Blue substrates may produce blue supernatants during the extraction process even though no α-amylase is present on them. If this is expected, results may be inconclusive (see below).

Inconclusive: The test is inconclusive if the blue color in the supernatant is lighter than that of the 1/100,000 human salivary α-amylase standard, but darker than that of the reagent blank. The test may also be inconclusive if the blue color in the supernatant is masked by another color generated during the extraction process (e.g., red-brown supernatant from a bloodstain).

Negative: The absence of α-amylase activity is indicated if the supernatant is clear.

REMARKS:
Amylase is an enzyme present in saliva at high concentrations, hence the use of amylase as a presumptive test for the presence of saliva. Amylase is responsible for the digestion of starches, first to oligosaccharides, then to maltose and glucose. The Phadebas tablets are composed of starch polymers conjugated to a blue dye. In the presence of amylase, the blue dye is liberated into the supernatant, generating a blue color in proportion to the level of amylase activity.

Amylase is also present in most other body fluids (e.g., urine) and tissues, albeit at much lower concentrations than that found in saliva. Feces, breast milk, and nasal secretions may have high levels of amylase.

**Method:** **Serology Test Interpretation Guidelines**

Date: 12/08/1999 Revision Date: 06/09/2025 Approved by: ARD

The following is meant as a guide for what conclusions can be drawn based on a given combination of serological test result. The inferences that may be drawn regarding the presence of the body fluid of interest are based only the serological test results obtained.

Inferences regarding the body fluids in question may also be influenced by other subjective criteria such as number of sperm cells observed, but the inferences presented herein only consider the serological test results. Any additional information regarding the sample (e.g., visual observations) must be documented in the technical record.

**BLOOD**

| Phenolphthalein/LMG | HemaTrace | Conclusion | Inference |
|---|---|---|---|
| **negative** | N/A | No blood indicated | Blood not found |
| **positive** | N/A | Hemoglobin indicated | Blood indicated |
| **positive** | negative | Hemoglobin not indicated | Blood not found |
| **positive** | positive | Hemoglobin detected | Blood indicated |
| **N/A** | negative | Hemoglobin not indicated | Blood not found |
| **N/A** | positive | Hemoglobin detected | Blood indicated * |
| **negative** | positive | Hemoglobin detected | Blood indicated * |
| **negative** | negative | No blood indicated | Blood not found |
| **inconclusive** | negative | Hemoglobin not indicated | Blood not found |
| **inconclusive** | positive | Hemoglobin detected | Blood indicated * |
| **negative** | inconclusive | Inconclusive | Presence of blood could not be determined |
| **positive** | inconclusive | Inconclusive | Presence of blood could not be determined |
| **inconclusive** | inconclusive | Inconclusive | Presence of blood could not be determined |

*It is necessary for a visible blood-like stain (i.e., reddish in color) to be present for an analyst to infer that blood is indicated.

**SEMEN**

| AP | p30 | Sperm | Conclusion | Inference |
|---|---|---|---|---|
| **negative** | N/A | N/A | AP not detected | Semen not found |
| **positive** | N/A | N/A | AP indicated | Semen/seminal fluid indicated |
| **inconclusive** | N/A | N/A | inconclusive | Presence of semen could not be determined |
| **negative** | negative | N/A | AP/p30 not detected | Semen not found |
| **negative** | positive | N/A | p30 detected | Semen/seminal fluid indicated |
| **positive** | negative | N/A | AP indicated/p30 not detected | presence of semen could not be determined |
| **positive** | positive | N/A | AP indicated/p30 detected | Semen/seminal fluid indicated |
| **inconclusive** | inconclusive | N/A | inconclusive | Presence of semen could not be determined |
| **inconclusive** | negative | N/A | p30 not detected | Semen not found |
| **inconclusive** | positive | N/A | p30 detected | Semen/seminal fluid indicated |
| **negative** | negative | negative | No semen indicated | Semen not found |
| **negative** | negative | positive | Sperm cells identified | Semen indicated |
| **negative** | negative | inconclusive | No semen indicated | Semen not found |
| **negative** | positive | negative | p30 detected | Seminal fluid indicated |
| **negative** | positive | positive | p30 detected/Sperm cells identified | Semen identified |
| **negative** | positive | inconclusive | p30 detected | Seminal fluid indicated |
| **positive** | negative | negative | AP indicated/p30 and sperm cells not detected | Semen not found |
| **positive** | negative | positive | AP indicated/ Sperm cells identified | Semen indicated |
| **positive** | negative | inconclusive | AP indicated | Presence of semen could not be determined |
| **positive** | positive | negative | AP indicated/p30 detected | Seminal fluid indicated |
| **positive** | positive | positive | AP indicated/p30 detected/Sperm cells identified | Semen identified |

| | | | | |
|---|---|---|---|---|
| **positive** | positive | inconclusive | AP indicated/p30 detected | Seminal fluid indicated |
| **positive** | inconclusive | negative | AP indicated | Seminal fluid indicated. Presence of semen could not be determined |
| **positive** | inconclusive | positive | AP indicated/Sperm cells identified | Semen indicated |
| **negative** | inconclusive | negative | No semen indicated | Semen not found |
| **negative** | inconclusive | positive | Sperm cells identified | Semen indicated |
| **inconclusive** | inconclusive | negative | AP/p30/sperm cells not detected | Semen not found |
| **inconclusive** | inconclusive | positive | Sperm cells identified | Semen indicated |
| **inconclusive** | inconclusive | inconclusive | AP/p30/sperm cell inconclusive | Presence of semen could not be determined |
| **inconclusive** | negative | negative | inconclusive | Presence of semen could not be determined |
| **inconclusive** | negative | positive | Sperm cells identified | Semen indicated |
| **inconclusive** | negative | inconclusive | p30 not detected | Semen not found |
| **inconclusive** | positive | negative | p30 detected | Seminal fluid indicated |
| **inconclusive** | positive | positive | p30 detected/Sperm cells identified | Semen identified |
| **inconclusive** | positive | inconclusive | p30 detected | Seminal fluid indicated |

For the purposes of the above table, an inconclusive result and not performing a test are considered equivalent.

**Differential Extractions**

| | |
|---|---|
| Enhanced male signal over NS fraction | Semen indicated |
| No enhancement of male signal over NS fraction | Presence of semen could not be determined |
| No male DNA in sperm fraction | No semen indicated |

Enhancement of male signal will be based on the human:male ratio differences between the sperm and non-sperm fractions.

### Saliva

| Phadebas | Conclusion | Inference |
|---|---|---|
| **negative** | amylase not detected | Saliva not found |
| **positive** | amylase detected | Saliva indicated |
| **inconclusive** | inconclusive | Presence of saliva could not be determined |

### Additional information

When providing inferences regarding the presence of a particular body fluid, it is recommended that analysts provide their opinions based upon a comparison of the serological results indicating the body fluid of interest versus the results not indicating the body fluid of interest.

For example, the observed red-brown color of the stain along with the positive phenolphthalein and HemaTrace results are more likely to have resulted from blood being present than if blood was not present.

The DNA Technical Manager will be consulted for any scenarios not listed in the above tables, or where additional guidance is needed.

Method: **Sampling from Microscope Slides for DNA Extraction**

Date: 09/08/2020 Revision Date: 06/29/2023 Approved by: ARD

REAGENTS:

1. XYLENE Fisher Scientific histological grade (or similar)
2. ETHANOL Fisher Scientific (or similar)

EQUIPMENT:

1. -20 FREEZER
2. STERILE SCALPEL BLADE

PROCEDURE:

Note 1: It may be advisable to attempt to perform microscopic examination on slides if there is no information available from a previous microscopic examination, or if no previous microscopic examination has been performed.

Slides from the Medical Examiner's office and wet mount slides will likely require phase contrast microscopic examination as they may not have used any staining techniques.

**Wet mount slides**

1. The slide cover can be removed using a scalpel blade.
2. The slide surface and coverslip can be swabbed with a cotton-tipped swab.
3. Proceed with extraction of the swab as appropriate (either differential extraction or non-differential extraction with DTT).

**Slides mounted with permount**

*Option 1*

1. Place the slide in the freezer for several hours to overnight.

2. Remove the slide from the freezer and on a clean surface use a sterile scalpel blade to pop (or pry) off the coverslip.

3. Once the coverslip is removed scrape the top of the slide and put scrapings into a microcentrifuge tube.

4. A few microliters of xylene (≤100µL) can be used to rinse the material into the microcentrifuge tube. If xylene is not used to wash mounting material into the microcentrifuge, xylene must be added to the microcentrifuge tube to dissolve the permount.

    a. The slide surface and coverslip may also be swabbed using a cotton-tipped swab moistened with xylene.

    b. If the slide/coverslip is swabbed, any liquid cellular material from the swab can be recovered through use of a spin basket. The swab may also be added into the digest when processed for DNA extraction.

Note 2: If a hair is being removed from a mounted slide, after removal of the coverslip the hair should be rinsed in xylene to remove and mounting media, then rinsed with ethanol prior to performing the hair extraction protocol.

5. Centrifuge the material at maximum speed for 10 minutes to pellet the cells.

6. Wash the cell pellet with 500-1000µL ethanol to remove the xylene and centrifuge again for 5 minutes.

7. Remove the supernatant.

8. Optional: residual ethanol may be evaporated though use of a heat block.

9. Proceed with extraction of the cell pellet as appropriate (either differential extraction or non-differential extraction with DTT).

*Option 2*

1. Place the slide in a shallow container and cover with xylene.
2. Soak the slide for several hours to overnight.
3. Remove the slide from the container and on a clean surface use a sterile scalpel blade to remove the coverslip.
4. Proceed with step 3 from Option 1.

REFERENCES

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Method: **General DNA Extraction**

Date: 03/30/2000 Revision Date: 10/21/2023 Approved by: ARD

REAGENTS:

1. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
2. PROTEINASE K, 10 mg/mL
3. DTT (1M DTT, 10mM Sodium Acetate, pH= 5.2)

EQUIPMENT:

1. Eppendorf Thermomixer C
2. Microcentrifuge (capable of 10,000-15,000 x g)

PROCEDURE:

1. Place a portion of the stain, substrate, or swab in a sterile 1.5mL microcentrifuge tube (or a 2.0mL collection tube) and add 500µL of digest buffer and 20µL of proteinase K.

   [The Large Volume protocol on the EZ1, EZ1 Advanced XL, and EZ2 Connect Fx can accommodate up to 900µL of volume in the sample tubes. Analysts may choose to add more or less digest buffer to accommodate larger or smaller substrates as long as the ratio of digest buffer to ProK remains 25:1.]

   [15µL of 1M DTT may be added to aid in the digestion bloodstains. If a tissue sample is embedded in paraffin wax, please refer to reference 2 for the process of removing the wax.]

   [For samples which may contain sperm cells, see Differential DNA Extraction, Differential DNA Extraction using the QIAcube, or Non-Differential DNA Extraction of Sexual Assault Type Evidence]

2. Incubate at 56°C for at least two hours at 900rpm in a thermomixer. Following incubation, the samples can be gently centrifuged to remove moisture from the lids.

3. At this point any samples can be purified using the BioRobot® EZ1, EZ1 Advanced XL, and/or EZ2 Connect Fx. Please refer to the desired protocol for the procedure.

REFERENCES:

1. Cetus Amplitype User Guide 1990
2. Fregeau CJ *et al.* AmpFlSTR Profiler Plus and AmpFlSTR COfiler Analysis of Tissue Stored in GenoFix, a New Tissue Preservation Solution for Mass Disaster DNA Identification. J Forensic Sci 2001; 46(5): 1180-1190.

Method: **Cartridge Case/Cartridge Extraction**

Date: 06/16/2014 Revision Date: 06/09/2025 Approved by: ARD

REAGENTS:

1. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
2. PROTEINASE K, 10 mg/mL (ProK)

EQUIPMENT:

1. Eppendorf Thermomixer C
2. Microcentrifuge (capable of 10,000-15,000 x g)

PROCEDURE:

***It is highly recommended that cartridge cases/cartridges are worked in small batches (6-12 cartridge cases/cartridges) separate from other types of samples.***

1. Place the cartridge case/cartridge in a sterile 5mL tube with the headstamp at the bottom of the tube. Most cartridge cases including some longer rifle cartridge cases (e.g., .223 Remington) should fit into a 5mL tube. For some longer cartridge cases (e.g., .308 Winchester) and many longer *unfired* cartridges, a 15mL or larger tube may be needed.

2. Add 800µL of digest buffer and 32µL of ProK.

   [The Large Volume protocol on the EZ1, EZ1 Advanced XL, and EZ2 Connect Fx can accommodate up to 900µL of volume in the sample tubes. Analysts may choose to add more or less digest buffer to accommodate larger or smaller caliber cartridges/ cartridge cases as long as the ratio of digest buffer to ProK remains 25:1. *The top of the digest buffer/proteinase K should not go over the top of the cartridge.* For example, .357 Magnum cartridge cases typically require twice the normal volume and .223 cartridge cases typically require three times the normal volume. If the total volume of digest buffer and ProK is >900µL, the liquid should be split

into multiple tubes for step 5 below. If a larger volume of digest buffer is used, ensure that the reagent blank has the equivalent volume.]

3. Incubate at 56°C for 30 minutes. The thermomixers have 5mL tube adapters that can be used. The speed of the thermomixers must be reduced to 500rpm or lower during incubation to avoid getting liquid inside the cartridge case. The heated thermomixer lids will not fit onto the thermomixers with the 5mL adapters.

4. Use a pair of forceps or wooden sticks to remove the cartridge case/cartridge from the 5mL tube, taking care to not scratch the outside of the cartridge case/cartridge (tip: place the forceps inside the opening of the cartridge case and place your finger inside the two prongs of the tweezers so that the tension of the tweezers inside the cartridge case allows you to pick up the cartridge case) and swab the entire outside of the cartridge case/cartridge with a sterile cotton-tipped swab.

5. Place the swab and remaining digest buffer/ProK solution into a sterile 2.0ml EZ1 sample tube or QIAGEN Lyse&Spin basket tube.

6. Wash the cartridge case/cartridge in PCR grade water to remove any digest buffer before repacking (the original 5mL tube from the lysis step can be used).

7. Incubate at 56°C for at least 90 minutes. (*Use of the thermomixers is highly recommended*)

8. At this point any samples can be purified using the BioRobot® EZ1, EZ1 Advanced XL, and/or EZ2 Connect Fx. Please refer to the desired protocol for the procedure.

REFERENCES:

1. Cetus Amplitype User Guide 1990
2. De Knijff et al., A sensitive method to extract DNA from biological traces present on ammunition for the purpose of genetic profiling. Int J Leg Med (2011) 125:597-602

Method: **DNA Extraction with Microscopic Examination**

Date: 03/30/2000 Revision Date: 10/21/2023 Approved by: ARD

REAGENTS:

1. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
2. PROTEINASE K, 10 mg/mL (ProK)

EQUIPMENT:

1. Microcentrifuge (capable of 10,000-15,000 x g)
2. Eppendorf Thermomixer C

PROCEDURE:

Note: This procedure is designed for evidence samples. Reference mouth swabs do not require microscopic analysis (see "BioRobot EZ1 DNA Purification of Reference Samples" procedure).

1. Place a portion of the substrate or swab in a 1.5mL microcentrifuge tube and add 0.75 to 1mL of sterile PCR grade water.

2. Incubate the sample at room temperature for at least 30 minutes with occasional vortexing.

3. Remove the substrate and place into a second sterile 1.5mL microcentrifuge tube and retain.

4. Centrifuge the sample in a microcentrifuge for 3-5 minutes at maximum speed.

5. Without disturbing the pellet, remove and discard all but 50-100µL of the supernatant. Resuspend the cell pellet.

6. A portion of the cells pellet can now be removed for microscopic examination.

Refer to the procedure "Identification of Cellular Material Using Xmas Tree Staining."

7. Add 500µL of digest buffer and 20µL of proteinase K. [The original substrate can be added back to increase the DNA yield from the sample].

   [The Large Volume protocol on the EZ1, EZ1 Advanced XL, and EZ2 Connect Fx can accommodate up to 900µL of volume in the sample tubes. Analysts may choose to add more or less digest buffer to accommodate larger or smaller substrates as long as the ratio of digest buffer to ProK remains 25:1.]

8. Incubate the sample at 56°C for at least 2 hours at 900rpm in a thermomixer.

9. At this point any samples can be purified using the BioRobot® EZ1, EZ1 Advanced XL, and/or EZ2 Connect Fx. Please refer to the desired protocol for the procedure.

REFERENCES:
Cetus Amplitype User Guide 1990

Method: **Differential DNA Extraction**

Date: 06/29/2000 Revision Date: 10/21/2023 Approved by: ARD

REAGENTS:

1. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
2. PROTEINASE K, 10 mg/mL
3. DTT (1M DTT, 10mM Sodium Acetate, pH= 5.2)
4. Sterile PCR grade water.

EQUIPMENT:

1. Microcentrifuge (capable of 10,000-15,000 x g)
2. Eppendorf Thermomixer C

PROCEDURE:

Note: Microscopic analysis of samples is optional and will be scenario-dependent. Observations obtained from microscopic analyses may provide information that may be useful in some instances (e.g., male-on male assaults). The general steps for preparing microscope slides is present in an Annex A at the end of the protocol. The protocol will identify the points when microscopic analysis may be performed and will direct the analyst to the Annex.

1. Place the sample(s) (swab or other substrate) to be extracted in a sterile 1.5mL tube or 2mL Lyse & Spin tube(s).

***Optional water-wash microscopy. If no dH2O microscopy is performed, skip to step 3***

2. If water-wash microscopic examination is desired, add 1mL of sterile PCR grade water and incubate at room temperature for at least 30 minutes vortexing the tube a couple of times during the incubation to aid in removing the cells from the substrate. Refer to Annex A for microscope slide preparation.

***Start of protocol without-water wash microscopic examination.***

3. Add 500µL of digest buffer and 20µL of proteinase K to the substrate and/or resuspended cell pellet. If the substrate is especially large it may require additional digest buffer and proteinase K (e.g., 900µL digest buffer and 36µL proteinase K[i]) or the use of multiple tubes.

4. Incubate at 56°C for 1-2 hours[ii] to lyse non-sperm cells at 900rpm in a thermomixer.

5. Remove the substrate and recover the liquid using a spin basket (do not use the Lyse & Spin basket), centrifuging 3-5 minutes at 10,000 x g (maximum speed). The liquid can be recovered using either a new sterile spin basket tube or the Lyse & Spin tube. If necessary, attempt to remove additional liquid from the substrate via a second centrifugation step to maximize cellular recovery from the sample. If separate tubes were used to recover liquid, combine the recovered liquids to a single tube. [Note: the substrate may be discarded at this point.]

6. Microcentrifuge the sample for 3-5 minutes at maximum speed. Without disturbing the cell pellet, remove all but 30-100µL of the supernatant and place in a sterile 2.0mL sample tube for purification. This supernatant contains DNA from any non-sperm cells present in the sample. [The reagent blank supernatant will act as a control for the non-sperm fraction] The sample can be put aside until DNA purification.

7. If step 4 was incubated for less than 2 hours, perform a 2nd digest: add 400-500µL of digest buffer (depending on how much volume remains after step 6) and 20µL of proteinase K to cell pellet. Incubate at 56°C for as much time as needed to ensure 2-4 hour total non-sperm cell lysis time (see end note ii) at 900rpm in a thermomixer. If a second digest is performed, repeat step 6, but discard the supernatant from the second digest.

Note: An analyst may elect to skip directly to step 10 in order to immediately perform the microscopic analysis. Steps 8 and 9 should be performed prior to step 12 if sperm cells are observed in the samples. In this instance steps 10 and 11 do not need to be repeated.

8. For microscopic examination see Annex A step 3. Wash the pellet as follows: Resuspend the pellet in 0.5-1.0mL digest buffer and vortex briefly. Spin the sample in a microcentrifuge for 5 minutes at maximum speed. Remove all but 30-100µL and discard.

9. Repeat wash step 8 a total of three or four times.

10. Resuspend the pellet in 0.5-1.0mL sterile PCR grade water and vortex briefly. Spin the cells in a microcentrifuge for five minutes at maximum speed. Remove and discard all but 50-100µL of the supernatant. Resuspend the cells in the remaining supernatant.

11. For microscopic examination see Annex A step 3.

12. Add 400-500µL digest buffer, 20µL 1M DTT and 15-20µL of 10 mg/mL proteinase K solution to the re-suspended sperm cell pellet.

13. Incubate for at least 2 hours at 56°C, or alternatively for at least 15 minutes at 70°C.

14. At this point any samples (either non-sperm or sperm fraction) can be purified using the BioRobot® EZ1, EZ1 Advanced XL, and/or EZ2 Connect Fx. Please refer to the desired protocol for the procedure.

## Annex A: Steps for Preparing Microscope slides (if applicable).

1. Remove the substrate from the water or digest buffer mixture with a pipette tip or plain wood applicator and transfer it to the spin basket of a secondary sterile microfuge tube. Attempt to remove additional liquid from the substrate via centrifugation (3-5 minutes at 10,000 x g (maximum speed) at room temperature) to maximize cellular recovery from the sample prior to microscopic analysis. If only a water wash was performed, retain the substrate for later testing. Transfer any liquid obtained to the original sample tube.

2. Spin the primary sample tube in a microcentrifuge for 3-5 minutes at 10,000 x g (maximum speed) at room temperature. Without disturbing the cell

pellet, remove and discard all but 50-100µL of the supernatant.

3. If the sample was in sterile PCR grade water, proceed with step 4. If the sample was in digest buffer, resuspend the cell pellet in at least 500µL of sterile PCR grade water and repeat step 2 and the first part of step 3.

4. Vortex the cell pellet to mix. Place 3-5µL of the sample (depending on volume of cell pellet) onto a well of a slide (different amounts may be used). Place the slide in an incubator or on a heat block to fix any cells to the slide.

5. Proceed to the procedure "Identification of Cellular Material Using Xmas Tree Staining". Microscopic examination of slides is considered optional and will be scenario-dependent.

If only a water-wash has been performed, the substrate should be placed back in the primary tube and the protocol can be continued from the previous point.

[i]*Other volumes of digest buffer and proteinase K must be in 25:1 proportions (i.e., 1µL of proteinase K for every 25µL digest buffer).*
[ii] *Option: The total incubation time for non-sperm cell lysis must be at least 2 hours. Incubation time may be longer to ensure lysing of the non-sperm cells. Proteinase K activity diminishes after approximately 2 hours. Non-sperm lysis times in excess of 4 hours are generally unnecessary.*

<u>REFERENCES</u>: Cetus Amplitype User Guide 1990

Method: **Differential DNA Extraction using the QIAcube or QIAcube Connect**

Date: 06/25/2018 Revision Date: 06/09/2025 Approved by: ARD

REAGENTS:

1. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
2. DTT (1M DTT, 10mM Sodium Acetate, pH= 5.2)
3. PROTEINASE K, 10 mg/mL
4. Sterile PCR grade water.

EQUIPMENT:

1. QIAcube or QIAcube Connect
2. QIAGEN QIAcube Tubes 1.5mL Tubes (QIAGEN Cat. 1050875)
3. 30mL Reagent Bottles (QIAGEN Cat. 990393)
4. 1000µL Wide-bore Tips (QIAGEN Cat. 990452)
5. Rotor Adapters (QIAGEN Cat. 990394)
6. Eppendorf Thermomixer C

PROCEDURE:

Note: Microscopic analysis of samples is optional and will be scenario-dependent. Observations obtained from microscopic analyses may provide useful information in some scenarios (e.g., male-on male assaults). This protocol has many points within it where microscopic examination could be performed. The general steps for preparing microscope slides is present in an Annex A at the end of the protocol. The protocol will identify the points when microscopic analysis may be performed and will direct the analyst to the Annex.

1. Place the sample(s) (swab or other substrate) to be extracted into appropriately labeled sterile 1.5mL tube(s) or 2mL Lyse & Spin tube(s).

***Optional water-wash microscopy. If no dH2O microscopy is performed, skip to step 3.***

2. If water-wash microscopic examination is desired, add 1mL of sterile PCR

grade water and incubate at room temperature for at least 30 minutes vortexing the tube a couple of times during the incubation to aid in removing the cells from the substrate. Refer to Annex A for microscope slide preparation.

***Start of protocol without-water wash microscopic examination.***

3. Add 500µL of digest buffer and 20µL of proteinase K to the substrate and/or resuspended cell pellet. If the substrate is especially large it may require additional digest buffer and proteinase K (e.g., 900µL digest buffer and 36µL proteinase K[i]) or the use of multiple tubes. Larger volumes than 520µL will require a second round of digestion in step 7.

4. Incubate at 56°C for 1-2 hours[ii] to lyse non-sperm cells at 900rpm in a thermomixer. If incubation is less than 2 hours, a 2nd digest must be performed in step 7.

5. Remove the substrate and recover the liquid using a spin basket (do not use the Lyse & Spin basket), centrifuging 3-5 minutes at 10,000 x g (maximum speed). The liquid can be recovered using either a new sterile spin basket tube or the Lyse & Spin tube. If necessary, attempt to remove additional liquid from the substrate via a second centrifugation step to maximize cellular recovery from the sample. If separate tubes were used to recover liquid, combine the recovered liquids to a single tube. [Note: the substrate may be discarded at this point.]

6. If step 4 was incubated for at least two hours, if the total volume of digest buffer and proteinase K is 520µL, if microscopy will <u>not</u> be performed, and if the QIAcube (Connect) will be used to separate the non-sperm fractions, skip to step 10. Otherwise, remove and retain the non-sperm fractions:

   Microcentrifuge the sample for 3-5 minutes at maximum speed. Without disturbing the cell pellet, remove all but 30-100µL of the supernatant and place in a sterile 2.0mL sample tube for purification. This supernatant contains DNA from any non-sperm cells present in the sample. [The reagent blank supernatant will act as a control for the non-sperm fraction]. The sample can be put aside until DNA purification.

7. If step 4 was incubated for less than 2 hours, perform a 2nd digest: add 400uL-500 of digest buffer (depending on how much volume remains after step 6) and 20µL of 10mg/mL proteinase K to cell pellet. Incubate at 56°C for as much time as needed to ensure 2-4 hours total non-sperm cell lysis time (see end note ii) at 900rpm in a thermomixer.

8. For microscopic examination see Annex A step 3. Ensure that there is at least 520µL of liquid in the sample tubes prior to using the QIAcube(s). Volumes larger than 520µL will reduce the effectiveness of the QIAcube wash steps.

9. After the incubation from step 7 is complete, remove the substrate (if not already removed) and spin down the original samples briefly to remove condensation from the tube caps.

10. Select the separation protocol on the QIAcube or QIAcube Connect. To navigate through the menus on the QIAcube, use the arrows on the touch screen and press "Select" or "Next" if necessary. To navigate through the menus on the QIAcube Connect, select the appropriate choice on the touch screen and press "Next" if necessary. The QIAcube Connect may require the analyst to log into the instrument. Use the appropriate account and password located on the instrument.

    a. From the main menu, select "DNA".
    b. Select "Pipetting" from the DNA menu.
    c. Select "Epithelial and Sperm Cell" from the Pipetting menu.
    d. Select the desired protocol. There are options which stop after the water washes (no-lysis) and options which will add the lysis buffer to digest the sperm fractions (lysis). The QIAcube Connect has greater flexibility in sample numbers per run than QIAcube. The options and compatible protocols are listed in the table below. **If using multiple instruments, use the same or compatible protocols for all instruments and run them concurrently (not consecutively on the same or separate instruments).**
    e. On the QIAcube Connect, select "Next" on the Define Parameters menu and select the appropriate number of samples on the sample number menu.

| Instrument | Samples per instrument | Add lysis buffer* | Select this protocol | Protocol is compatible with |
|---|---|---|---|---|
| QIAcube | 2-6 | Yes | 6 lysis buffer | QIAcube Connect Protocol 1 |
| | 2-6 | No | 6 no lysis buffer | QIAcube Connect Protocol 2 |
| | 7-12 | Yes | 12a lysis buffer | QIAcube Connect Protocol 3A |
| | | | 12b lysis buffer** | QIAcube Connect Protocol 3B |
| | 7-12 | No | 12a no lysis buffer | QIAcube Connect Protocol 4A |
| | | | 12b no lysis buffer** | QIAcube Connect Protocol 4B |
| QIAcube Connect | 2-9 | Yes | Protocol 1 | QIAcube 6 lysis buffer |
| | 2-10 | No | Protocol 2 | QIAcube 6 no lysis buffer |
| | 2-12 | Yes | Protocol 3A | QIAcube 12a lysis buffer |
| | | | Protocol 3B*** | QIAcube 12b lysis buffer |
| | 2-12 | No | Protocol 4A | QIAcube 12a no lysis buffer |
| | | | Protocol 4B*** | QIAcube 12b no lysis buffer |

* If microscopy is to be performed after washes, do not select add lysis buffer options
** 12b options are the 2nd half of corresponding 12a options. Refill tips before run
*** 3B and 4B options are 2nd half of corresponding 3A and 4A options. Refill tips before run

11. Set up QIAcube and/or QIAcube Connect instrument(s) as described in Annex B. The instrument should prompt you for loading.

12. If the samples are not in 1.5 mL Spin tubes or QIAGEN snap cap tubes, transfer them 1.5 mL Spin tube or QIAGEN snap cap tubes. Place each tube containing the lysate into position 3 of the rotor adapters. Bend the cap of each tube back and insert it into the cap holder in position L3 (see Figure 1 below).

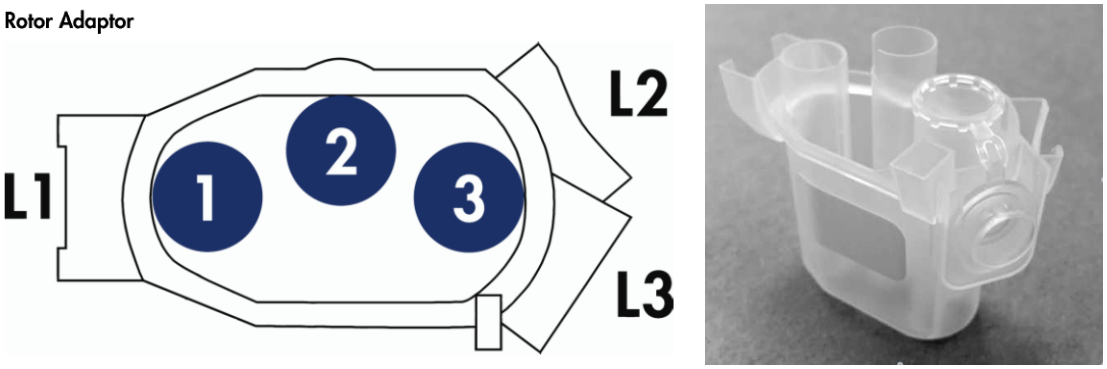



*Figure 1 - QiaCube Rotor Adapter*

The QIAcube Connect recommends swapping the order of steps 13 and 14.

13. Refer to "QIAcube Loading Chart" (Annex C) for correct tube positioning based on sample number and place the rotor adapters containing the sample tube into the QIAcube centrifuge.

14. Next, load the corresponding 2.0mL QIAGEN Lyse and Spin snap cap tube[iii] into the QIAcube (Connect) shaker rack (Figure 2) based on the correct tube positioning from the "QIAcube Loading Chart" (see Annex C). Repeat steps 12 through 14 for all samples. Check to make sure that tubes are pressed all the way down and the tube caps are fitted properly into their holders (see Figure 2). The QIAcube instruments cannot accommodate batch sizes of 1 or 11. If the batch size is 1 or 11 samples, additional blank tube(s) containing water may be placed in a rotor adapter(s) to balance to centrifuge.

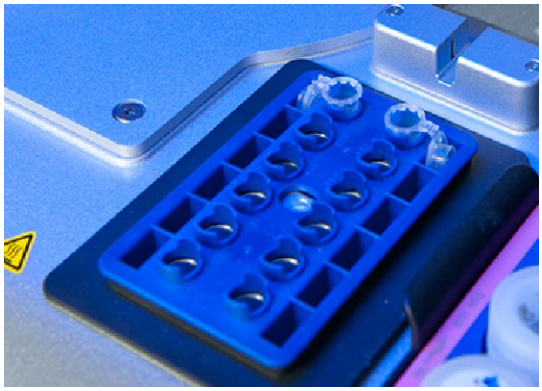


*Figure 2 - QIAcube Shaker Rack*

15. Close the instrument door and select "Start" to start the protocol.

16. At the end of the protocol, if not running a "b" protocol, skip to step 17. If running a "b" protocol, remove and cap the 2.0mL QIAGEN Lyse and Spin snap cap tubes from the QIAcube shaker rack. Repeat steps 10-15, this time selecting the appropriate "b" protocol.

    If non-sperm fractions were <u>not</u> removed in step 6, the tubes from the shaker rack are non-sperm fraction tubes and should be retained and purified. If non-sperm fractions were removed in step 6, the tubes from step 6 should be purified and the tubes from the shaker rack can be discarded.

17. After the protocol is complete, remove and retain the sperm fractions in the 1.5 mL Spin basket or QIAGEN snap-cap tubes originally placed in the rotor adapter. If not already removed, remove the 2.0mL QIAGEN Lyse and Spin tubes from the shaker rack. Verify whether these tubes contain the NS fractions (see note in step 16). These may be purified or discarded at this time as appropriate.

18. If a "Lysis" Protocol was used on the QIAcube, skip to step 17. If a "No Lysis" protocol was selected:

    a. If performing microscopic examination after a "no lysis protocol" see Annex A step 4.
    b. Add 400-500µL digest buffer, 20µL 1M DTT and 20µL of 10 mg/mL proteinase K solution to the re-suspended sperm cell pellet.

19. Incubate the sperm fractions for at least 2 hours at 56°C, or alternatively for at least 15 minutes at 70°C. The total volume of the sperm fractions must be transferred into a 2.0mL EZ1 Sample Tube Lyse & Spin tube prior to purification on the BioRobot EZ1, EZ1 Advanced XL, and/or EZ2 Connect Fx. It is recommended that they be transferred to EZ1 compatible tubes prior to sperm fraction incubation.

20. At this point the sperm and non-sperm fractions may be purified using the BioRobot® EZ1, EZ1 Advanced XL, or EZ2 Connect Fx. Please refer to the desired protocol for the procedure.

## Annex A: Steps for Preparing Microscope slides (if applicable).

1. Remove the substrate from the water or digest buffer mixture with a pipette tip or plain wood applicator and transfer it to the spin basket of a secondary sterile microfuge tube. Attempt to remove additional liquid from the substrate via centrifugation (3-5 minutes at 10,000 x g (maximum speed) at room temperature) to maximize cellular recovery from the sample prior to microscopic analysis. If only a water wash was performed, retain the substrate for later testing. Transfer any liquid obtained to the original sample tube.

2. Spin the primary sample tube in a microcentrifuge for 3-5 minutes at 10,000 x g (maximum speed) at room temperature. Without disturbing the cell pellet, remove and discard all but 50-100µL of the supernatant.

3. If the sample was in sterile PCR grade water, proceed with step 4. If the sample was in digest buffer, resuspend the cell pellet in at least 500µL of sterile PCR grade water and repeat step 2 and the first part of step 3.

4. Vortex the cell pellet to mix. Place 3-5µL of the sample (depending on volume of cell pellet) onto a well of a slide (different amounts may be used). Place the slide in an incubator or on a heat block to fix any cells to the slide.

5. Proceed to the procedure "Identification of Cellular Material Using Xmas Tree Staining". Microscopic examination of slides is considered optional and will be scenario-dependent.

6. If only a water-wash has been performed, the substrate should be placed back in the primary tube and the protocol can be continued from the previous point.

## Annex B: QIAcube Setup.

***Plasticware and QIAcube Setup***

1. Prepare the tubes and reagents required for the QIAcube fraction separation

and washing. Tubes and rotor adapters can be labeled with pre-made labels. For the QIAcube and subsequent EZ1 purification you will need the following tubes/plasticware:

a. 1.5 mL Spin basket tube or QIAGEN snap cap tube – **this is the unseparated sperm cell/non-sperm lysate tube**
b. Rotor adapters – **this is where the tube from a. is placed**
c. 2.0mL QIAGEN Lyse and Spin snap cap tubes – **this is the non-sperm lysate tube** (if labels are used, ensure that the tubes are seated properly and completely prior to starting the protocol)
d. 2.0mL EZ1 Sample Tube – **these are the input tubes used for purification on the EZ1s for the sperm fractions.**
e. Two 1.5 mL QIAGEN EZ1 Elution or Sarstedt screw cap tubes – **these are the elution tubes for the EZ1s for both fractions** (place any labels below the ridges on the side of the tube)

2. QIAcube setup:

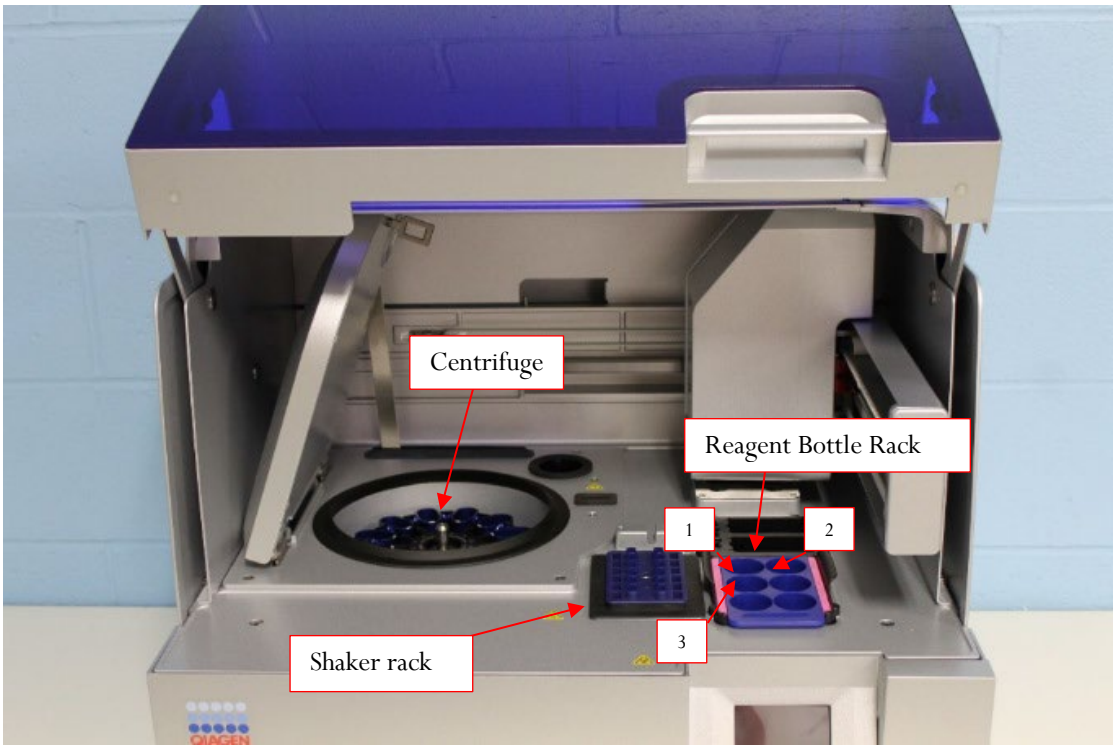



*Figure 3 - QIAcube Deck*

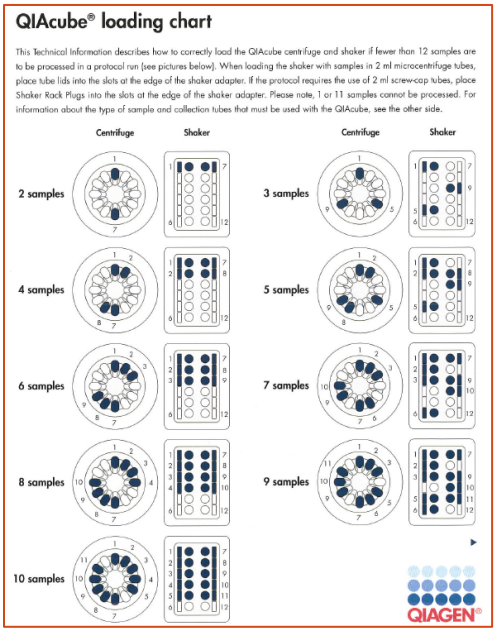
The instruments should prompt you for loading. The QIAcube Connect recommends step c *before* step b.

a. Check that the waste drawer is empty and the liner is clean (can be wiped with 70% ethanol if needed).
b. Fill the tip racks with wide bore 1000µL Disposable Filter-Tips.
c. Put ~30mL of PCR grade water into a 30mL reagent bottle and place it on the QIAcube (Connect) worktable in Reagent Bottle Rack position 1. If running 12b "no-lysis" protocol on QIAcube or Protocol 3B or 4B on the QIAcube Connect, refill the bottle in position 1 with PCR grade water. If running the 12b "lysis" protocol on the QIAcube, put ~30mL of PCR grade water into a 30mL reagent bottle and place it on the QIAcube worktable in Reagent Bottle Rack position 2.
d. If running a QIAcube (Connect) protocol that will add the sperm lysis buffer, use the table below to prepare the appropriate amount of lysis buffer in a 15mL tube, based on the number of samples on each instrument. Add the lysis buffer for each instrument to 30mL reagent bottles. Care should be taken to avoid creating bubbles in the sperm lysis buffer as QIAcube pipetting can be affected by the presence of bubbles in the reagent bottle.
e. Once the sperm lysis buffer is prepared, seat the 30mL reagent bottle on the QIAcube worktable in Reagent Bottle Rack position 3.

Note: Do not fill the 30mL reagent bottles above the fill line on the side of the bottle.

| Sperm Lysis Buffer reagents | | | |
|---|---|---|---|
| **# samples** | **SDPD digest buffer (µL)** | **Proteinase K (µL)** | **1M DTT (µL)** |
| **2** | 5630 | 225 | 225 |
| **3** | 6130 | 245 | 245 |
| **4** | 6630 | 265 | 265 |
| **5** | 7130 | 285 | 285 |
| **6** | 7630 | 305 | 305 |
| **7** | 8130 | 325 | 325 |
| **8** | 8630 | 345 | 345 |
| **9** | 9130 | 365 | 365 |
| **10** | 9630 | 385 | 385 |
| **12** | 10630 | 425 | 425 |

## Annex C: QIAcube Loading Chart.




***QIAcube Protocol End Notes***

[i]*Other volumes of digest buffer and proteinase K must be in 25:1 proportions (i.e., 1µL of proteinase K for every 25µL digest buffer).*

[ii] *Option: The total incubation time for non-sperm cell lysis must be at least 2 hours. Incubation time may be longer to ensure lysing of the non-sperm cells. Proteinase K activity diminishes after approximately 2 hours. Non-sperm lysis times in excess of 4 hours are generally unnecessary.*

References:

QIAcube User Manual 02/2016

Method: **Non-differential DNA Extraction of Sexual Assault Type Evidence**

Date: 02/17/2023 Revision Date: 01/31/2024 Approved by: ARD

REAGENTS:

1. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
2. PROTEINASE K, 10 mg/mL
3. DTT (1M DTT, 10mM Sodium Acetate, pH= 5.2)

EQUIPMENT:

1. Eppendorf Thermomixer C
2. Microcentrifuge (capable of 10,000-15,000 x g)

PROCEDURE:

1. Place a portion of the stain, substrate, or swab in a sterile 1.5mL microcentrifuge tube (or a 2.0mL collection tube) and add 500µL of digest buffer, 20µL of proteinase K, and 20µL of 1M DTT.

   [The Large Volume protocol on the EZ1, EZ1 Advanced XL, and EZ2 Connect Fx can accommodate up to 900µL of volume in the sample tubes. Analysts may choose to add more or less digest buffer to accommodate larger or smaller substrates as long as the ratios of digest buffer to ProK and digest buffer to DTT remain 25:1.]

2. Incubate at 56°C for at least two hours at 900rpm in a thermomixer. Following incubation, the samples can be gently centrifuged to remove moisture from the lids.

3. At this point any samples can be purified using the BioRobot® EZ1, EZ1 Advanced XL, and/or EZ2 Connect Fx. Please refer to the desired protocol for the procedure.

REFERENCES:

1. Cetus Amplitype User Guide 1990

2. Fregeau CJ *et al.* AmpFlSTR Profiler Plus and AmpFlSTR COfiler Analysis of Tissue Stored in GenoFix, a New Tissue Preservation Solution for Mass Disaster DNA Identification. J Forensic Sci 2001; 46(5): 1180-1190.

Method: **DNA Extraction from Hair**

Date: 03/30/2000 Revision Date: 01/31/2024 Approved by: ARD

REAGENTS:

1. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
2. 10 mg/mL PROTEINASE K
3. DTT (1M DTT, 10mM Sodium Acetate, pH= 5.2)

MATERIALS/ EQUIPMENT:

1. Microcentrifuge (capable of 10,000-15,000 x g)
2. Xylene and 100% ethanol (for mounted hairs)
3. Diamond scribe (for mounted hairs)
4. 50mL sterile disposable plastic tubes
5. Eppendorf Thermomixer C

PROCEDURE:

For mounted hairs, refer to the protocol on sampling from slides.

Hairs should typically be evaluated/examined by the Trace Evidence Unit prior to DNA extraction to confirm that the evidence is a human hair and whether the hair is suitable for DNA analysis. Evidence hairs that will be entirely consumed during DNA analysis must be evaluated/examined by trace prior to consumption.

Reference hairs tested may be pooled and examined together

1. Photograph the hair.

2. Examine the hair under a stereomicroscope/dissecting microscope. Note the possible presence of body fluids on the hair.

3. Wash each evidence hair separately to reduce surface dirt and potential contaminants as follows (pooled reference hairs may be washed as a group):

If DNA testing of possible body fluids on the surface of the hair is not required, fill a sterile 50mL plastic screw-topped tube with sterile PCR grade water and place the hair in the tube using a pair of sterile forceps.

If DNA testing of possible body fluids on the surface of the hair is required, place hair into an appropriately labeled sterile 1.5mL tube, 2mL Lyse & Spin tube, or EZ1 sample tube using a pair of sterile forceps. Wash the hair with 500-800µL of digest buffer. Place the hair in a 50mL plastic screw-topped tube filled with sterile PCR grade water to remove any residual digest buffer. Perform the applicable DNA extraction method on the tube containing the washed possible body fluids.

4. Cut off 0.5cm to 1cm of the proximal (root) end of the hair for digestion. Because the hair may contain cellular material on the surface that may or may not originate from the hair donor, it is advisable to cut off 0.5 to 1cm of the shaft adjacent to the root as a control. The remaining shaft should be retained. Pooled reference hairs may have a shaft control prepared from pooled shaft portions adjacent to the sampled roots. **See the section of the GlobalFiler procedure on amplifying shaft controls.**

5. Place the hair sample and its shaft control into separate sterile 2.0mL EZ1 sample tubes. Add 500µL of digest buffer, 20µL of proteinase K, and 20µL of 1M DTT.

6. Incubate at 56°C for at least 6 hours at 900rpm in a thermomixer.

7. Add an additional 15µL of proteinase K and 20µL 1M DTT to the samples.

8. Incubate at least 6 hours or until the hair is dissolved.

9. At this point any samples can be purified using the BioRobot® EZ1, EZ1 Advanced XL, and/or EZ2 Connect Fx. Please refer to the desired protocol for the procedure.

REFERENCES:

Cetus Amplitype User Guide 1990

Method: **The Processing and DNA Extraction of Bone Samples**

Date: 06/21/2005 Revision Date: 06/29/2023 Approved by: ARD

REAGENTS:

1. LIQUID NITROGEN (16 liters is needed as a minimum) obtained from the 50 gallon tank located in the Trace Evidence section.
2. BLEACH, 10-20% (Sodium Hypochlorite)
3. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
4. PROTEINASE K, 10 mg/mL
5. DTT (1M DTT, 10mM Sodium Acetate, pH= 5.2)
6. AMMONIUM OXALATE (saturated, pH= 3.0)
7. EDTA (0.5M, pH= 8.0)

EQUIPMENT:

1. 6850 Freezer Mill with grinding accessories
2. 50mL plastic sterile tubes

PROCEDURE:
A bone kit has been assembled for the analysts that require the tools needed to process bone samples for casework. The kit includes a vise, clamps to secure the vise to a table, a hacksaw with a supply of blades, sandpaper, and a Dremel™ tool with accessories. It is intended that the vise be used to secure the bone sample while sectioning a portion for analysis.

1. Clean the two metal end plugs, the metal impactor, and the plastic vial with soap and water being careful to remove all of the bone debris. Following this cleaning soak the components in bleach solution for no longer than 30 minutes.

2. Rinse in water and dry all the components (If the metal plugs and impactor are not dried they will rust).

3. Swab all components using a PCR grade water moistened cotton-tipped swab. This swab will serve as a control during the extraction procedure and

should be extracted alongside the bone sample.

4. Remove a portion of the evidence bone (the amount of bone sectioned will depend on the age and condition of the bone as well as the environmental exposure) using a clean hacksaw blade. Clean the bone piece by removing adhering tissue, decaying marrow, and any other debris. Use the provided sandpaper to remove the approximately 1mm of the outermost surfaces of the bone; including the central area where the marrow was (the bone section may need to be cut in half length-wise to accomplish this).

5. Soak the bone sample in bleach solution for approximately 15 minutes. Rinse the bone section twice with PCR grade water.

6. Wrap the bone piece in a latex glove and use a hammer to break the bone into several smaller fragments.

7. Tightly place one steel end plug on the plastic grinding vial. Place bone fragments and metal impactor inside vial. Close the vial with the other metal end plug.

8. Open the freezer mill lid by pressing the Lid Up/Down switch. Lift the metal gate handle up and turn it clockwise to line it up with the slot on the gate. Pull the gate down to insert the vial. Insert the vial, close, and lock the gate (loosely tighten the gate).

9. Wearing insulated gloves and other appropriate protective gear gradually fill the tub with 16 liters of liquid nitrogen. This requires the carboy that is used to transport liquid nitrogen to be filled completely four times.

10. Lower the lid slowly by pressing the Lid Up/Down switch until the coil enters the liquid nitrogen. Pause to allow boiling of the nitrogen around the warm coil. When the boiling subsides, close the lid the rest of the way.

11. When the vapor stream has slacked off (30 seconds or so) check the liquid nitrogen level with the internal sensor by pressing control keys 2 and 3 simultaneously.

12. When you press the RUN key (with sufficient liquid nitrogen in the tub, a

loaded vial in the coil, and the factory settings in place), the Freezer/Mill will perform the following steps:

a. The mill cools for 8 min., while the TIME display counts down the minutes remaining in the pre-cooling period. The T3 indicator light should be on.
b. The first grinding period begins and lasts for 2 minutes, while the TIME display counts down the time remaining in that grinding period and the RUN indicator light is lit. The CYCLE display shows 3.
c. The first cooling period begins and lasts for 2 minutes, while the TIME display counts down and the T2 indicator light is on. The CYCLE display still shows 3.
d. The second grinding/cooling cycle takes place, the same as in steps b and c combined. The TIME display will count down and the appropriate RUN and COOL indicator lights will be on in turn, while the CYCLE display shows 2.
e. The third grinding/cooling cycle is like the second (step d) except that the CYCLE display shows 1.
f. At the end of the third grinding cycle, the TIME display will flash END for several seconds and then the control panel will return to Program Mode, exhibiting the same parameters as when the mill was first started.

13. Wearing protective gear, raise the lid by pressing the Lid Up/Down switch and remove the vial by opening the locked gate.

14. Fit the metal end plug extractor over one end of the vial and turn knob clockwise several turns until the screw engages the metal end plug. Squeeze the lever, then turn the knob clockwise and repeat until the end plug is removed. (If the screw locks up wait briefly for vial to warm up before attempting to open again.)

15. When the end-plug is removed from the vial, empty the contents of the vial into a suitable container. Perhaps the easiest means of gathering the ground bone is first to pour the contents of the vial into a disposable plastic weigh boat and then in turn transfer the contents to one, or several, 5ml plastic tubes.

16. Clean the two metal end plugs, the metal impactor, and the plastic vial with soap and water being careful to remove all of the bone debris. Following this cleaning soak the components in 10% bleach solution for no longer than 30 minutes.

17. Rinse in water and dry all the components (If the metal plugs and impactor are not dried they will rust).

18. If running another bone sample, ensure the liquid nitrogen level is appropriate and proceed.

19. After the milling of the bone is complete (refer to 6850 Freezer Mill Procedure), pour the ground bone into a clean weigh boat and then transfer the ground bone into a 5mL plastic tube (or multiple 5mL tubes depending on volume of bone powder).

20. Decalcifying is accomplished by adding 4mL of 0.5M EDTA, pH = 8.0 to the 5mL plastic tube containing the ground bone. Place the tube on a rocking shaker overnight and the following morning spin the tube in the QIAcube at approximately 3000xg for 5 minutes.

    Instructions for centrifuging using the QIAcube:

    Open the QIAcube lid and remove the number of centrifuge buckets from the rotor required to spin the bone samples and balance the rotor. The 5mL tubes should be secured in the buckets using parafilm. Place the buckets back in the rotor.

    On the QIAcube select the Tools menu. Use the up/down arrow buttons to navigate to “Centrifuge”. Press “Select”. The total time centrifuged should be 5 minutes at 3000xg. If the settings are different, press the “Edit” button. Use the up/down arrow buttons to move between the Time1, Speed 1, Time 2, Speed 2, and Acceleration options. Ensure the total time between the Time 1 and Time 2 options equals 5 minutes. Ensure the Speed 1 and Speed 2 (if used) are set to 3000xg. The Acceleration option should be set to 5. Press “Save”. Press “Back”. Check the settings to ensure they are correct. If correct, press “Start”.

    Transfer the supernatant off into another 5mL plastic tube. Save this tube for step #21. Add 4mL of 0.5M EDTA to the bone powder and resuspend the pellet. Place the tube on the rocking shaker. The 0.5M EDTA should be changed at least once per day until such time as the decalcification process is complete.

21. The decalcification process should be monitored by the addition of saturated ammonium oxalate pH= 3.0, to the decanted supernatants. The addition of the ammonium oxalate to the decanted supernatant will produce a cloudy precipitate if calcium is present. If the supernatant remains clear after the addition of ammonium oxalate the decalcification is complete. The supernatant can be discarded.

22. Following decalcification, wash the bone pellet three times by adding 4mL of sterile PCR grade water, resuspending the pellet and centrifuge the sample in the QIAcube (see above for instructions) for 5 minutes at approximately 3000xg. Discard the supernatant.

    The QIAcube rotor should be removed and cleaned. The area the rotor was removed from within the QIAcube should also be cleaned. No bleach should be used on the rotor, buckets, or QIAcube surfaces.

23. To the bone powder pellet (after decalcification), add 450µL of extraction buffer, 150µL of 10 mg/mL proteinase K, and 75µL of 1M DTT and mix. Incubate the tube at 56°C for at least 4 hours.

24. To the initial incubation add another 150µL of 10 mg/mL proteinase K and 75µL of 1M DTT. Incubate the tube at 56°C at least 6 hours.

Please refer to the BioRobot® EZ1 DNA, EZ1 Advanced XL, or EZ2 Connect Fx purification procedure for steps on purifying the DNA from the bone sample(s).

25. If needed, the purified DNA sample can now be concentrated. Please refer to the protocol for the use of the Micorcon-100 centrifugal filter devices (Concentration Using Microcon YM-100 Centrifugal Filter Devices) to concentrate the purified samples.

REFERENCES:

Hochmeister MN *et al.* "Typing of Deoxyribonucleic Acid (DNA) Extracted from Compact Bone from Human Remains. J Forensic Sci. 1991 Nov;36(6):1649-61.

Method: **BioRobot® EZ1, EZ1 Advanced XL, or EZ2® Connect Fx DNA Purification of Evidence Samples**

Date: 01/01/2007 Revision Date: 06/09/2025 Approved by: ARD

**If using multiple purification instruments (EZ1, EZ1Advanced XL, and EZ2 Connect Fx) all instruments must be run concurrently (not consecutively)**

REAGENTS:

BioRobot® EZ1 DNA Investigator reagent cartridges. The cartridges contain all reagents required for the DNA extraction and purification of samples on the BioRobot® EZ1. The reagents in the cartridges includes guanidine thiocyanate/guanidine hydrochloride, the paramagnetic silica coated beads, ethanol, TE buffer, and sterile water.

MATERIALS/EQUIPMENT:

1. EZ1 DNA Investigator kit (QIAGEN Catalogue #952034)

2. BioRobot® EZ1, BioRobot® EZ1 Advanced XL, or EZ2® Connect Fx

PROCEDURE:

1. Remove the substrate (if applicable), and transfer the liquid digest to the EZ1 sample tubes provided in the EZ1 DNA Investigator kit (alternatively, the 2.0ml collection tube can be used in the instrument).

   If the 2.0mL collection tubes are used, centrifuge the tubes at maximum speed to collect the lysate.

**The following steps deal with the set-up and operation of the BioRobot® EZ1 and EZ1 Advanced XL.**

2. If applicable, insert the DNA Investigator v1.2 (EZ1) or EZ1 DNA Investigator Advanced XL v1.1 (EZ1 Advanced XL) protocol card into the card slot on the

appropriate instrument and turn the instrument on.

3. If the protocols are not displayed, press "START" to display the protocols menu. On the EZ1 Advanced XL select "ESC" when prompted to create a report file. If a maintenance reminder displays on the EZ1 Advanced XL, select the appropriate number before pressing "START".

4. Select the number for the desired protocol. The Large Volume protocol should be selected if evidence samples are being extracted.

5. Select the number for the desired elution buffer (TE or water).

6. Select the number for the desired elution volume based on the expected yield; 40, 50, 100, and 200µL are options. Note that the elution volume will be the same for all samples on the same BioRobot® EZ1 or EZ1 Advanced XL.

7. Review the selections from steps 4-6. If any are incorrect, select "ESC" to return to step 4. If the selections from steps 4-6 were correct, press any other key.

8. The text in the LCD prompts the loading of the BioRobot® EZ1 or EZ1 Advanced XL. As you complete steps 19-24, below, press any key (other than "ESC") to proceed through the text displayed in the LCD.

**The following steps deal with the set-up and operation of the EZ2 Connect Fx.**

9. Turn the instrument on. It may take a few moments to load the software.

10. If necessary, log into the software by entering the User ID and Password for the instrument using the touch screen on the front of the instrument. Select the data entry sections for User ID or Password and use the barcode scanner to scan the User ID/password on the front of the instrument or manually type them in using the keyboard on the screen. After typing the appropriate User ID or password, select "Accept". After typing both, select "Log in".

11. If a shortcut is present in the shortcut pane for the large volume protocol with the desired eluent (TE or water) and elution volume (40, 50, 100, or 200µL), select it from the shortcut pane, then select "Edit sample positions"

from the "Review samples" window and skip to step 16.

12. If a shortcut is not present in the shortcut pane for the large volume protocol with the desired eluent (TE or water) and elution volume (40, 50, 100, or 200µL), select "DNA" from the "Applications" pane on the "Setup" tab.

13. Select "DNA Investigator Kit" from the "Select kit" pane and select "Next".

14. Select the desired protocol from the "Select Protocol" pane. The "DNA Investigator Large Volume" protocol should be selected if evidence samples are being extracted. Select "Next".

15. Select the appropriate drop-down lists on the Define parameters pane to select the desired Elution buffer (TE or water), select "Tip rack" for the Rack type, and select the desired elution volume based on the expected yield; 40, 50, 100, and 200µL are acceptable options from the list. Note that the elution volume will be the same for all samples on the same EZ2 Connect Fx. Select "Next".

16. Select the sample positions that will be used by the instrument. These can be selected or de-selected individually, or the slider at the bottom of the menu can be changed to select or de-select all samples. After all applicable sample positions are selected, select "Next".

17. On the "Enter sample IDs" pane, select "Generate missing sample IDs" to fill in Sample IDs, if necessary. If entering the specific sample information (e.g., case number and barcode) and/or a note for a sample is desired, the sample ID section and/or note for each position number can be selected and the Sample ID or note can be manually entered. Select "Next".

18. Select "Next" to proceed through the loading prompts for the EZ2 Connect Fx as you complete steps 19-24, below.

The following steps deal with the loading of samples and reagents into the BioRobot® EZ1, EZ1 Advanced XL, or EZ2 Connect Fx.

19. Invert the reagent cartridges (1-6 per extraction run per instrument on the

EZ1, 1-14 per extraction run on the EZ1 Advanced XL, or 1-24 per extraction run on the EZ2 Connect Fx) to mix and then tap the cartridges to deposit the reagents to the bottom of their wells. Insert the appropriate number of reagent cartridges in the desired positions of the cartridge rack(s). Additional samples can be accommodated in additional instruments.

20. Load the cartridge rack(s) (with cartridges) into the appropriate instrument(s). Ensure that the reagent cartridges are seated properly in the cartridge rack(s) and that each cartridge rack is seated properly in its instrument.

Steps 21-23 can be performed in an alternate order as long as each column with a sample tube has a reagent cartridge, tip holder with a filter-tip, and an elution tube corresponding to the sample.

21. Load uncapped, appropriately labeled elution tubes into row 1/row D (front row) of the tip rack(s).

22. Load tip holders containing the filter-tips into row 2/row C of the tip rack(s) in each position with a corresponding reagent cartridge.

23. Load opened sample or collection tubes (from Step 1.) into row 4/row A (back row) of the tip rack(s) in each position with a corresponding reagent cartridge and tip holder/filter-tip. Check to ensure that the sample tubes are in the positions corresponding to the matching elution tubes so that no sample mix-ups occur.

24. Review the loading of the instrument/run setup overview for accuracy. The EZ2 Connect Fx has an optional (approximately 4-6 minute) load check to verify that all components are in the appropriate columns of the instrument. Do not perform the load check if samples are in lyse and spin tubes. If load check is not desired, select "Skip load check".

25. Close the instrument door/hood and select "START"/"Start" to start the extraction protocol. If a load check was performed on the EZ2 Connect Fx, select "Start Run" when successfully completed.

26. When the protocol ends, the screen displays "Protocol Finished".

27. Open the workstation door/hood. Remove and cap the elution tubes containing your purified DNA. Remove and discard the sample tubes, tip holders, filter tips, and used reagent cartridges in their appropriate waste containers.

28. If you intend to run another extraction protocol, press any key on the BioRobot® EZ1 or EZ1 Advanced XL to perform cleaning/maintenance and press any key return to step 3. The EZ2 Connect Fx provides instructions for cleaning and maintenance. Select the check box and indicate when finished.

The BioRobot® EZ1, EZ1 Advanced XL, and EZ2 Connect Fx can be cleaned by briefly wiping the tip racks and cartridge racks in a solution of 70-95% ETOH. The BioRobot® EZ1, EZ1 Advanced XL, and EZ2 Connect Fx components may also be cleaned using a diluted neutral soap followed by water. The UV cross linker may be used to decontaminate the BioRobot® EZ1 components. The EZ1 Advanced XL and EZ2 Connect Fx have built in UV cross linkers that can be used to decontaminate the instruments for between 20 and 60 minutes. The UV decontamination can be selected on the EZ2 Connect FX from the "Maintenance" tab. The piercing units of the EZ2 Connect Fx should be wiped with an ETOH solution after use.

Unless another extraction protocol is being performed the BioRobot® EZ1, EZ1 Advanced XL, or EZ2 Connect Fx may be switched "OFF".

<u>REFERENCES:</u>

1. BioRobot® EZ1 Genomic DNA Kit Handbook. QIAGEN February 2003
2. Magtration® System 6GC Operation Manual Version 1.1. Precision System Science Co. 2002.
3. Montpetit SA, Fitch IT, O'Donnell PT. A simple automated instrument for DNA extraction in forensic casework, J Forensic Sci. 2005 May;50(3):555-63.
4. Kishor R, *et al.* Optimization of DNA extraction from low yield and degraded samples using the BioRobot EZ1 and BioRobot M48. J Forensic Sci. 2006 Vol. 51(5): 1055.
5. EZ2 Connect and EZ2 Connect Fx User Manual. QIAGEN

Method: **DNA Extraction from Reference Samples Using the BioRobot® EZ1, EZ1 Advanced XL, or EZ2® Connect Fx**

Date: 06/02/2003 Revised: 06/09/2025 Approved by: ARD

**If using multiple purification instruments (EZ1, EZ1Advanced XL, and EZ2 Connect Fx) all instruments must be run concurrently (not consecutively)**

REAGENTS:

1. DIGEST BUFFER (Teknova Org Sperm Wash Buffer)
2. PROTEINASE K, 10 mg/mL
3. BioRobot EZ1 DNA Investigator reagent cartridges.
   The cartridges contain all reagents required for the DNA extraction and purification of samples on the BioRobot EZ1. The reagent in the cartridges included guanidine thiocyanate/guanidine hydrochloride, the paramagnetic silica coated beads, ethanol, TE buffer, and sterile water.

MATERIALS/EQUIPMENT:

1. EZ1 DNA Investigator kit (QIAGEN Catalogue #952034)
2. BioRobot® EZ1 or BioRobot® EZ1 Advanced XL

PROCEDURE:

1. To a sterile 1.5mL tube or to a 2.0ml collection tube add 190µL of digest buffer, 10µL of 10mg/mL proteinase K and the appropriate amount of the reference sample (bloodstain/liquid blood/reference mouth swab).

2. Mix gently and incubate at 56°C for at least 30 minutes. During the incubation, the robot may be set-up for extraction.

3. Remove the substrate (if applicable) and transfer the liquid digest to the EZ1 sample tubes provided in the EZ1 DNA Extraction kit. If the 2.0mL collection tubes were used, centrifuge them at maximum speed for 5min to collect the lysate.

**The following steps deal with the set-up and operation of the BioRobot® EZ1 and EZ1 Advanced XL.**

4. If applicable, insert the DNA Investigator v1.2 (EZ1) or EZ1 DNA Investigator Advanced XL v1.1 (EZ1 Advanced XL) protocol card into the card slot on the appropriate instrument and turn the instrument on. Note that the elution volume will be the same for all samples on the same BioRobot® EZ1 or EZ1 Advanced XL.

5. If the protocols are not displayed, press "START" to display the protocols menu. On the EZ1 Advanced XL select "ESC" when prompted to create a report file. If a maintenance reminder displays on the EZ1 Advanced XL, select the appropriate number before pressing "START".

6. Select the number corresponding to the "Trace", or "Norm" protocol. The Normalization protocols will limit the DNA yield of the samples by employing a limited amount of paramagnetic beads in the extraction.

7. Select the number for the desired elution buffer (TE or water).

8. Select the number for the desired elution volume; 200µL is recommended for most reference sample extractions (additional elution volumes available are 40, 50, and 100µL). Note that the elution volume will be the same for all samples on the same BioRobot® EZ1 or EZ1 Advanced XL.

9. Review the selections from steps 6-8. If any are incorrect, select "ESC" to return to step 6. If the selections from steps 6-8 were correct, press any other key.

10. The text in the LCD prompts the loading of the BioRobot® EZ1 or EZ1 Advanced XL. As you complete steps 20-25 below, press any key (other than ESC to proceed through the text displayed in the LCD.

**The following steps deal with the set-up and operation of the EZ2 Connect Fx.**

11. Turn the instrument on. It may take a few moments to load the software.

12. If necessary, log into the software by entering the User ID and Password for the instrument using the touch screen on the front of the instrument. Select the data entry sections for User ID or Password and use the barcode scanner to scan the User ID/password on the front of the instrument or manually type them in using the keyboard on the screen. After typing the appropriate User ID or password, select "Accept". After typing both, select "Log in".

13. If a shortcut is present in the shortcut pane for the desired combination of protocol (DNA Investigator Trace or DNA Investigator Normalization), eluent (TE or water), and elution volume (40, 50, 100, or 200µL), select it from the shortcut pane, then select "Edit sample positions" from the "Review samples" window and skip to step 18.

14. If a shortcut is not present in the shortcut pane for the desired combination of protocol (DNA Investigator Trace or DNA Investigator Normalization), eluent (TE or water), and elution volume (40, 50, 100, or 200µL), select "DNA" from the "Applications" pane on the "Setup" tab.

15. Select "DNA Investigator Kit" from the "Select kit" pane and select "Next".

16. Select the desired protocol from the "Select Protocol" pane. Select the "DNA Investigator Trace" or "DNA Investigator Normalization" protocol. The Normalization protocol will limit the DNA yield of the samples by employing a limited amount of paramagnetic beads in the extraction. Select "Next".

17. Select the appropriate drop-down lists on the Define parameters pane to select the desired Elution buffer (TE or water), select "Tip rack" for the Rack type, and select the desired elution volume based on the expected yield; 40, 50, 100, and 200µL are acceptable options from the list. Note that 200µL is recommended for most reference sample extractions (additional elution volumes available are 40, 50, and 100µL) and that the elution volume will be the same for all samples on the same EZ2 Connect Fx. Select "Next".

18. Select the sample positions that will be used by the instrument. These can be

selected or de-selected individually, or the slider at the bottom of the menu can be changed to select or de-select all samples. After all applicable sample positions are selected, select "Next".

19. On the "Enter sample IDs" pane, select "Generate missing sample IDs" to fill in Sample IDs, if necessary. If entering the specific sample information (e.g., case number and barcode) and/or a note for a sample is desired, the sample ID section and/or note for each position number can be selected and the Sample ID or note can be manually entered. Select "Next".

20. Select "Next" to proceed through the loading prompts for the EZ2 Connect Fx as you complete steps 21-26, below.

**The following steps deal with the loading of samples and reagents into the BioRobot® EZ1, EZ1 Advanced XL, or EZ2 Connect Fx.**

21. Invert the reagent cartridges (1-6 per extraction run per instrument on the EZ1, 1-14 per extraction run on the EZ1 Advanced XL, or 1-24 per extraction run on the EZ2 Connect Fx) for your extraction to mix, then tap the cartridges to deposit the reagents to the bottom of their wells. Insert the appropriate number of reagent cartridges in the desired positions of the cartridge rack(s). Additional samples can be accommodated in additional instruments.

22. Load the cartridge rack(s) (with cartridges) into the appropriate instrument(s). Ensure that the reagent cartridges are seated properly in the cartridge rack(s) and that each cartridge rack is seated properly in its instrument.

Steps 23-25 can be performed in an alternate order as long as each column with a sample tube has a reagent cartridge, tip holder with a filter-tip, and an elution tube corresponding to the sample.

23. Load uncapped, appropriately labeled elution tubes into row 1/row D (front row) of the tip rack(s).

24. Load tip holders containing the filter-tips into row 2/row C of the tip rack(s)

in each position with a corresponding reagent cartridge.

25. Load opened sample or collection tubes (from Step 3.) into row 4/row A (back row) of the tip rack(s) in each position with a corresponding reagent cartridge and tip holder/filter-tip. Check to ensure that the elution tubes are in the positions corresponding to the matching sample tube so that no sample mix-ups occur.

26. Review the loading of the instrument/run setup overview for accuracy. The EZ2 Connect Fx has an optional (approximately 4-6 minute) load check to verify that all components are in the appropriate columns of the instrument. Do not perform the load check if samples are in lyse and spin tubes. If load check is not desired, select "Skip load check".

27. Close the workstation door/hood and select "START"/ "Start" to start the extraction protocol. If a load check was performed on the EZ2 Connect Fx, select "Start Run" when successfully completed.

28. When the protocol ends, the screen displays "Protocol Finished".

29. Open the workstation door/hood. Remove and cap the elution tubes containing your purified DNA. Remove and discard the sample tubes, tip holders, filter tips, and used reagent cartridges in their appropriate waste containers.

30. If you intend to run another extraction protocol, press any key on the BioRobot® EZ1 or EZ1 Advanced XL to perform cleaning/maintenance and press any key return to step 3. The EZ2 Connect Fx provides instructions for cleaning and maintenance. Select the check box and indicate when finished.

The BioRobot® EZ1, EZ1 Advanced XL, and EZ2 Connect Fx can be cleaned by briefly wiping the tip rack and cartridge rack in a solution of 70-95% ETOH. The BioRobot® EZ1, EZ1 Advanced XL, and EZ2 Connect Fx components may also be cleaned using a diluted neutral soap followed by water. The UV cross linker may be used to decontaminate the BioRobot® EZ1 components. The EZ1 Advanced XL and EZ2 Connect Fx have built in UV cross linkers that can be used to decontaminate the instruments for between 20 and 60 minutes.

The UV decontamination can be selected on the EZ2 Connect FX from the "Maintenance" tab. The piercing units of the EZ2 Connect Fx should be wiped with an ETOH solution after use.

Unless another extraction protocol is being performed, the BioRobot® EZ1, EZ1 Advanced XL, or EZ2 Connect Fx may be switched "OFF".

REFERENCES:

1. BioRobot® EZ1 Genomic DNA Kit Handbook. QIAGEN February 2003
2. Magtration® System 6GC Operation Manual Version 1.1. Precision System Science Co. 2002.
3. Montpetit SA, Fitch IT, O'Donnell PT. A simple automated instrument for DNA extraction in forensic casework, J Forensic Sci. 2005 May;50(3):555-63.
4. EZ2 Connect and EZ2 Connect Fx User Manual. QIAGEN

Method: **qPCR with the Quantifiler™ Trio DNA Quantification Kit using the NIST SRM Human DNA Quantitation Standard (Manual setup)**

Date: 11/16/2022 Revision Date: 7/31/2024 Approved by: ARD

REAGENTS:

1. TE Buffer: Teknova TE Buffer Low EDTA (0.1 mM EDTA, pH 7.6)
2. Quantifiler™ Trio DNA Quantification Kit: Applied Biosystems (Ref 4482910)

    A. Quantifiler™ Trio Primer Mix
    B. Quantifiler™ THP PCR Reaction Mix
    C. Quantifiler™ THP DNA Standard
    D. Quantifiler™ Automation Enhancer (contact HID Sales and Support to order this product)

**Before master mix preparation, 1µL of Quantifiler™ Automation Enhancer should be added to each 1000µL PCR Reaction Mix tube** (expiration - 6 months after addition of the Automation Enhancer or the expiration date printed on the kit, whichever comes first). Automation Enhancer can prevent bubbles from being introduced during robotic mixing and pipetting. The kit should be labeled with the date that the Automation Enhancer was added.

The components of the kit should be stored at -15 to -25°C upon receipt and stored at 2 to 8°C after initial use. The primer sets contain a light sensitive dye and should be stored protected from light. The expiration date is printed on the kit.

MATERIALS:

1. MicroAmp ® Optical 96-Well Reaction Plate: Applied Biosystems (P/N N8010560)

2. MicroAmp™ Optical Adhesive Film: Applied Biosystems (P/N 4311971)

3. MicroAmp® Splash Free 96-Well Base: Applied Biosystems (P/N 4312063)

EQUIPMENT:

1. QuantStudio™ 5 Real-Time PCR Instrument: Applied Biosystems

PROCEDURE:

1. If necessary, thaw the components of Quantifiler™ Trio DNA Quantification Kit and add 1µL of Quantifiler™ Automation Enhancer to each 1000µL PCR Reaction Mix tube. The kit should be labeled with the date that the Automation Enhancer was added. After initial use, the kit should be stored refrigerated (2 to 8°C).

2. Vortex the Quantifiler™ Trio Primer Mix, Quantifiler™ THP PCR Reaction Mix, and Quantifiler™ THP DNA Standard for at least 3-5 seconds. Spin the tubes briefly in a microcentrifuge to remove any liquid from the caps.

3. Prepare a 1/10 and a 1/50 dilution of the Quantifiler™ THP DNA Standard in TE buffer. These dilutions will serve as calibrators for the quantitation assay.

4. Determine the total number of samples to be amplified including the set of DNA calibrators and one qPCR blank. Create a master mix by combining the following volumes of reagents into a 2ml sample tube:

| | | |
|---|---|---|
| 8µL of Quantifiler™ Trio Primer Mix | x | # of Samples |
| 10µL of Quantifiler™ THP PCR Reaction Mix | x | # of Samples |

   Note: Additional master mix (~10% suggested) should be created to allow for pipetting error.

5. Vortex the master mix at medium speed for at least 5 seconds and spin the tube briefly in a microcentrifuge to remove any liquid from the cap.

6. With the 96-well optical reaction plate in a support base dispense 18µL of the master mix into each sample well.

7. Add 2µL or 8µL of sample, 2µL of calibrators, and the appropriate volume of controls (2 or 8uL) to the appropriate wells.

Note: For potential discontinuation of samples, add 8µL of sample to the appropriate wells.

8. Carefully seal the reaction plate with the optical adhesive cover. Use the applicator to ensure the cover forms a smooth airtight seal over the entire 96-well surface.

9. Transport the reaction plate to the amplification area, spin down the plate.

10. Turn on the computer (if necessary) and then the QuantStudio™ 5 instrument.

11. To load the plate, touch "eject icon" on the Instrument Touch Screen (upper right corner) to open the instrument drawer. DO NOT PRESS the instrument drawer to open it.

12. Position the reaction plate in the plate adapter so that well A1 is in the upper left corner and the notched corner is in the upper right corner. Touch "eject icon" on the Instrument Touch Screen (upper right corner) to close the instrument drawer. DO NOT PUSH the instrument drawer to close it.

Instrument Touch Screen, "eject icon" to open/close instrument is circled.


13. Open **Quant Trio Set-up** tab in SIMS, save as Text (Tab delimited) on FB H:\QuantStudio5 Files.

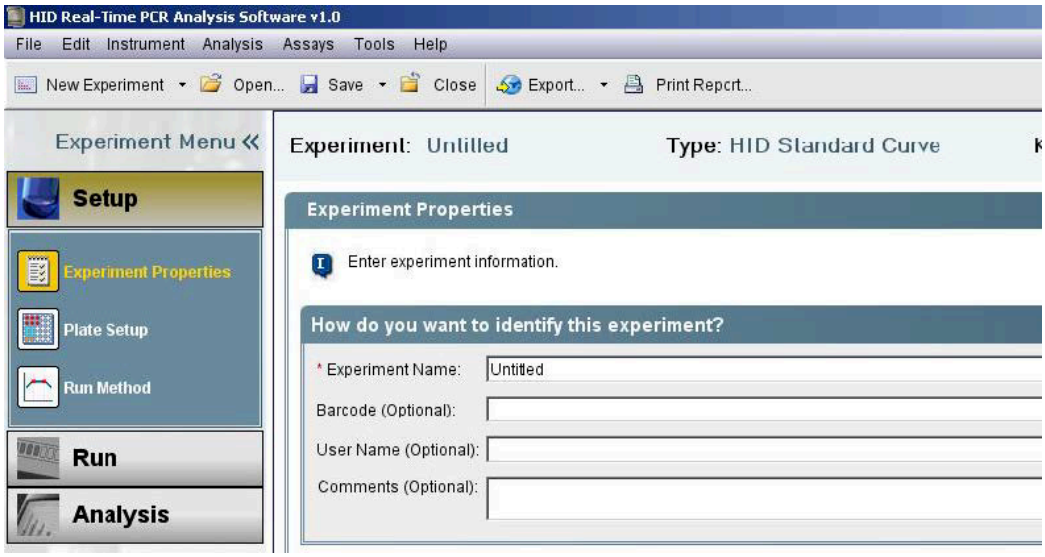
14. Launch the HID Real-Time PCR Analysis Software v1.3.1, Login (User Name – DNA).

15. Choose Quantifiler™ Trio icon (IMPORTANT: If you choose "New Experiment" - click the down arrow next to New Experiment to open the drop-down list and select Quantifiler Trio.

16. On HID Real-Time PCR Analysis Software experiment screen, in the Experiment Menu, select **Setup>Experiment Properties** (on the left): type the experiment name (no special characters are allowed), make sure that the QuantStudio 5 instrument is selected experiment.

    The following parameters are automatically set:
    - Instrument: QuantStudio 5 (96 wells)
    - Experiment Type: Quantitation-HID Standard Curve
    - Reagents: TaqMan Reagents
    - Ramp Speed: Standard (~1 hour to complete a run)

    Use the Experiment Menu at the left of any screen to navigate the software.




17. Import sample text file by selecting **File>Import>** browse for the appropriate file (.txt) that was saved on FB H:\QuantStudio5 Files. Select the file, press Start Import, Yes, OK.

18. In the Experiment Menu, click **Setup>Plate Setup**. Select the **Define Targets and Samples** tab. Sample names will be in the **Define Samples** area on the right side of the pane, make sure that all samples (including calibrators and TE blank) have **Sample type "unknown"**. You can add a sample if needed, click **Add New Sample** and a new line appears in the Sample Name field, type a new sample name (make sure to define sample type) and click **Save Sample**.

19. Experiment Menu: **Setup>Plate Setup**. Select the **Assign Targets and Samples** tab to see the plate layout (**View Plate Layout** tab). If new sample was added, select the sample location in the **Plate Layout** and then find the sample name on the left under **Assign sample(s) to the selection wells** tab and check the box (on the left) next to the sample name.

20. In the Experiment Menu, click **Setup>Run Method** to confirm the automatically set conditions:

    **Holding Stage: 95.0C-2 min**
    **Cycling Stage (40 Cycles):**
    **95.0C - 9 sec**
    **60.0C - 30sec**
    Ramp speeds set to 2.5°C/s
    Reaction volume set to 20 µL

21. Experiment Menu: **Setup>Analysis>Virtual Standard Curve> Add Virtual Standard Curve to Experiment>** Choose **SDPD Virtual Curve> Add Selected Virtual Standard Curve**.

22. Press the green **Start Run** button. From Experiment Menu: Setup, the **Start Run** button is near the upper right corner of the screen. From Experiment Menu: Run, the **Start Run** button is near the upper left corner of the screen. Save as .eds file (on FB H:\QuantStudio5 Files).

23. When the run is done, touch "eject icon" on the QuantStudio™ 5 Touch Screen (upper right corner) to open the instrument drawer. Discard the plate, touch "eject icon" on the Instrument Touch Screen to close the instrument drawer.

24. From Experiment Menu: **Analysis>View Well Table** tab, select **Group by> Well Position (Column)** near the middle of the screen. Ensure all samples and controls in the plate are highlighted. To export data to SIMS, on the main screen (HID Real-Time PCR Analysis Software v1.3.1) **File>Export> Export Properties** tab> Click checkbox next to **Results** (default). Click **Browse** and open Export File Location (on FB H:\QuantStudio5 Files), export data as .xls file. Press **Start Export**. Close the program after the Export.

25. Press "Done" on the QuantStudio 5 instrument and close the HID Real-Time PCR Analysis Software.

26. Open the exported .xls file from step 23. Copy and paste .xls export file data into SIMS **Quant Trio Data** tab (cell A1).

27. View data under **Quant Trio Results** tab in SIMS. The "Quant Trio Results" worksheet should be printed (File>Print or use the print icon) and included in the analytical record.

Assessment of DNA calibrators:

The concentration of the Human DNA standard received with each lot of kit will be determined during the QC process. The values obtained during this QC will determine the expected values for the calibrators.

Values obtained that are significantly outside the expected quantitation values of calibrators (i.e., more than ±0.5 $C_T$) may indicate a problem with the qPCR run. If problems are indicated the quantitation assay should be interpreted with caution and a second assay may be required. If multiple sets of calibrators are run with an assay, values for each set of calibrators should be evaluated.

Assessment of sample quality:

If a sample has a high small autosomal $C_T$ value and a normal IPC $C_T$ value (see **Quant Trio Results** tab for normal and high range) the sample has a low amount of DNA.

If a sample has a low small autosomal $C_T$ value and a normal or slightly high IPC $C_T$ value, the sample has a high amount of DNA. If the sample has a high amount

of human DNA it will often out compete the IPC for amplification components thus decreasing its amplification efficiency.

If a sample has a high small autosomal $C_T$ value and a high IPC $C_T$ value, the sample is likely inhibited. Appropriate troubleshooting measures should be employed with the sample to overcome the potential inhibitors.

To determine the Quality Index, evaluate the Degradation Index in conjunction with the IPC $C_T$ to assess the potential presence of PCR inhibitors and degradation that may have an impact on downstream sample processing

| IPC $C_T$ flag triggered? | Degradation Index | Quality Index interpretation† |
|---|---|---|
| No | <1 | Typically indicates that DNA is not degraded or inhibited. |
| | 1 to 10 | Typically indicates that DNA is slightly to moderately degraded. PCR inhibition is also possible, however not enough to significantly suppress IPC amplification. |
| | >10 or blank (no value) | Typically indicates that DNA is significantly degraded. PCR inhibition is also possible, however not enough to significantly suppress IPC amplification. Values >10 can be obtained from some samples with very low small autosomal quantitation values due to stochastic effects. |
| Yes | <1 | Although theoretically possible, this result is unlikely because PCR inhibitors in sufficient concentration to trigger the IPC $C_T$ flag typically would affect the large autosomal target as well. |
| | >1 or blank (no value) | Typically indicates that the DNA is affected by degradation and/or PCR inhibition. |

† These are general guidelines that may not apply to all samples depending on the inhibitors present, the varying quantity of contributor DNA in mixed samples and the STR kit used.

The relative amount of male and female DNA in a mixture is determined by dividing the total human DNA quantitation (SA) by the male DNA quantitation (Y).

REFERENCE:

Quantifiler™ Trio DNA Quantification Kit User Guide. Applied Biosystems.

QuantStudio™ 5 Real-Time PCR Instrument (for Human Identification) User Guide.

HID RealTime PCR Analysis Software Version 1.3 Manual.

Method: **Quantifiler™ Trio Setup Using the Hamilton Microlab® Nimbus 4**

Date: 11/16/2022 Revision Date: 02/17/2023 Approved by: ARD

REAGENTS:

1. TE Buffer: Teknova TE Buffer Low EDTA (0.1 mM EDTA, pH 7.6)
2. Quantifiler™ Trio DNA Quantification Kit: Applied Biosystems (Ref 4482910)
    A. Quantifiler™ Trio Primer Mix
    B. Quantifiler™ THP PCR Reaction Mix
    C. Quantifiler™ THP DNA Standard
    D. Quantifiler™ Automation Enhancer (contact HID Sales and Support to order this product)

**Before master mix preparation, 1µL of Quantifiler Automation Enhancer should be added to each 1000µL PCR Reaction Mix tube** (expiration - 6 months after addition of the Automation Enhancer or the expiration date printed on the kit, whichever comes first). Automation Enhancer can prevent bubbles from being introduced during robotic mixing and pipetting. The kit should be labeled with the date that the Automation Enhancer was added.

The components of the kit should be stored at -15 to -25°C upon receipt and stored at 2 to 8°C after initial use. The primer sets contain a light sensitive dye and should be stored protected from light. The expiration date is printed on the kit.

MATERIALS:

1. MicroAmp® Optical 96-Well Reaction Plate: Applied Biosystems (P/N N8010560)

2. MicroAmp™ Optical Adhesive Film: Applied Biosystems (P/N 4311971)

EQUIPMENT:

1. Hamilton Microlab® Nimbus 4 Liquid Handling Workstation: Hamilton Robotics

2. QuantStudio™ 5 Real-Time PCR Instrument: Applied Biosystems

PROCEDURE:

1. This protocol is to setup the 96-well optical plate for qPCR DNA quantitation using Quantifiler™ Trio quantitation kit and the NIST Quantitation Standard Curve.

Important notes to remember when using the Microlab Nimbus for setup:

   a. The pre-run maintenance should be performed prior to the first run of the day (refer to maintenance protocol in the FB Unit Policy Manual).
   b. It is highly recommended that all tip racks are full prior to the start of a run. At minimum, complete columns of tips must be present sufficient to perform the run.
   c. Sarstedt 1.5mL screw-cap tubes are required for use on the instrument for all samples and controls. 2.0mL screw-cap tubes are required for master mix. Caps should be removed after tubes are placed in the racks and replaced prior to removing tubes from the racks.

2. If necessary, thaw the components of Quantifiler Trio DNA Quantification Kit and add 1µL of Quantifiler Automation Enhancer to each 1000µL PCR Reaction Mix tube. The kit should be labeled with the date that the Automation Enhancer was added. After initial use, the kit should be stored refrigerated (2 to 8°C).

3. Vortex the Quantifiler Trio Primer Mix, Quantifiler THP PCR Reaction Mix and Quantifiler THP DNA Standard for at least 3-5 seconds. Spin the tubes briefly in a microcentrifuge to remove any liquid from the caps.

4. Prepare a 1/10 and a 1/50 dilution of the Quantifiler THP DNA Standard in TE buffer. These dilutions will serve as calibrators for the quantitation assay.

5. Determine the total number of samples to be amplified including the set of DNA calibrators and one qPCR blank (TE). Create a master mix by combining

the following volumes of reagents into a 2ml screw-cap tube:

| | | |
|---|---|---|
| 8µL of Quantifiler Trio Primer Mix | x | # of Samples |
| 10µL of Quantifiler THP PCR Reaction Mix | x | # of Samples |

Note: The above is the minimum volume required per sample. The Nimbus requires additional master mix to perform the setup. The volumes calculated in the current version of the SDPD SIMS should be prepared.

Note: If the total number of samples is greater than 72, then two tubes of master mix are required. The first 2.0mL screw-cap tube should contain 950µL of master mix and the second tube should contain the remaining volume.

6. Open the **Quant Sheet** in the most current version of the SDPD SIMS (found on the FB network H:\Worksheets).
   a. The default spreadsheet shows the locations for the DNA calibrators and blank.
   b. Fill out the "Sample Name" column with a list of the sample names (Autofill from SampleIDs). If appropriate, edit the volume for the samples from 2 or 8µL (do not change the volume of the standards). Calibrators and TE blank – 2 µL.
   c. Save the SDPD SIMS workbook to a location accessible on the FB network (the Nimbus cannot read from a password protected Excel file).
   d. When finished, the **qPCR worksheet** should be printed for the case notes.
   e. Exit the SDPD SIMS.

7. Empty the tip waste (if necessary).

8. Refill the tip racks on the Nimbus.

9. Place the 96-well optical plate in the appropriate location on the deck.

10. Place the DNA samples in the appropriate order (according to the setup sheet filled in earlier). You may use your printed copy to ensure the sample positions are correct.

Note: The samples are to be placed in the racks in a left to right direction, filling each column completely before the next.

11. Place the Quantifiler Master Mix in the appropriate place on the deck.

Note: If more than 72 samples are being quantified then the master mix will need to be split into two tubes (see step 5 above).

12. Remove the caps from the samples and master mix.

13. Close the instrument door (the door must remain closed during operation).

14. Open the Hamilton Run Control software (on the desktop).

    a. Open the current version of the **SDPD Trio and GF PCR Setup** method within the software (File>Open> SDPD Trio and GFPCR Setup v.1.1). Alternatively, a two-step process can be employed: first "master mix only" (File>Open> SDPD Trio and GFPCR Setup v.1.1_master mix only) and second (after run for master mix is complete) "sample only" (File>Open> SDPD Trio and GFPCR Setup v.1.1_sample only).
    b. Click the "**Run**" button (►).
    c. In the user prompt, navigate to the worklist input file (from step 6 above) and select the SIMS file. For amplification type, select "**Quantifiler**" and click "**Continue**".
    d. Verify that sufficient 300µL and 50µL tips are loaded onto the deck and click "**Continue**".
    e. Enter the first and last position of the 300µL tips (1=A1, 2=A2, …, 96=H12) and click "**OK**".
    f. Enter the first and last position of the 50µL tips (1=A11, 2=A21, …, 96=H121, 97=A12, 98=A22, …, 192=H122) and click "**OK**".

15. The Nimbus will now add master mix to the 96-well optical plate and then add 2 (or 8)µL of the samples to the master mix.

16. Click "**OK**" to acknowledge completion of the run.

17. When the protocol has finished, the tubes can be re-capped and removed and

the 96-well optical plate can be covered with an optical adhesive cover and carried to the amplification room for loading into the QuantStudio 5 Real-Time PCR Instrument. Spin down the plate before loading into the instrument.

18. The qPCR analysis can be continued by following the "qPCR with the Quantifiler™ Trio DNA Quantification Kit using the NIST SRM Human DNA Quantitation Standard (Manual setup)" starting on step 9.

REFERENCES:

Microlab® Nimbus Independent Channel Operator's Manual. Hamilton Robotics.
Microlab® Nimbus Enclosed Version Operator's Manual. Hamilton Robotics.
Microlab® Nimbus Independent Channel Programmer's Manual. Hamilton Robotics.
Quantifiler® Trio DNA Quantification Kit User Guide. Applied Biosystems.

Method: **Target Ranges for Single Source Samples Using NIST Standard based Quantitation**

Date: 05/01/2012 Revision Date: 02/05/2018 Approved by: ARD

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The chart below represents the results obtained for the sensitivity study of the GlobalFiler and Yfiler Plus kits using data obtained with the NIST standard. The target ranges represent target amounts of DNA that should result in peak height between 3,000-10,000RFU.

This information is presented for analysts as a guide to determine the amplification targets for single source samples. Sample type, variation between lots of quantitation kits, and the expectation of obtaining a mixture will affect the amount of DNA amplified in casework samples.

| DNA Typing Kit | Quantification Range for Single Source Samples |
|---|---|
| GlobalFiler | 0.5 - 0.7ng |
| YFiler Plus | 0.4 - 0.8ng |

Method: **Concentrating DNA Extracts with Microcon MRCFOR100 Filters**

Date: 04/01/2011 Revision Date: 06/09/2025 Approved by: ARD

REAGENTS:

TE Buffer: Teknova TE Buffer Low EDTA (0.1 mM EDTA, pH 7.6)

MATERIALS:

1. Microcon MRCFOR100 Centrifugal Filter Devices (Millipore)

2. Microcentrifuge (capable of at 1,000 x g)

PROCEDURE:

Note: The capacity of the Microcon MRCFOR100 reservoir is approximately 500µL. If the purified extract has a larger volume it may require adding any remaining amount of sample that did not fit into the reservoir after the first spin. This protocol may also be used to further concentrate previously purified samples.

1. Assemble the Microcon MRCFOR100 unit by inserting the filter device with the clear reservoir toward the top into the filtrate tube.

2. Add approximately 50µL of TE buffer to the upper reservoir of the Microcon filter device.

3. Add the appropriate amount of extracted DNA sample to be concentrated on top of the TE buffer. Seal with the attached cap.

4. Centrifuge the assembled Microcon filter device in a microcentrifuge for 12-15 minutes at 2300rpm (~500 x g). The DNA sample will be concentrated in about 5-15µL of TE buffer toward the bottom of the upper Microcon reservoir (molecules with molecular weights of less than 100,000 Daltons will pass

through the filter). Check the Microcon assembly periodically during the spin to ensure that the filter has not dried out.

Important: Spinning the filter to dryness can cause irreversible binding of the DNA to the membrane.

5. Discard the effluent in the filtrate tube. Replace the Microcon filter device right-side up into the filtrate tube.

8. Add 3-6µL of TE buffer to the Microcon reservoir (depending on desired final volume). Gently vortex the filter assembly.

9. Collect the DNA sample by inverting the reservoir into a new Microcon filtrate tube and centrifuge at approximately 3200 rpm (approximately 1000 x g) for 3-5 minutes.

10. The concentrated purified DNA now resides in the tube. The volume can be further adjusted as necessary to achieve the desired concentration. Cap the tube and store frozen. Alternatively, transfer the purified DNA to a different tube, such as a sterile screw-cap tube for storage.

REFERENCE:

Microcon Centrifugal Filter Device User Guide, Revision J. Millipore 2022

Method: **DNA Concentration Using the DNA Speed-Vac DNA-120**

Date: 10/27/2014 Revision Date: 08/12/2019 Approved by: ARD

REAGENTS:

TE Buffer: Teknova TE Buffer Low EDTA (0.1 mM EDTA, pH 7.6)

EQUIPMENT:

Savant DNA Speed Vac DNA-120

NOTES:

Based on an internal study conducted on concentrating samples containing TE buffer, it is not recommended to concentrate samples containing TE by more than a factor of 8 if purified using the BioRobot EZ1s. Samples concentrated by factors greater than described above started exhibiting signs of inhibition. It is presumed that the concentration of the EDTA in the TE inhibits PCR by chelating the magnesium in the reaction. Concentration of samples eluted in water generally do not exhibit PCR inhibition at any level of volume reduction.

PROCEDURE:

1. Place the appropriately labeled open sample extract tubes in the Savant DNA Speed-Vac DNA-120.

2. Turn on the DNA Speed-Vac DNA-120. Select one of the three drying rate options using switch "D" (Low = ambient temperature, medium = 43°C, high = 65°C; medium or high is recommended). Generally, either medium or high are selected. The drying rate will influence how quickly evaporation occurs.

3. The Speed-Vac DNA 120 model is capable of both "Manual" mode and "Auto" mode (where you can set a timer). For MANUAL mode go to step 4. For AUTO MODE, skip to step 9.

**MANUAL MODE**

4. Switch "A" should be in the "OFF" position.
5. Choose the MANUAL setting using switch "A".
6. Choose the HEATER setting using switch "B".

7. Use switch "C" to adjust the heater from "OFF" to disable the heater, or to "CCC" to operate the heater for the duration of the run. A specified time can also be used.

8. The Speed-Vac will start as soon as you switch "A" to MANUAL and will continue to run until you turn OFF the run using switch "A".

**AUTO MODE**

9. Switch "A" should be in the "OFF" position.

10. Choose the RUN timer setting using switch B".

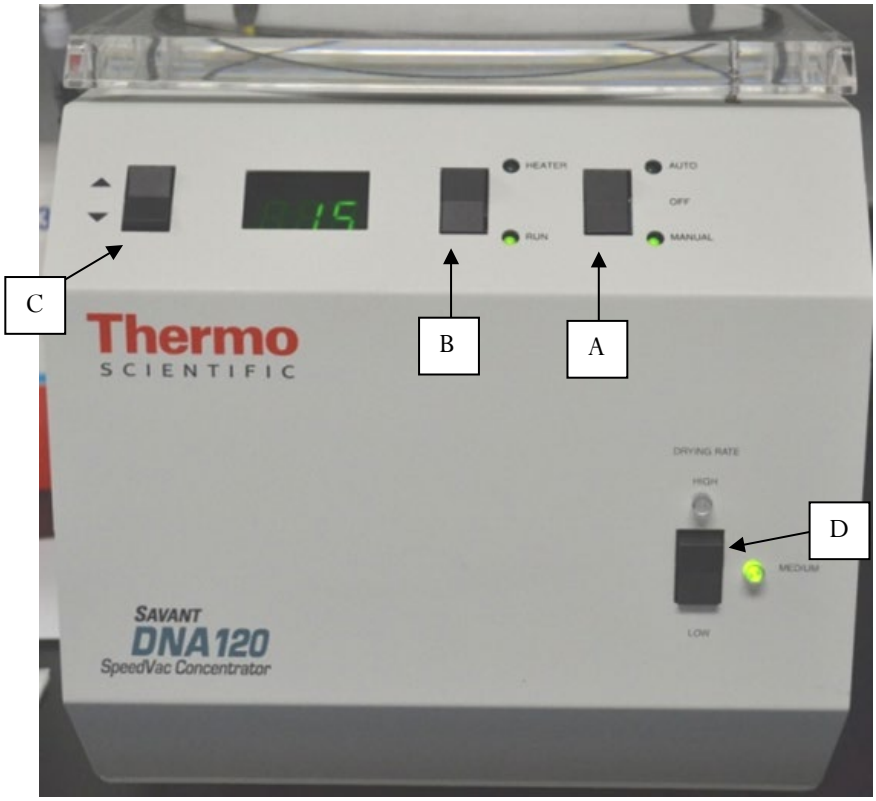
11. Use switch "C" to increase or decrease the amount of time you want the Speed-Vac to run. A decimal point separates hours from minutes (1.20 = 1 hour and 20 minutes).

12. Choose the HEATER timer setting using switch "B".

13. Use switch "C" to increase or decrease the amount of time you want the Speed-Vac to heat the sample. A decimal point separates hours from minutes (1.20 = 1 hour and 20 minutes). *Suggestion*: Make this the same time as the run time.

14. Choose the AUTO setting using switch "A". The Speed-Vac will start as soon as you switch "A" to AUTO and will shut off after the programmed time.

During any run, the display can be toggled between elapsed run time (manual mode) or remaining run time (auto mode), and remaining heat time through the use of switch "B".




| *Approximate Times50µL TE to 10uL* | Old Speed Vac | New Speed Vac |
|---|---|---|
| **Medium Heat** | 35 minutes | 20 minutes |
| **High Heat** | 15 minutes | 10 minutes |
| ***40µL TE to 10uL*** | **Old Speed Vac** | **New Speed Vac** |
| **Medium Heat** | 20 minutes | 10 minutes |
| **High Heat** | 15 minutes | 5-10 minutes |

**Method: Amplification with the GlobalFiler® kit**

Date: 10/05/2015 Revision Date: 06/09/2025 Approved by: ARD

REAGENTS:

1. TE Buffer: Teknova TE Buffer Low EDTA (0.1 mM EDTA, pH 7.6)
2. GlobalFiler PCR Amplification Kit: Applied Biosystems, 200 reactions (P/N 4476135) or 1000 reactions (P/N 4482815)

   A. GlobalFiler® Master Mix
   B. GlobalFiler® Primer Set
   C. GlobalFiler® Allelic Ladder
   D. 007 Control DNA

   Store all kit components at -15 to -25°C upon receipt. Store components at 2 to 8°C after initial use. The primer sets and the ladder are light sensitive and should be stored protected from light. The expiration date is printed on the kits.

MATERIALS:

1. MicroAmp Autoclaved 0.2mL Reaction Tubes with Caps: Applied Biosystems (P/N N801-0612)
2. MicroAmp 96-Well Tray: Applied Biosystems (P/N N8010541)
3. MicroAmp 96-well Plate Base: Applied Biosystems (P/N 4312063)

EQUIPMENT:

1. Veriti® 96-well Thermal Cyclers: Applied Biosystems

PROCEDURE:

1. Thaw the Master Mix and Primer Set.

   IMPORTANT! Thawing is required only during first use of the kit. After first use, reagents are stored at 2 to 8°C and, therefore, do not require subsequent thawing. Do not refreeze the reagents.

2. Fill out a GlobalFiler® Amplification Worksheet and determine the total number of samples to be amplified including the positive amplification control (007 DNA), the negative amplification control, and all samples.

3. Label a 0.2 mL MicroAmp Reaction Tube for each of the samples to be amplified.

4. Vortex the GlobalFiler® Master Mix, GlobalFiler® Primer Set, 007 Control DNA tubes at least 3 seconds and spin briefly in a microcentrifuge to remove any liquid from the caps.

5. Create a master mix by combining the following volumes of reagents into a 1.5 mL clear (non-colored) microcentrifuge tube:

| | | | | |
|---|---|---|---|---|
| a. | 7.5 µL | GlobalFiler® Master Mix | x | (# samples) + 10 % |
| b. | 2.5 µL | GlobalFiler® Primer Set | x | (# samples) + 10 % |

6. Vortex the master mix at least 5 seconds and then spin briefly in a microcentrifuge to remove any liquid from the cap.

*Note: Steps 6-8 must be performed in a biological hood using dedicated pipettes.*

7. Aliquot 10 µL of the master mix into each labeled 0.2 mL reaction tube.

8. If less than 15 µl of sample is added for any sample, the difference should be made up by adding TE buffer for a final volume of 15 µl.

9. Add sample to the labeled 0.2 mL reaction tubes as follows:

   - For all evidence and reference samples, add approximately 0.6-0.9 ng of target DNA in a volume equal to or less than 15 µL. *Note*: 0.6-0.9 ng is a suggested target amount. The actual amount added will depend on the nature of the sample and any additional information obtained from DNA quantification.
   - For reagent blanks, add a volume that is equal to the greatest volume from any of the evidence samples associated with it.
   - For evidence hairs, add a volume of the shaft control adjacent to the root (if applicable) that is equal to the volume used for the hair root sample.

- Add 4-7 µL (0.1 ng/µL) of 007 Control DNA to the positive amplification control tube. Add TE buffer to obtain a final volume of 15 µL.
- Add 15 µL of TE buffer to the negative control tube.

10. Transport the tubes to the Amplification Room. Place a MicroAmp tray containing the PCR reaction tubes into one of the thermal cyclers. Use the capping tool to firmly close the caps on all PCR reaction tubes.

11. Turn the thermal cycler on (if not already done).

12. Select and run the **GlobalFiler** program.

    a. Initial Incubation
       95°C, 1 minute

    b. 29 Cycles
       94°C, 10 seconds
       59°C, 90 seconds

    c. Final Extension
       60°C, 10 minutes

    d. Final Hold
       4°C, up to 24 hours

13. Remove the amplified products from the thermal cycler any time after reaching 4°C. Long term storage of amplified DNA should be at -15 to -25°C.

REFERENCES:
GlobalFiler® PCR Amplification Kit User Guide

Method: **Amplification with the Yfiler™ Plus Kit**

Date: 02/05/2018 Revision Date: 01/31/2024 Approved by: ARD

REAGENTS:

1. TE Buffer: Teknova TE Buffer Low EDTA (0.1 mM EDTA, pH 7.6)
2. Yfiler™ Plus PCR Amplification Kits: Applied Biosystems (P/N 4484678 and 4482730)
   A. Yfiler™ Plus Master Mix
   B. Yfiler™ Plus Primer Set
   D. DNA Control 007

   Store all kit components at -15 to -25°C upon receipt. Store components at 2 to 8°C after initial use. The primer sets and the ladder are light sensitive and should be stored protected from light. The expiration date is printed on the kits.

MATERIALS:

1. MicroAmp Autoclaved 0.2mL Reaction Tubes with Caps: Applied Biosystems (P/N N801-0612)
2. 96- Well Optical Reaction Plates: Applied Biosystems (P/N N801-0560)
3. MicroAmp® 8-cap Strip: Applied Biosystems (P/N N8010535)
4. MicroAmp 96-Well Tray: Applied Biosystems (P/N N8010541 or P/N 4379983)
5. MicroAmp 96-well Plate Base: Applied Biosystems (P/N 4312063)

EQUIPMENT:

1. Veriti® 96-well Thermal Cyclers: Applied Biosystems

PROCEDURE:

1. Fill out an Yfiler™ Plus Amplification Worksheet and determine the total number of samples to be amplified including the positive control (007), the negative amplification control, and all samples.

2. Label a 0.2mL MicroAmp Reaction tube for each of the samples to be amplified, or alternatively use an appropriately labeled 96-well optical reaction plate for amplification.

3. Vortex the Yfiler™ Plus Master Mix, Yfiler™ Plus Primer Set, and DNA Control 007 briefly. Spin the tubes briefly in a microcentrifuge to remove any liquid from the caps.

4. Create a master mix by combining the following volumes of reagents into a 1.5mL microcentrifuge tube:

    a. 10µL of Yfiler™ Plus Master Mix

    b. 5.0µL of Yfiler™ Plus Primer Set

    Note: Include volume for additional reactions to provide excess volume for the loss that occurs during reagent transfers.

5. Vortex the master mix at least 3 seconds then centrifuge briefly.

*Note: Steps 6 - 8 are performed in a biological hood using dedicated pipettes.*

6. Dispense 15µL of the master mix into each labeled 0.2mL reaction tube or required plate well.

7. If less than 10µL of sample is added for any sample, the difference should be made up by adding TE buffer.

8. Add sample to the labeled 0.2mL reaction tubes or plate well as follows:

    - For all single source evidence and reference samples, add approximately 0.6ng of male target DNA in a volume equal to or less than 10µL. Note: 0.6 ng is a suggested target amount of DNA. The actual amount added will depend on the nature of the sample and any additional information obtained from quantitation (or estimation of the concentration) of male DNA in a sample or from autosomal DNA testing.

- For reagent blanks add a volume that is equal to the greatest volume from any of the evidence or reference samples associated with it.

- The DNA Control 007 positive amplification control requires dilution prior to addition to any amplification reaction (suggestion 9µL into 291µL). Target 0.6ng of the DNA Control 007 (10µL of the suggested dilution). If less than 10µL of Control DNA is added, the difference is made up by adding TE buffer.

- Add 10µL of TE buffer to the negative control tube.

9. Transport the tubes or plate to the amplification area. Place a MicroAmp tray containing the PCR reaction tubes into one of the thermal cyclers, or the plate directly into the block. Use the capping tool to firmly close the caps on all PCR reaction tubes or plate.

10. Turn the thermal cycler on (if not already done).

11. Select the program named **Yfiler Plus**.

    a. Initial incubation at 95°C for 1 minute.

    b. 30 cycles of the following profile:
    Denature at 94°C for 4 seconds
    Anneal/Extend at 61.5°C for 1 minute

    c. Final extension at 60°C for 22 minutes

    d. Final hold at 4°C for up to 24 hours.

The amplified products can be removed from the thermal cycler at any time after reaching 4°C. Amplified samples are stored in the freezer.

REFERENCES:
Yfiler™ Plus PCR Amplification Kit User's Manual

Method: **GlobalFiler® and Yfiler™ Plus Setup Using the Hamilton Microlab® Nimbus 4**

Date: 04/17/2013 Revision Date: 06/29/2023 Approved by: ARD

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REAGENTS:

1. TE Buffer: Teknova TE Buffer Low EDTA (0.1 mM EDTA, pH 7.6)
2. GlobalFiler PCR Amplification Kit: Applied Biosystems, 200 reactions (P/N 4476135) or 1000 reactions (P/N 4482815)

    A. GlobalFiler® Master Mix
    B. GlobalFiler® Primer Set
    A. 007 Control DNA

    Store all kit components at -15 to -25°C upon receipt. Store components at 2 to 8°C after initial use. The primer sets and the ladder are light sensitive and should be stored protected from light. The expiration date is printed on the kits.

3. Yfiler™ Plus PCR Amplification Kit: Applied Biosystems (P/N 4484678 and 4482730)

    A. Yfiler™ Plus Master Mix
    B. AmpF*l*STR® Yfiler™ Plus Primer Set
    C. DNA Control 007

    Store all kit components at -15 to -25°C upon receipt. Store components at 2 to 8°C after initial use. The primer sets and the ladder are light sensitive and should be stored protected from light. The expiration date is printed on the kits.

MATERIALS:

1. 96-Well Optical Reaction Plates: Applied Biosystems (P/N N8010560)
2. MicroAmp 8-cap strips: Applied Biosystems (P/N N801-0535)

EQUIPMENT:

1. Hamilton Microlab Nimbus 4 Liquid Handling Workstation: Hamilton Robotics
2. Veriti® Thermal Cyclers: Applied Biosystems

PROCEDURE:

1. This protocol is to setup the 96-well optical plate for PCR DNA amplification using GlobalFiler and Yfiler™ Plus.

Important notes to remember when using the Microlab Nimbus for setup:

   a. The pre-run maintenance should be performed prior to the first run of the day (refer to maintenance protocol in the FB Unit Policy Manual).
   b. It is highly recommended that all tip racks are full prior to the start of a run. At minimum, complete columns of tips must be present sufficient to perform the run.
   c. Sarstedt 1.5mL screw-cap tubes are required for use on the instrument for all samples, controls, and TE buffer. 2.0mL screw-cap tubes are required for master mix. Caps should be removed after tubes are placed in the racks and replaced prior to removing tubes from the racks.

2. **GlobalFiler** – Vortex the GlobalFiler® Master Mix, GlobalFiler® Primer Set, and DNA Control 007 briefly then spin the tubes in a microcentrifuge.

   **Yfiler Plus** – Vortex the Yfiler™ Plus Master Mix, Yfiler™ Plus Primer Set, and DNA Control 007 briefly then spin the tubes in a microcentrifuge.

3. Determine the total number of samples to be amplified including the positive control and one amplification blank. Create a master mix by combining the following volumes of reagents in a 2.0mL screw-cap tube.

| | | |
|---|---|---|
| **GlobalFiler** | | |
| 7.5 µL GlobalFiler® Master Mix | x | # of Samples |
| 2.5 µL GlobalFiler® Primer Set | x | # of Samples |
| **Yfiler Plus** | | |
| 10µL Yfiler™ Plus Master Mix | x | # of Samples |
| 5µL Yfiler™ Plus Primer Set | x | # of Samples |

Note: The above is the minimum volume required per sample. The Nimbus requires additional master mix to perform the setup. The volumes calculated in the current version of the SDPD SIMS should be prepared.

4. Open the **Amp Sheet** in the most current version of the SDPD SIMS (found on the FB network H:\Worksheets).

    a. The default spreadsheet shows the deck position of all samples and their destination positions.
    b. Fill out the "Sample Name" column with a list of the sample IDs and the DNA conc. [ng/µL] column. Ensure that the VolumeSample and VolumeTE are completed appropriately.

NOTE: Choose the appropriate kit on this sheet so that the volume of TE in the negative amplification control reflects the different volumes that are needed by the GlobalFiler® kit (total to 15 µL) vs. Yfiler™ Plus (total to 10 µL).

    c. Save the SDPD SIMS workbook to a location accessible on the FB network (the Nimbus cannot read from a password protected Excel file or files saves as .xlsx files).
    d. When finished, the appropriate PCR worksheet should be printed for the case notes.
    e. Exit the SDPD SIMS.

5. Empty the tip waste (if necessary).

6. Refill the tip racks on the Nimbus.

7. Place the 96-well optical plate in the appropriate location on the deck.

8. Place the DNA samples in the appropriate order (according to the setup sheet filled in earlier). You may use your printed copy to ensure the sample positions are correct.

Note: The samples are to be placed in the racks in a left to right direction, filling each column completely before the next.

9. Place the master mix in the appropriate place on the deck.

10. Place four 1.5mL screw-cap tubes each containing 300µL of TE in the appropriate place on the deck.

11. Remove the caps from the samples, master mix, and TE.

12. Close the instrument door (the door must remain closed during operation).

13. Open the Hamilton Run Control software (on the desktop).

    a. Open the current version of the **SDPD Trio and GF PCR Setup** method within the software (File>Open> SDPD Trio and GFPCR Setup v.1.1). Alternatively, a two-step process can be employed: first "master mix only" (File>Open> SDPD Trio and GFPCR Setup v.1.1_master mix only) and second (after run for master mix is complete) "sample only" (File>Open> SDPD Trio and GFPCR Setup v.1.1_sample only).
    b. Click the "**Run**" button (►).
    c. In the user prompt, navigate to the worklist input file (from step 4 above), select "**GlobalFiler**" for GlobalFiler amplification setup, or "**Minifiler/Yfiler**" for Yfiler amplification, and click "**Continue**".
    d. Verify that sufficient 300µL and 50µL tips are loaded onto the deck and click "**Continue**".
    e. Enter the first and last position of the 300µL tips (1=A1, 2=A2, …, 96=H12) and click "**OK**". Note: This step is omitted for the samples only version of the method.

f. Enter the first and last position of the 50 µL tips (1=$A1_1$, 2=$A2_1$, …, 96=$H12_1$, 97=$A1_2$, 98=$A2_2$, …, 192=$H12_2$) and click "**OK**". Note: This step is omitted for the master mix only version of the method.

14. The Nimbus will now add master mix, TE buffer, and sample DNA to the 96-well optical plate.

15. Click "**OK**" to acknowledge completion of the run.

16. When the protocol has finished, the tubes can be re-capped and removed and the 96-well optical plate can be covered with strip caps and carried to the amplification room for loading into the Veriti® thermal cycler.

17. Turn the thermal cycler on (if not already done).

18. Select and run the corresponding amplification protocol for the amplification kit. In summary:

    **GlobalFiler**: 95 °C 1 min→29 cycles of 94 °C 10 sec, 59 °C 90 sec→ 60 °C 10 min→4 °C ∞
    **Yfiler Plus**: 95 °C 1 min→30 cycles of 94 °C 4sec, 61.5 °C 1 min→ 60 °C 22 min →4 °C ∞

19. Remove the amplified products from the thermal cycler any time after reaching 4°C. Long term storage of amplified DNA should be at -15 to -25°C.

REFERENCES:

Microlab® Nimbus Independent Channel Operator's Manual. Hamilton Robotics.
Microlab® Nimbus Enclosed Version Operator's Manual. Hamilton Robotics.
Microlab® Nimbus Independent Channel Programmer's Manual. Hamilton Robotics.
GlobalFiler® PCR Amplification Kit User's Manual. Applied Biosystems.
Yfiler™ Plus PCR Amplification Kit User's Manual. Applied Biosystems.

Method: **3500 Genetic Analyzer Instrument Preparation and Calibration**
Date: 7/29/15 Revision Date: 06/29/2023 Approved by: ARD

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REAGENTS:

1. Conditioning Reagent 3500 Series: Applied Biosystems, (P/N 4393718), store at 2-8°C.

2. Anode Buffer Container 3500 Series: Applied Biosystems (P/N 4393927), store at 2-8°C.

3. Cathode Buffer Container 3500 Series: Applied Biosystems (P/N 4408256), store at 2-8°C.

4. Performance Optimized Polymer (POP-4™ (384)) 3500 Series: Applied Biosystems (P/N 4393715), store at 2-8°C.

5. DS-36 Matrix Standard (Dye Set J6): Applied Biosystems (P/N 4425042), store at 2-8°C, protected from light. Stable for one year.

   This is the matrix standard set used when analyzing amplified DNA fragments generated using the GlobalFiler and Yfiler™ Plus kits and labeled with 6 dyes: 6-FAM, VIC, NED, TAZ, SID, and LIZ).

6. HIDI Formamide: Applied Biosystems (P/N 4311320)

MATERIALS:

1. Septa for Cathode Buffer: Applied Biosystems (P/N 4410715)

2. Luer lock syringe: Applied Biosystems (in the PDP Cleaning kit, P/N 4359572)

3. MicroAmp Optical 96-Well Reaction Plates: Applied Biosystems (P/N N8010560)

4. MicroAmp Splash Free Support Base: Applied Biosystems (P/N 4312063)

5. Septa for 3500 Genetic Analyzers, 96 well: Applied Biosystems (P/N 4412614)

6. Retainer and Base for 3500 Genetic Analyzer, 96 well: Applied Biosystems (P/N 4410228)

7. 96-well cooling block: Stored in the reagent freezer

8. 3500 Genetic Analyzer 8-Capillary Array (36 cm): Applied Biosystems (P/N 4404683)

9. Kit PDP Cleaning: Applied Biosystems (Pump Cleaning Kit) (P/N 4414007)

EQUIPMENT:

Veriti® Thermal Cyclers: Applied Biosystems
3500 Genetic Analyzer: Applied Biosystems

PROCEDURE:

Note: Refer to the policy manual section on equipment (Section 6.10) for additional information on 3500 maintenance procedures. The following procedures are to be performed as required outside of normal maintenance procedures.

**Powering on the 3500 and launching the Data Collection software**

1. Power on the computer. At the Log On prompt, log in to the computer.

2. Before turning on the 3500 instrument, ensure that the oven door is closed and locked, that the instrument doors are closed, and the computer is powered on.

3. Power on the instrument by pressing the on/off button on the front of the instrument. Ensure that the green status light is on and not flashing before proceeding.

4. Click on the **3500 Series Data Collection Software 2** icon on the desktop. Log in

to the data collection software with the password taped to the monitor.

5. Use the Dashboard to review maintenance notifications. When you complete a task, click the **green check mark** to indicate that it was completed, or the **red X** to mark it as dismissed.

6. Click on Maintenance on the top panel. Use the left panel to navigate to the schedule calendar or Maintenance Wizards to complete the needed maintenance.

**Restarting the computer and instrument**

1. Close data collection software.

2. Shut down computer.

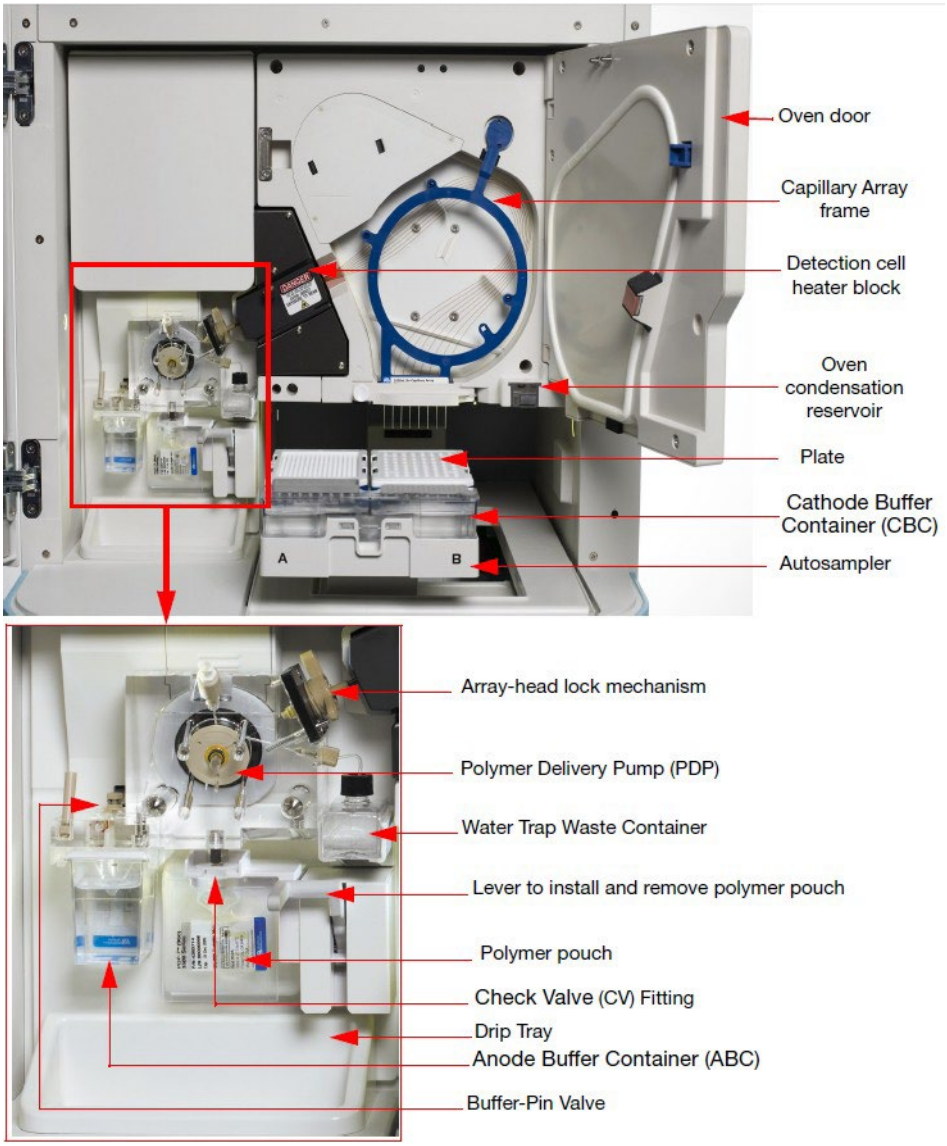
3. Once computer is shut down, press the **power** button on the instrument.

4. Restart computer, but don't log in.

5. Once the computer is on, turn the 3500 back on by pressing the **power** again. Let the instrument initialize and wait for the solid green light to appear before logging on to the computer.

6. Click on the **3500 Series Data Collection Software 2** icon on the desktop. Log in to the data collection software (it may take a minute for the connections to be reestablished before the software can open).

INSTRUMENT INTERIOR COMPONENTS:




## Replacing the polymer

If there isn't sufficient polymer to complete your run or if the polymer has exceeded 4 weeks use on the instrument, it is necessary to place a new polymer pouch on the instrument. Get a new polymer pouch and let it

equilibrate to room temperature before replacing the old one. Use the **Replenish Polymer wizard** to complete this. Follow the wizard prompts in order to flush the pump of the old polymer and refill the capillaries with the new polymer. This wizard takes 10-20 minutes to complete

## Spatial Calibration

The 3500 Series Data Collection software uses images collected during the spatial calibration to establish a relationship between the signal emitted by each capillary and the position where that signal is detected by the CCD camera. A spatial calibration is required to ensure correct alignment of the capillaries and optimal detection of the signal in each capillary.

A new spatial calibration is recommended when:

- A capillary array is installed, replaced or temporarily removed from the detection block
- The instrument is moved
- The detection cell door is opened

### Perform Spatial Calibration and Evaluate Results

1. Click "**Maintenance**" on the Dashboard. Click "**Spatial**" under the Calibrate heading on the left.

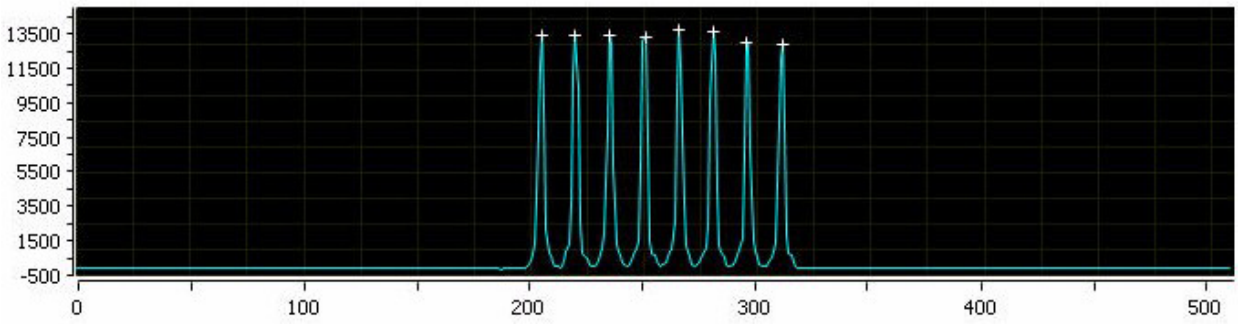
2. Select **No Fill**, or select **Fill** to fill the array with polymer before starting the calibration.

3. Click "**Start Calibration**". The display updates as the run progresses.

4. When the run is complete, evaluate the spatial calibration profile to ensure that you see the following:
   - One sharp peak for each capillary. Small shoulders may be acceptable.
   - One marker (+) at the apex of every peak. No off-apex markers.
   - An even peak profile (all peaks about the same height).
   - The Max Spacing – Min Spacing should be no more than 2 pixels.

5. If the results meet the criteria above, click "**Accept Results**".

Note: If any peaks are lower than usual for the instrument or the heights drastically slope up or down, repeat the calibration with a fill. See below for an example of an acceptable Spatial Profile.




6. Click "**View Spatial Calibration Report**".

## Spectral Calibration

A spectral calibration creates a matrix that corrects for the overlapping fluorescence emission spectra of the dyes. Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the dyes. The goal of multicomponent analysis is to effectively correct for spectral overlap and minimize the presence of artifacts, such as spectral pull-up, in the data.

A new spectral calibration is recommended/required when:

- The capillary array is changed (required) or temporarily removed from the detection block.
- The instrument is moved.
- The laser or CCD camera has been realigned/replaced by a service engineer.
- An increase in (pull-up and/or pull-down peaks) is observed.
- A new dye set is used on the instrument.
- The capillary array length or polymer type is changed.
- The detection cell door is opened (not required by the software, but recommended).

Perform Spectral Calibration and Evaluate Results

1. Pre-heat the oven by clicking "**Start Pre-heat**".

*Note*: Preheating for approximately 30 minutes helps mitigate subtle first-run migration rate effects. The pre-heat function automatically turns off after 2 hours.

2. Click "**Maintenance**" on the Dashboard. Click "**Spectral**" under the Calibrate heading on the left panel.

3. Check the pump assembly for bubbles and run the Remove Bubble wizard if needed.

4. Vortex the appropriate Matrix Standard. Prepare the Master Mix:

   For GlobalFiler® and Yfiler™ Plus analysis, use **Dye Set J6.**
   a. Standard: 10 µL
   b. HIDI Formamide: 290 µL

*Caution*: Formamide is a known teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a fume hood and wear chemical resistant gloves and safety glasses when handling.

*Note:* Volumes other than above may be used to obtain spectral peaks between 3000-10000 RFU. The total volume should not exceed 300µL.

*Note:* After a capillary array is changed, a new spectral calibration must be run for each dye set.

5. Load 10 µL of matrix standard/HIDI mixture into wells A1-H1 of a 96-well reaction plate. Note: multiple matrix samples (e.g., J6 or G5) can be run in subsequent columns on this plate if needed)

6. Cover the plate with a 3500 septa mat and centrifuge to ensure that the samples are at the bottom of the wells and place the reaction plate in the Veriti® thermal cycler.

7. Denature the samples by running the **spectraldenature** protocol on the 9700 (95˚C, 5 min).

Note: The cover plate of the Veriti® thermal cycler may be closed onto the septa mat-covered reaction plate, but do not clamp the cover down. Although the lid is not heated during the denature protocol, it may stick to the septa mat and pull it up when the cover is opened if it is clamped down.

8. After the denaturation protocol is finished, chill the reaction plate immediately in the 96-well cooling block (-20˚C) for at least 3 minutes.

9. Place the plate into a 3500 Series 96-well plate base (it will only fit in one orientation) and cover with a 3500 Genetic Analyzer 96-well plate retainer. Check to ensure that the retainer clip is properly seated.

10. Press the **tray** button on the front of the 3500 Genetic Analyzer. When the autosampler tray presents itself, open the door of the instrument and place the tray onto the either position of the autosampler (the tray will only fit in one orientation). Tilt the plate toward the middle, first before lowering into the side clip. Close the instrument door.

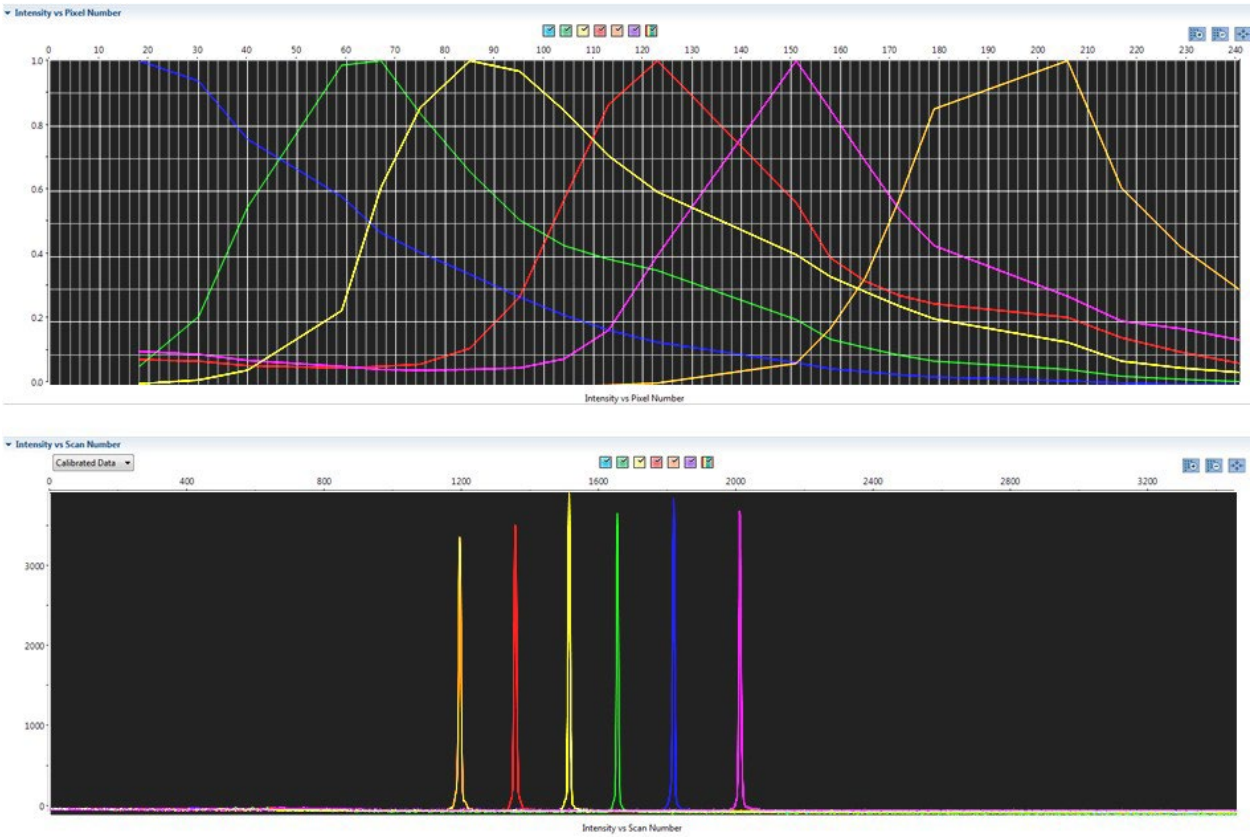
11. In the 3500 Series Data Collection Software 2, Click "**Maintenance**" on the dashboard. Click "**Spectral**" under the Calibrate heading on the left.

12. Select the number of wells (96), plate position, chemistry standard (Matrix Standard) and dye set (J6 or G5). Select allow borrowing.

13. Click "**Start Run**". Run takes ~30 minutes to complete. Passing capillaries are shown in green and failing capillaries are shown in red. Borrowed capillaries are shown in yellow with an arrow indicating the adjacent capillary from which results were borrowed.

14. Evaluate each capillary for the following:
    - Order of the peaks in the spectral profile from left to right:
      J6: blue, green, yellow, red, purple, orange (an example is below)
      G5: blue, green, yellow, red, orange

- Peak heights are sufficiently robust (>2,000 RFU) and do not contain extraneous peaks, gross overlaps, dips, or irregular morphology. If peak heights exceed 10,000 RFU, set up another plate with a reduced amount of matrix standard. If peak heights are below 2,000 RFU, set up another plate with an increased amount of matrix standard. See below for an example of an acceptable J6 spectral profile.G5 is similar, but without the purple.




15. If the results meet the criteria above, click "**Accept Results**". If the results do not meet the criteria, set up another plate, adjusting amount of matrix standard, if necessary, and run again.

## Installing or replacing the capillary array

It is recommended that the capillary array be replaced after 160 injections per capillary, or removed from the instrument for storage if the instrument is to

be shut down for an extended period of time; however, capillary life may be extended based on the quality of the data obtained from the array.

Select the "**Install Array Wizard**" for guidance. This wizard takes 15-45 minutes to complete.

*Note*: A spatial calibration AND spectral calibration are required after a capillary array change. The instrument will not run without a new spectral calibration associated with that capillary RFID tag.

## Removing bubbles from the pump block

Periodically, check the pump chamber, channels and tubing for air bubbles. Press the button on the right hand side of the front of the instrument to turn on the lamp inside the instrument. This will help locate any air bubbles.

Select the "**Bubble Removal Wizard**" for guidance. This wizard takes 5-15 minutes to complete.

## Pump Cleaning

In nearly all circumstances, the Water Wash Wizard is very effective in cleaning the pump assembly. Occasionally, a situation in which the Water Wash Wizard is not sufficiently effective may arise. If polymer has dried in the channels of the lower block, or if some contamination in the pump assembly is suspected, use the Pump Cleaning Kit to thoroughly clean the pump assembly.

If this procedure is needed, refer to the Pump Cleaning Kit Protocol for 3500 Series Analyzers.

REFERENCES:

Applied Biosystems 3500/3500xL Genetic Analyzer User Guide
Applied Biosystems 3500/3500xL Genetic Analyzer User Bulletin
Product insert sheet from Dye Set J6 (Pub. Number 4426042 Rev. A)
Pump Cleaning Kit Protocol for 3500 Series Analyzers

Method: **Preparation of samples for analysis on the Applied Biosystems 3500 Genetic Analyzer**

Date: 7/29/15 Revision Date: **06/29/2023** Approved by: ARD

REAGENTS:

1. HIDI Formamide: Applied Biosystems (P/N 4311320)

2. GeneScan™ 600 LIZ® Size Standard v2.0: Applied Biosystems (P/N 4408399) The tubes may be stored for up to six months at 2-8˚C.

3. The ladders used will be dependent on the kit:
   a) GlobalFiler® Allelic Ladder: (included in the GlobalFiler® kits) Store at −15 to −25˚C upon receipt and at 2 to 8˚C after initial use. Store protected from light up to the expiration date.
   b) Yfiler™ Plus Allelic Ladder: (included in the Yfiler™ Plus kits) Store at −15 to −25˚C upon receipt and at 2 to 8˚C after initial use. Store protected from light up to the expiration date.

MATERIALS:

1. MicroAmp Optical 96-Well Reaction Plates: Applied Biosystems (P/N N8010560)
2. MicroAmp Splash Free Support Base: Applied Biosystems (P/N 4312063)
3. Septa for 3500 Genetic Analyzers, 96 well: Applied Biosystems (P/N 4412614)
4. Retainer and Base for 3500 Genetic Analyzer, 96 well: Applied Biosystems (P/N 4410228)
5. 96-well cooling block: Stored in the reagent freezer

EQUIPMENT:

1. Veriti® Thermal Cyclers: Applied Biosystems
2. 3500 Genetic Analyzer: Applied Biosystems

PROCEDURE:

IMPORTANT: STRmix™ analysis requires the use of different analysis methods to analyze evidence and reference samples. To have multiple analysis methods within one project, each requires at least one ladder associated with it. To accomplish this, two ladders per analysis method should be prepared for electrophoresis on the 3500, or a single set of ladders can be prepared and the .hid files of the ladders can be duplicated within the run folder and imported into GeneMapper ID-X.

Note: Prior to preparing samples, the oven on the 3500 Genetic Analyzer can be preheated by clicking "**Start Preheat"** and the Veriti® thermal cycler can be turned on so that the top plate can come to temperature.

1. Create a master mix by combining the following volumes of reagents into a 1.5 mL microcentrifuge tube.

   | | | |
   |---|---|---|
   | 9.6 µL formamide | x | (# of samples + 10%) |
   | 0.4 µL GS600 [LIZ] | x | (# of samples + 10%) |

   Caution: Formamide is a known teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a fume hood and wear chemical resistant gloves and safety glasses when handling.

2. Vortex the master mix.

3. Take out a new 96-well reaction plate.

4. Aliquot 11 µL of formamide into the formamide blank well (suggestion: A1) if used as a control.

5. Aliquot 10 µL of the master mix into each of the appropriate wells on the 96-well reaction plate to accommodate the numbers of amplified samples and allelic ladders samples.

6. Add 1 µL of the desired Allelic Ladder to the appropriate wells on the reaction plate. An allelic ladder following all samples is also recommended. Additional ladders are recommended when setting up over 3 injections.

7. Add 1µL of amplified sample to each sample well of the reaction plate.

**Note:** If setting up only selected samples from an amplification plate for analysis on the 3500 Genetic Analyzer, analysts should be aware that the *amplification plate map* should be used for the well locations of the PCR product in the amplification plate and the 3500 sample setup plate map should be used for the destination.

**Note:** The 3500 instrument samples wells in sets of eight (A through H). Any extra wells in a set of eight should have either formamide or formamide/internal size standard included in them so that the capillaries are not descending into empty wells.

8. Place a 3500 septa mat onto the reaction plate. It is recommended that the 3500 septa mats are not trimmed. If the septa mat is trimmed down, it must be balanced on the opposite side so that the capillaries are not damaged. The plate may be spun down prior to denaturing.

9. Place the reaction plate in the Veriti® thermal cycler. Denature the samples by running the **denature** protocol (95˚C, 3 min, 11 µL).

**Note**: The cover plate of the Veriti® thermal cycler may be closed onto the septa mat-covered reaction plate, but do not clamp the cover down. Although the lid is not heated during the denature protocol, it may stick to the septa mat and pull it up when the cover is opened if it is clamped down.

10. Immediately after the denaturation protocol is finished, chill the reaction plate by immediately placing it the 96-well cooling block (-20˚C) for three minutes.

11. Place the plate into a 3500 Series 96-well plate base (it will only fit in one orientation) and cover with a 3500 Genetic Analyzer 96-well plate retainer. Check to ensure that the retainer clip is properly seated.

12. Press the **tray** button on the front of the 3500 Genetic Analyzer. When the autosampler tray presents itself, open the door of the instrument and place the tray onto the either position of the autosampler (the tray will only fit in one orientation). Close the instrument door.

REFERENCES:
GlobalFiler® PCR Amplification Kit User's Manual
Applied Biosystems 3500/3500xL Genetic Analyzer User Guide

Method: **Preparation of amplified samples for analysis on the Applied Biosystems 3500 using the QIAgility**

Date: 7/29/15 Revision Date: **10/21/2023** Approved by: ARD

REAGENTS:

1. HIDI Formamide: Applied Biosystems (P/N 4311320)

2. GeneScan™ 600 LIZ® Size Standard v2.0: Applied Biosystems (P/N. 4408399) The tubes may be stored for up to six months at 4°C.

3. The ladders used will be dependent on the kit:
   a) GlobalFiler® Allelic Ladder: (included in the GlobalFiler® kits) Store at −15 to −25°C upon receipt and at 2 to 8°C after initial use. Store protected from light up to the expiration date.
   b) Yfiler™ Plus Allelic Ladder: (included in the Yfiler™ Plus kits) Store at −15 to −25°C upon receipt and at 2 to 8°C after initial use. Store protected from light up to the expiration date.

MATERIALS:

1. MicroAmp Optical 96-Well Reaction Plates: Applied Biosystems (P/N N8010560)
2. MicroAmp Splash Free Support Base: Applied Biosystems (P/N 4312063)
3. Septa for 3500 Genetic Analyzers, 96 well: Applied Biosystems (P/N 4412614)
4. Retainer and Base for 3500 Genetic Analyzer, 96 well: Applied Biosystems (P/N 4410228)
5. 96-well cooling block: Stored in the reagent freezer

EQUIPMENT:

1. QIAgility: QIAGEN Corporation
2. Veriti® Thermal Cycler: Applied Biosystems
3. 3500 Genetic Analyzer: Applied Biosystems

PROCEDURE:

IMPORTANT: STRmix™ analysis requires the use of different analysis methods to analyze evidence and reference samples. To have multiple analysis methods within one project, each requires at least one ladder associated with it. To accomplish this, two ladders per analysis method should be prepared for electrophoresis on the 3500, or a single set of ladders can be prepared and the .hid files of the ladders can be duplicated within the run folder and imported into GeneMapper ID-X.

Note: Prior to preparing samples, the oven on the 3500 Genetic Analyzer can be preheated by clicking "**Start Preheat"** and the Veriti® thermal cycler can be turned on so that the top plate can come to temperature.

1. Create a master mix by combining the following volumes of reagents into a 5 mL flat tube:

| | | |
|---|---|---|
| 19.2 µL formamide | x | (# of samples + 20%) |
| 0.8 µL GeneScan-600 [LIZ] | x | (# of samples + 20%) |

   Caution: Formamide is a known teratogen and is harmful by inhalation, skin contact, and ingestion. Use in a fume hood and wear chemical resistant gloves and safety glasses when handling.

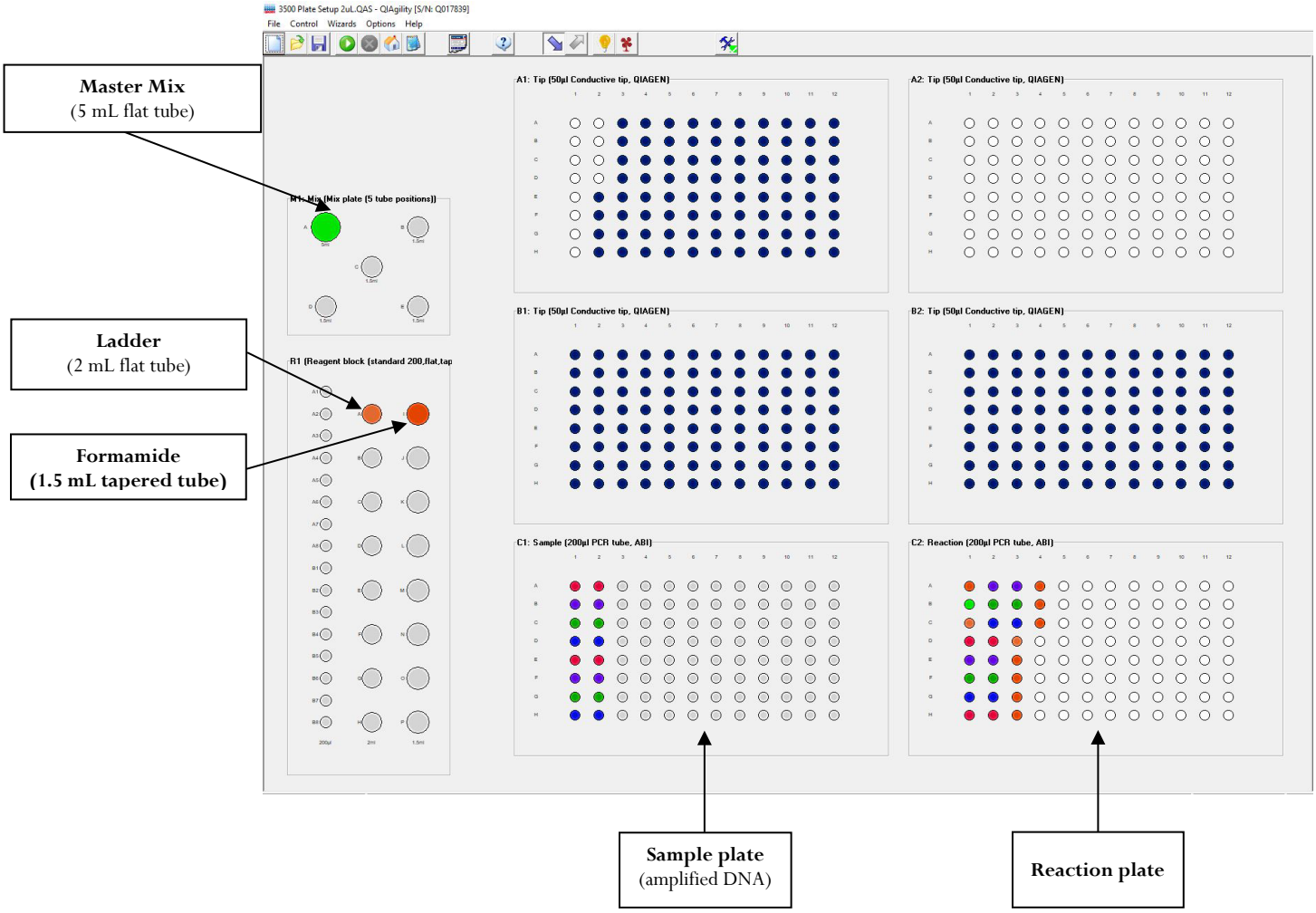
2. Vortex the master mix. Place the tube of master mix into Well A on the M1 (Mix Plate) deck on the QIAgility. **See diagram for deck layout and proper tube placement.**

3. Open the **3500 Plate Setup 2 µL** icon on the desktop to run the protocol for preparing GlobalFiler samples.

4. Place the amplified sample plate on the C1 Sample plate area on the deck. Place a new 96-well optical reaction plate on the C2 Reaction plate area on the deck.

5. Remove the cover from the 96-well optical reaction plate that contains amplified samples.

## Deck Layout Diagram




6. Place a 2 mL flat tube containing ample kit-dependent Allelic Ladder in Well A on the R1 (Reaction Plate) deck. Make sure the Ladder has been mixed (via vortexing) and spun down (in a microcentrifuge) prior to loading on the deck.

7. Place a 1.5 mL tapered tube containing at least **250 µL** formamide into Well I on the R1 (Reaction Plate) deck.

8. Look at the C1 Sample Plate on the computer monitor.

**8A. If you have more samples than are lit up with the colored indicators on the Sample Plate:**

Select the "**Sample Plate**" (there should be a red square around the plate). Highlight (click and drag or Ctrl and select the desired wells) to add the extra wells that you need to correspond with the number of samples that were amplified, including the positive and negative controls (a red square around the well indicates that the well has been selected).
Right click and select "**Add selected wells to sample bank**"

In the Update Sample Bank window, select "**Existing Bank**". If "Amplified Samples (# wells)" is not selected below "Existing Bank", select it. Click "Add Selection and Close". The added wells should now light up with color indicators and the Reaction Plate should automatically correlate with the Sample Plate. There will be 11 additional wells that are color indicated on the Reaction Plate: 1 for formamide only, 1 for master mix only, 2 for ladders (one in the well C1 and one in the well after the last sample), and 7 additional formamide only wells at the end to ensure that the capillaries are not descending into empty wells.

**8B. If you have fewer samples than are lit up with the colored indicators on the Sample Plate:**

Select the "**Sample Plate**" (there should be a red square around the plate). Highlight (click and drag) to select the wells that you need to remove to correspond with the number of samples that were amplified, including the positive and negative controls (a red square around the well indicates that the well has been selected).
Right click and select "**Remove selected wells from sample bank**"

Click "Delete Selection and Close". The previously lit wells should turn clear and the Reaction Plate should automatically be updated to correlate with the Sample Plate. There will be 11 additional wells that are color indicated on the Reaction Plate: 1 for formamide only, 1 for master mix only, 2 for ladders (one in the well C1 and one in the well after the last sample), and 7 additional formamide only wells at the end to ensure that the capillaries are not descending into empty wells.

9. Ensure that there are enough 50 µL tips for the run:

   Refill the tip racks in plate positions A2, B1, and B2 (if necessary). *If there are not enough tips for the run, the instrument will prompt you in the Checklist window when you attempt to start the run.*
   To replace the tips, remove the empty tip rack from the tip rack holder on the instrument and replace with a full rack of tips (the full tip rack will only seat one way on the tip rack holder). Using the mouse, highlight (click and drag) the tip positions that are now available based on where the tips were just added. Right click and choose "**Set selected tips to 'Available'**".

10. Either click on the **green dot with the arrow in it** on the shortcut tool bar, or choose "**Control"** on the tool bar and select "**Start**". In the Checklist window, click on each message box, such that there's an X in each box. Verify that the tip disposal box is not too full (empty it, if necessary) and click **OK**.

11. The robot will now add the master mix, formamide blanks, ladders and DNA samples to the 96-well optical reaction plate.

12. When the protocol has finished, the sample plate on C1 on the deck (the plate containing the original amplified samples) can be covered with a foil cover or strip caps for freezer storage.

    Remove the reaction plate from the C2 deck position and place a 3500 plate septa mat on it. It is recommended that the 3500 septa mats are not trimmed. If the septa mat is trimmed down, it must be balanced on the opposite side so that the capillaries are not damaged. The plate may be spun down prior to denaturing.

13. Place the reaction plate in the Veriti® thermal cycler. Denature the samples by running the **denature** protocol (95˚C, 3 min, 20 µL).

**Note**: The cover plate of the Veriti® thermal cycler may be closed onto the septa mat-covered reaction plate, but do not clamp the cover down. Although the lid is not heated during the denature protocol, it may stick to the septa mat and pull it up when the cover is opened if it is clamped down.

14. Immediately after the denaturation protocol is finished, chill the reaction

plate by immediately placing it the 96-well cooling block (-20°C) for three minutes.

15. Place the plate into a 3500 Series 96-well plate base (it will only fit in one orientation) and cover with a 3500 Genetic Analyzer 96-well plate retainer. Check to ensure that the retainer clip is properly seated.

16. Press the **tray** button on the front of the 3500 Genetic Analyzer. When the autosampler tray presents itself, open the door of the instrument and place the tray onto the either position of the autosampler (the tray will only fit in one orientation). Close the instrument door.

Select sample re-preparation:
In order to re-prepare select samples from the 96-well plate of amplified samples, highlight all of the wells that you **do not** want re-prepared and delete them from the sample plate following step 8B.

REFERENCES:
GlobalFiler® PCR Amplification Kit User's Manual
QIAgility Users Manual

Method: **Decontaminating the QIAgility Using the Automated UV Protocol**

Date: 10/21/2023 Approved by: ARD

EQUIPMENT:

1. QIAgility - QIAGEN Corporation

PROCEDURE:

1. With the "3500 Plate Setup 2µL" program open, click on the UV Light icon (the yellow light bulb).
2. In the "UV Lamp Control" window, choose the length of time you wish the UV lamp to be active by moving the arrows up or down in the "Timed Control" section (a minimum of 5 minutes is recommended).
3. Select Start.
4. An alert will display asking to ensure the tip ejector is present and the lid is closed. Ensure the lid is closed and select "Yes"
5. When the UV light operation has completed, click "OK".

REFERENCES:
QIAgility Users Manual

Method: **Capillary Electrophoresis of Amplified Samples on Applied Biosystems 3500 Genetic Analyzer**

Date: 7/29/15 **Revision Date: 02/05/2018** Approved by: ARD

MATERIALS:

1. 3500 Data Collection Software (Series 2)
2. Optional: Plate record file (.txt) from SIMS worksheet

PROCEDURE:

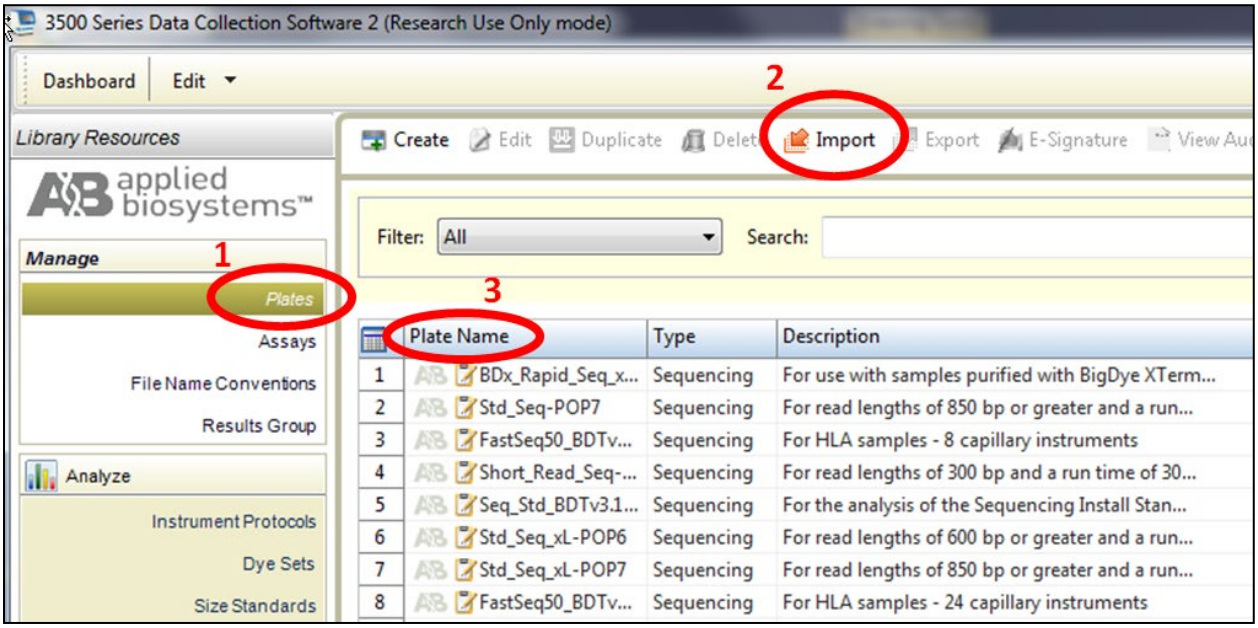
1. Launch the **3500 Series Data Collection Software 2**.

2. Check the dashboard to verify that maintenance has been done, consumables are not expired, and buffer levels are at fill line. If they are not at the fill line (or within a few millimeters), they need to be replaced with a new container of buffer.

3. If not already done during plate setup, preheat the oven by clicking **Start Pre-Heat**.

4. Check pump assembly for bubbles and run the Remove Bubble wizard if needed by clicking the Maintain Instrument button, the blue Maintenance Wizards button on the left, and the Remove bubbles button.

5. To import a plate record (.txt file created from the SDPD SIMS), click on **Library** (on the top panel). Make sure that **Plates** is selected on the left panel and click **Import** (top panel). Navigate to where the .txt file is saved (if you don't see it, ensure that it is searching for .txt files).

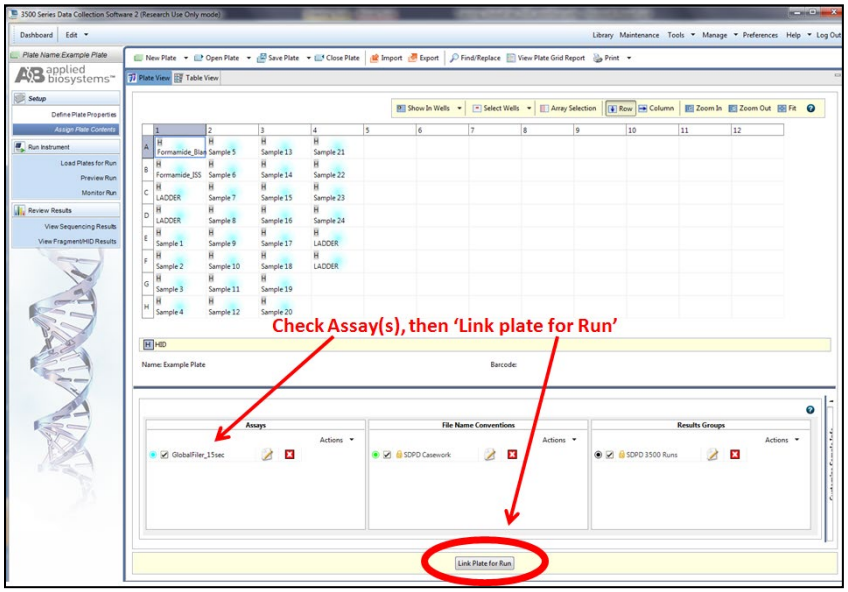

6. After creating the plate file, find it on the list of plates, click on it, and then click **Edit**. This will bring up the plate view, so that sample names and Assays can be edited.

7. Once file is imported, check sample type and assay information for each well using Plate View or Table View (***see Figure below*** for an example of Plate View). In plate view, hover over each well to check that all fields are correct.

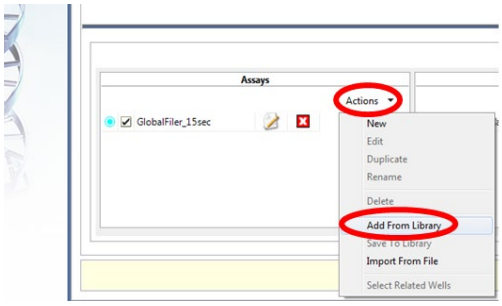
**The colored dot next to the sample should correspond to the assay listed in the Assays box.** Details of Assay names are listed below.

*Alternatively*, a plate can be created by clicking **Create New Plate** on the dashboard screen, manually entering plate name and assigning plate contents.

a. Sample Name: Analyst designated name for each sample and control
b. Sample Type: Each sample should be designated appropriately
    i. Allelic Ladder
    ii. Positive Control
    iii. Negative Control
    iv. Sample
c. Assay Name:
    i. **GlobalFiler_15sec** - (Default protocol)
    ii. **GlobalFiler_24sec** - (For use when samples need to be injected for a longer period of time)
    iii. **GlobalFiler_10sec** - (For use when samples need to be injected for a shorter period of time)
    iv. **GlobalFiler_5sec** - (For use when samples need to be injected for a shorter period of time)
    v. **Yfiler Plus_10sec** - (Default protocol)
    vi. **Yfiler Plus_16sec** - (For use when samples need to be injected for a longer period of time)
    vii. **Yfiler Plus_5sec** - (For use when samples need to be injected for a shorter period of time)
d. File Convention Name: SDPD Casework
e. Results Group Name: SDPD 3500 Runs




8. If changes need to be made to any of the assays, click **Actions** in the Assays box, and then **Add from library**. Add the Assay(s) you need from the list in step 7c.




Once the relevant assay is listed in the Assays box, you can highlight the well(s) that need to be corrected, and uncheck the incorrect Assay, and click a check mark next to the relevant one for the corresponding highlighted samples. Use the color indicator and hovering the mouse to verify the correct parameters are being used for your samples, OR use Table view in the top left for a list of parameters associated with that sample.

**Note:** ***Make sure that the empty wells do NOT have an Assay assigned to them. A colored dot indicates that data collection for that well is desired, and the capillary electrodes will descend into empty wells.

9. Load the plate by pressing the **tray** button on the front of the 3500 Genetic Analyzer. When the autosampler tray presents itself, open the door of the instrument and place the plate (clipped into the plate retainer) onto the either position of the autosampler (the tray will only fit in one orientation).

**Note**: Make sure to slide the plate in towards the stationary center clip first and then lower it in to the side clip. Ensure that it is seated properly and clipped in. Close the instrument door.

10. Once plate is loaded and sample details are assigned, click **Link Plate for Run.** Make sure that plate is assigned to the corresponding position on the autosampler (A or B).

**Note**: If two plates need to be started at the same time, make sure they are both loaded and on the appropriate position, click Link "Plate for Run". Plate file needs to be correct or pre-edited, because once it is linked, there is no option to edit this second plate.

11. Click **Start Run**. The run will continue until all specified injections are completed. It is not necessary to rename the run, because files will be stored on the local computer in a folder that is named by: ***Plate name.***

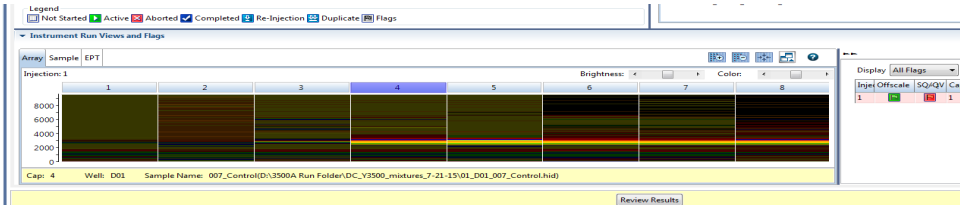
**To Review samples on the 3500 software and Re-inject *with the same Assay***

Results can be viewed on the 3500 software by clicking **View Fragment/HID Results.** This can help to determine if any samples need to be re-injected without having to transfer them to a computer with GeneMapper ID-X. Peak heights and shape can be assessed with this viewer. Use the cursor on the axes to zoom. Note: OL alleles will not be flagged in this view.

If a sample needs to be re-injected ***with the same Assay***, (for example, a sample is identified with poor resolution of the size standard), this can be accomplished within the same run IF the run is still going. Once the injections have completed, or if a re-injection with a different Assay is required, a new run should be started for that re-injection.

To re-inject (with the same assay), select the sample of interest on the plate. It is also necessary to select only that particular sample on the array view (see below), so that re-injection data are not collected for well in that injection. In, injection, or in the array view select the capillary that

corresponds to the well or sample of interest, then click Re-inject. In the Re-injection dialog box, select **original protocol**. Do not use this method for any other type of re-injection. The re-injection will be added to the bottom of the list and saved into the same results folder. The file will have the same name, but a number in parenthesis at the end to distinguish it from the original injection.




12. Run will automatically complete.

13. Data files (.HID) are located on the computer connected to the 3500 Genetic Analyzer (D:AB SW & DATA>3500 Run Folder). Move the Run Folder into the individual Analyst's Folder on the FB network (H:) prior to any analysis using GeneMapper ID-X (v1.4).

**Re-injections**

If a re-injection is necessary, either using the same assay or different ones, set up a new run.

14. Click the blue button in the dashboard **Edit Existing Plate**.

15. Find your previous plate and open it.

16. Re-name the plate if you want the .HID files to go into a different folder. If not, the re-injected files will go into the same folder and will have *(2)* after them.

17. On the plate view page in the Assays box in the lower left, add all the assays you want to use by clicking **Actions** in the assay box below and choose **Add from Library**.
    a. If a longer injection is desired, choose GlobalFiler_24sec
    b. If a shorter injection is desired, choose GlobalFiler_10sec or GlobalFiler_5sec.

18. Highlight all wells and clear the existing Assay from them.

19. Highlight only the wells you want to re-inject and use the check box (under Assays) to assign the correct Assay. There should only be colored dots in the wells that you want to collect data from.

20. Click **Link Plate for Run** and **Start Run**.

21. Examine data and proceed as described above.

REFERENCES:
GlobalFiler® PCR Amplification Kit User's Manual
Applied Biosystems 3500/3500xL Genetic Analyzer User Guide
Applied Biosystems 3500/3500xL Genetic Analyzer User Bulletin

Method: **GeneMapper ID-X™ Analysis of Data**

Date: 06/01/2006 **Revision Date: 10/05/2015** Approved by: ARD

INTRODUCTION:

GeneMapper™ ID-X software is an automated genotyping software package that will genotype samples based on allelic bin definitions stored within the Panel Manager. The AmpFlSTR panels and bins are pre-installed in the Panel Manger within the software. Custom SDPD Analysis Methods have also been created in the GeneMapper Manager for use in analyzing raw data files for GlobalFiler®, Identifiler Plus, Identifiler, Yfiler™, and Yfiler™ Plus amplifications, respectively. An analysis method defines the analysis parameters to be used for the analysis of samples imported into the Project Window. When the GeneMapper™ ID-X software is installed on any computer, the Panel, Bin Set and Analysis Method have to be defined prior to the analysis of raw data files. See also the procedure for importing panels and bins.

MATERIALS:

Applied Biosystems GeneMapper™ ID-X Software

PROCEDURE:

IMPORTANT: STRmix™ analysis requires the use of different analysis methods to analyze evidence and reference samples. To have multiple analysis methods within one project, each requires at least one ladder associated with it. To accomplish this, the .hid files of the ladders can be duplicated within the run folder and imported into GeneMapper ID-X, or two ladders per analysis method can be prepared for electrophoresis on the 3500.

1. Open the GeneMapper™ ID-X software by clicking on the icon located on the computer's desktop and log-in. Type in or select your user ID from the drop-down menu as the User Name. The password is case sensitive. Upon successful log-in the **Project Window** will open. The project window is the primary window for the user interface.

2. To add samples to the Project Window. Select **Edit>Add Samples to Project** or click the icon.
   For samples run on the 3130:
   - Navigate to Forensic Biology on FB server H:\3500 Run Folders to locate the desired run folder.
   - Select the desired run folder and click **Add To List**. The selected folder will appear in the **Samples To Add** box on the right. Note: Multiple run folders can be added to the same project. Click **Add** to import the files into the project and close the dialog box.

3. For each sample within the **Project Window** select the appropriate **Sample Type**, **Analysis Method**, **Panel**, **Size Standard** (see below). Unknowns should be designated with Sample Type "Sample", Allelic Ladders should be designated with Sample Type "Allelic Ladder", the positive PCR control should be designated with Sample Type "Positive Control", and any reagent blanks, amplification blanks, or formamide blanks should be designated with sample type as "Negative Control".

| Kit | Analysis Method | Panel | Size Standard |
|---|---|---|---|
| **GlobalFiler** | GF_reference | GlobalFiler_2019 | GS600_LIZ |
| **GlobalFiler** | STRmix_GF_evidence | GlobalFiler_2019 | GS600_LIZ |
| **Yfiler Plus** | YF+_evidence | Yfiler Plus 2018 | GS600_LIZ |
| **Yfiler Plus** | YF+_reference | Yfiler Plus 2018 | GS600_LIZ |

Note: In general, analysis parameters will not change with the exception of the analysis range which will be dependent on the electrophoresis of each run.

4. Click ► (Analyze) and the Save project dialog box opens. Save the project with a uniquely identifying name [e.g., initials(Case #)]. After analysis, the project is automatically saved, and the Genotypes Tab becomes available. Analyzing the data sizes and genotypes the samples in the project. There must be at least one ladder in each run folder analyzed for the analysis to be successful.

5. Accurate application of the size standard to the samples can be verified in a variety of ways. One method uses the Process Component Quality Values (PQVs) **Sizing Quality** flag as an efficient method of verifying the sizing of individual samples. In the Samples tab of the Project Window the sizing

quality of each sample is displayed as a symbol (■ = Pass, ▲ = Check sizing for problems, or ● = Low Quality, sizing failed).

6. The individual sample files can be reviewed and printed for the analytical record. If needed, labels can be edited within the samples. To edit labels, select the peak to be edited, right-click and select the applicable option of changing the artifact or allele label. An Edit Allele Comment dialog box will open and a reason for the change can be input. Sample plots with edited labels should be printed for the analytical record with the edit displayed in the plot. Labels may be changed but must not be deleted.

7. PQVs can be used to assess the quality of a variety of aspects of each sample. For a full list of the PQVs please use the GeneMapper™ ID-X help function within the software.

8. When the analysis of the GeneMapper ID-X project is completed the project file should be exported to the appropriate folder on the Forensic Biology network. To export the project: highlight the project in the **Projects** tab in **GeneMapper Manager** and click the **Export** button. Browse for the appropriate folder on the network (**Forensic Biology on FB server (**H:\GeneMapperID Projects) and save the exported project using the Project name. Use of analysts initials at the start of the file name will be useful in locating projects as the number of files increases and is highly recommended [e.g., inits(XX-XXXXXX; etc.)].

9. The original project should be deleted from the GeneMapper ID-X database once the project is exported. To delete the original project: highlight the project in the **Projects** tab in **GeneMapper Manager** and click the **Delete** button. Prior to deleting the project it is recommended to check that the file was exported to the appropriate folder.

10. To view exported GeneMapper ID-X projects. Open **GeneMapper Manager** and from the **Projects** tab click **Import**. Browse to **Forensic Biology on FB server (**H:\GeneMapperID Projects**)** and select the desired project. Click **OK**. The project will have been imported to the GeneMapper ID-X database and can be opened as usual. When the imported project is no longer needed it should be deleted from the database (see step 9).

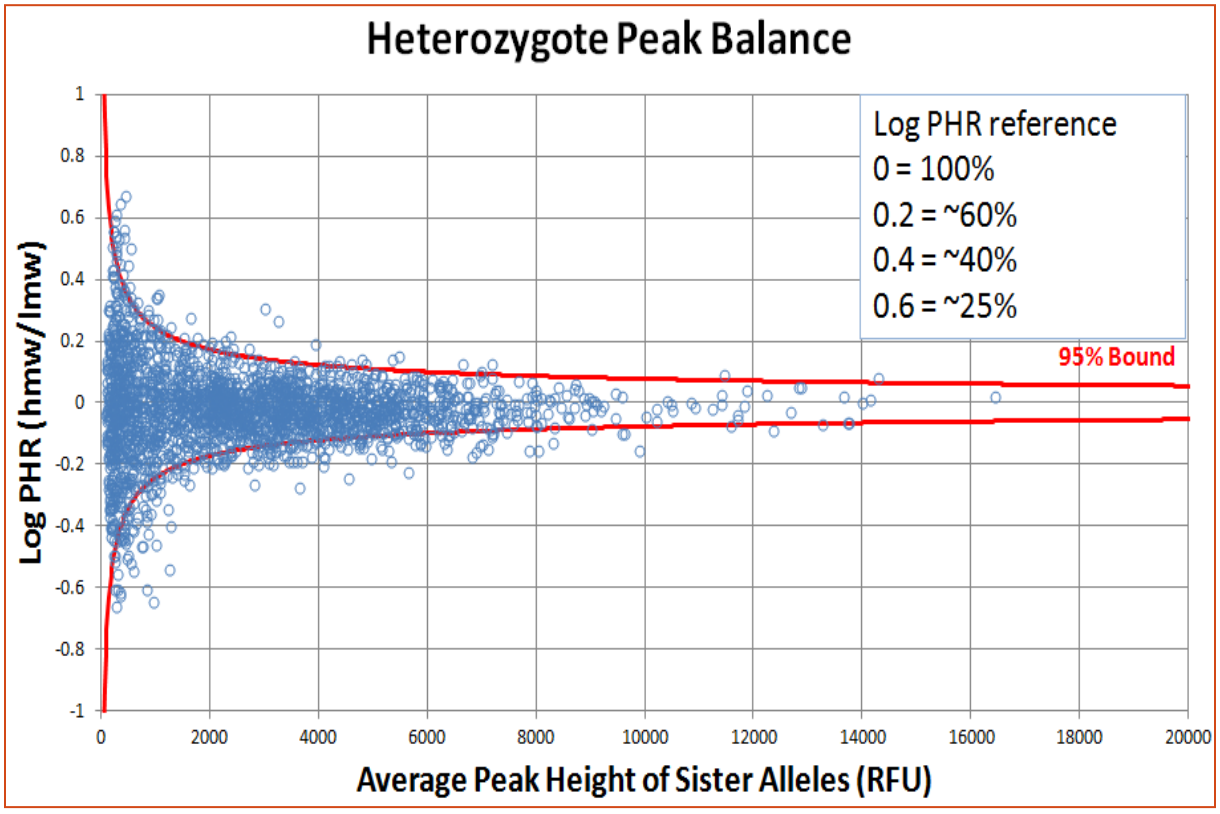
REFERENCES:

GlobalFiler PCR Amplification Kit User's Manual
AmpF*l*STR Identifiler Plus™ PCR Amplification Kit User's Manual
Yfiler™ Plus PCR Amplification Kit User's Manual
GeneMapper™ ID-X Software version 1.4 User Manual.
GeneMapper™ ID-X Software Tutorial.

Method: **GlobalFiler Interpretation Thresholds**

Date: 10/05/2015 **Revision Date: 08/12/2019** Approved by: ARD

- Analytical Threshold: A peak detection threshold of 100RFU will be used for analysis of GlobalFiler data. Peaks below 100RFU can be used qualitatively to determine the possible presence of a mixture or to determine the number of contributors.
- Homozygous allele peaks are approximately twice the height of heterozygous allele peaks as a direct result of allele dosage.
- Peak Height Ratio: At heterozygous loci, the ratio of the height of the lower RFU peak to the height of the higher RFU peak is generally above 60% (see Figure below where stutter ratios are presented in log format to also show the relationship between molecular weight), however, peak height ratios tend towards larger imbalances as the height of the peaks decreases toward the analytical threshold. Red lines are 95% boundaries.

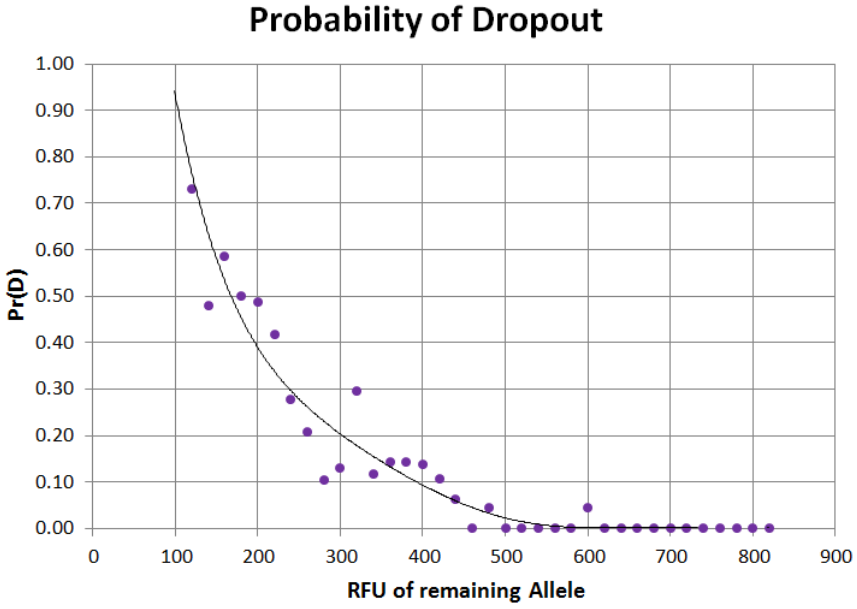


- Stutter Threshold: n-1 repeat stutter peaks will be filtered by the GMID-X software for reference samples when they are below the percent cutoff value for a locus. The software will also filter n+1 repeat stutter and n-1/2 repeat stutter for those loci that have those phenomena. Evidence samples will be analyzed in GMID-X with no stutter filters. Data from the SDPD GlobalFiler Stutter Study has demonstrated that true n+1 stutter peaks may occur above three standard deviations from the mean stutter ratio. More detailed information can be obtained by consulting the stutter data tables created in conjunction with the validation study. Please see the autosomal interpretation guidelines for more information regarding stutter. The stutter cutoff values used by the software for reference sample analysis are based on our internal validation and are presented below:

| Locus | Stutter Filter (%) | | | |
|---|---|---|---|---|
| | GMID-X *N-1* | GMID-X *N+1* | GMID-X *N-.5* | GMID-X *N-2* |
| D3S1358 | 14.25 | 2.75 | 0 | 3.05 |
| vWA | 13.70 | 6.30 | 0 | 2.25 |
| D16S539 | 13.65 | 3.00 | 0 | 2.80 |
| CSF1PO | 12.85 | 3.35 | 0 | 1.30 |
| TPOX | 5.50 | 0 | 0 | 0.40 |
| D8S1179 | 12.70 | 5.85 | 0 | 2.00 |
| D21S11 | 12.45 | 9.40 | 0 | 2.05 |
| D18S51 | 15.65 | 8.95 | 0 | 3.65 |
| DYS391 | 14.45 | 0 | 0 | 3.15 |
| D2S441 | 8.70 | 2.20 | 0.65 | 1.15 |
| D19S433 | 13.10 | 2.65 | 0 | 2.55 |
| TH01 | 5.75 | 1.70 | 0 | 0.50 |
| FGA | 16.40 | 4.00 | 0 | 3.30 |
| D22S1045 | 19.10 | 7.50 | 0 | 3.00 |
| D5S818 | 12.80 | 9.05 | 0 | 1.50 |
| D13S317 | 11.80 | 3.35 | 0 | 1.25 |
| D7S820 | 13.65 | 2.35 | 0 | 2.80 |
| SE33 | 20.45 | 9.10 | 5.55 | 6.00 |
| D10S1248 | 15.25 | 2.80 | 0 | 3.50 |
| D1S1656 | 14.25 | 4.45 | 6.85 | 3.05 |
| D12S391 | 16.40 | 2.10 | 0 | 4.05 |
| D2S1338 | 17.65 | 13.20 | 0 | 4.70 |

Another type of stutter artifacts includes n-2 repeat stutter. n-2 repeat stutter general is detected when amplification of the parent peak is robust. The SDPD validation studies have established that the expectations for the ratio of the n-2 repeat stutter peak to the n-1 repeat stutter peak is equivalent or less than the ratio of the n-1 repeat stutter peak to the parent peak. In general, if the parent peak is robust, a peak in an n-2 position may be stutter if it is less than 1.5 times the n-1 repeat stutter peak's stutter percentage. STRmix v2.9 now models n-2 stutter, so the guideline above should be used in determining number of contributors, but the n-2 stutter peaks do not need to be edited out.

- Stochastic Threshold: Validation data have been used by STRmix™ to establish a probability of drop-out for alleles of various peak heights. This probability of drop-out is used in lieu of a traditional stochastic threshold (or homozygote peak height threshold). Analysts should be aware of what a theoretical stochastic threshold may be when evaluating GlobalFiler DNA profiles. As such, the following graphical representation (histogram) is an approximation of the STRmix™ probability of drop-out graph.



Probability of Identity using the GlobalFiler kit.

The Probability of Identity ($P_I$) [Sensabaugh 1982] is the probability that two individuals selected at random will have an identical GlobalFiler kit genotype. For the GlobalFiler kit the $P_I$ are as follows:

1 in 26 septillion in the U.S. Caucasian population,
1 in 299 sextillion in the Asian population,
1 in 16 septillion in the African American population,
1 in 32 septillion in the Hispanic population.

The $P_I$ expresses the average 21 locus DNA profile frequencies using the Applied Biosystems GlobalFiler kit. This data was taken from the GlobalFiler user's manual page 114.

REFERENCES:
SDPD GlobalFiler Validation studies
GlobalFiler User's Manual

Method: **Yfiler™ Plus Interpretation Thresholds**

Date: 02/05/2018 Approved by: ARD

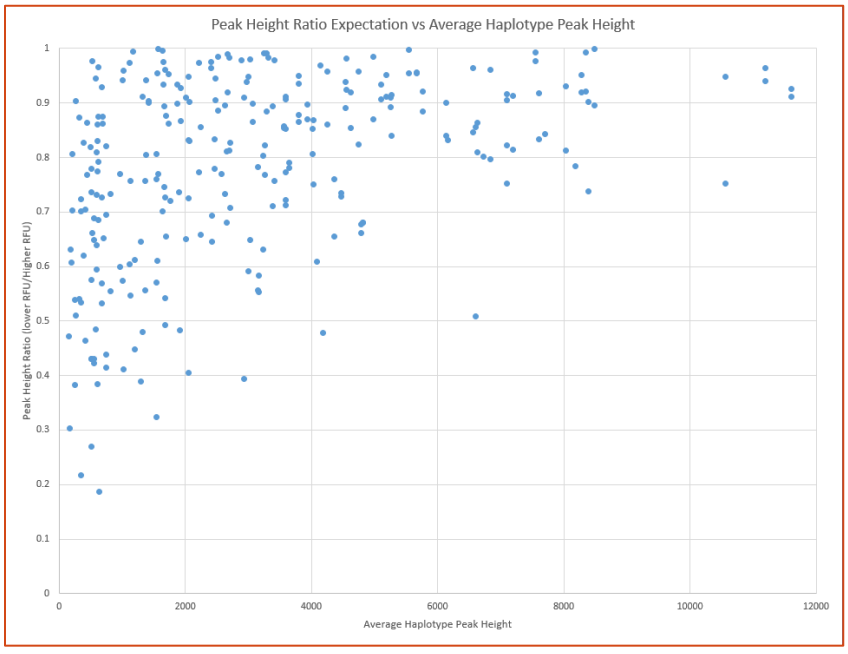
- Analytical Threshold: A peak detection threshold of 100RFU will be used for Yfiler™ Plus amplification kit. Peaks below 100RFU can be used qualitatively to determine the possible presence of a mixture or to determine the number of contributors.

- Stutter Thresholds: n+1/n-1 repeat stutter products are common in the tri- and tetranucleotide repeat loci. n-1 repeat stutter does occur to a lesser degree in the penta- and hexanucleotide repeat markers while n+1 repeat stutter is rarer. Additional stutter products or artifacts other than full repeat stutter have been observed at various loci in the Yfiler™ Plus kit. Reference sample analysis will use a global 30% cutoff and the stutter thresholds below will not apply. The stutter cutoff values used by the software for evidence samples are presented below:

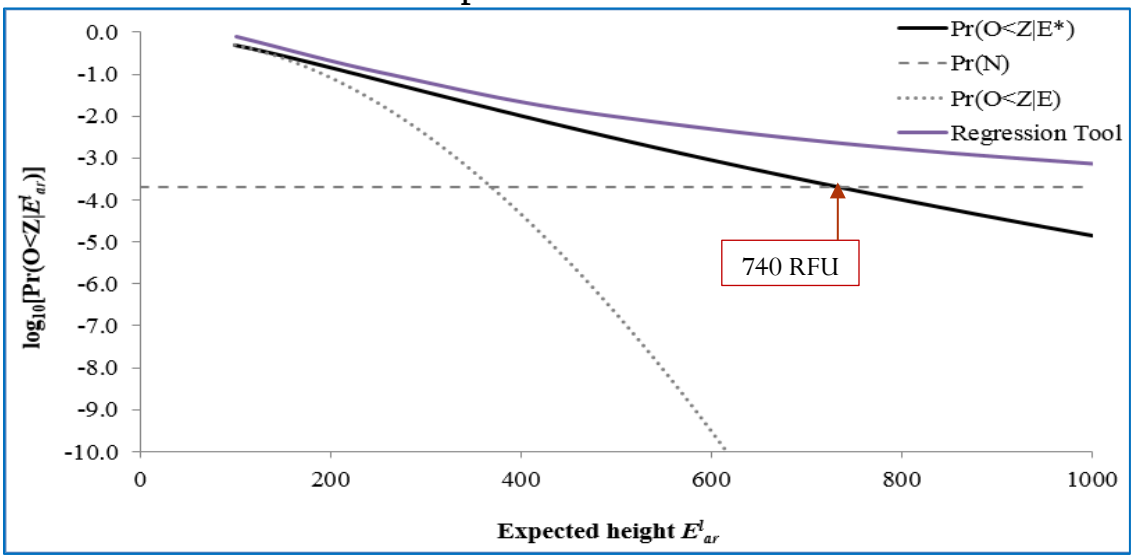
| Yfiler Plus GMID-X Stutter Filters | | | | | | |
|---|---|---|---|---|---|---|
| Locus | n-1 Repeat Stutter Filter | n+1 Repeat Stutter Filter | n-2nt Stutter Filter | n+2nt Stutter Filter | n-1nt Filter | n-5nt Stutter Filter |
| DYS576 | 0.1473 | 0.0401 | | | | |
| DYS389I | 0.0880 | 0.024 | | | | |
| DYS635 | 0.1209 | 0.0175 | | | | |
| DYS389II | 0.1831 | 0.0447 | | | | |
| DYS627 | 0.1465 | 0.0330 | 0.0365 | | | |
| DYS460 | 0.1136 | 0.0211 | | | | |
| DYS458 | 0.1493 | 0.0230 | | | | |
| DYS19 | 0.1259 | 0.0210 | 0.1219 | 0.03 | | |
| YGATAH4 | 0.1127 | 0.0232 | | | | |
| DYS448 | 0.042 | n/a | | | | |
| DYS391 | 0.1006 | 0.0133 | | | | |
| DYS456 | 0.1511 | 0.0306 | | | | |
| DYS390 | 0.1383 | 0.0153 | | | | |
| DYS438 | 0.0562 | insufficient data | | | | |
| DYS392 | 0.1779 | 0.1090 | | | | |
| DYS518 | 0.2402 | 0.0471 | | | | |
| DYS570 | 0.1448 | 0.0220 | | | | |
| DYS437 | 0.0843 | 0.0170 | | | | 0.022 |
| DYS385 | 0.1603 | 0.0162 | | | | |
| DYS449 | 0.2255 | 0.0583 | | | | |
| DYS393 | 0.1368 | 0.0346 | | | | |
| DYS439 | 0.1013 | 0.0247 | | | 0.016 | |
| DYS481 | 0.2893 | 0.0470 | 0.1836 | | | |
| DYF387S1 | 0.1339 | insufficient data | | | | |
| DYS533 | 0.1177 | 0.0360 | | | | |

Another type of stutter artifacts includes n-2 repeat stutter. n-2 repeat stutter general is detected when amplification of the parent peak is robust. The SDPD validation studies have established that the expectations for the ratio of the n-2 repeat stutter peaks in the stutter study for Yfiler™ Plus. As with autosomal STR markers, a peak in an n-2 position may be stutter if it is less than 1.5 times the n-1 repeat stutter peak's stutter percentage. Analysts should use caution when making determinations on n-2 stutter peak removal from mixtures. Analysts are encouraged to use the data in the stutter study as a guide for making determinations on n-2 stutter.

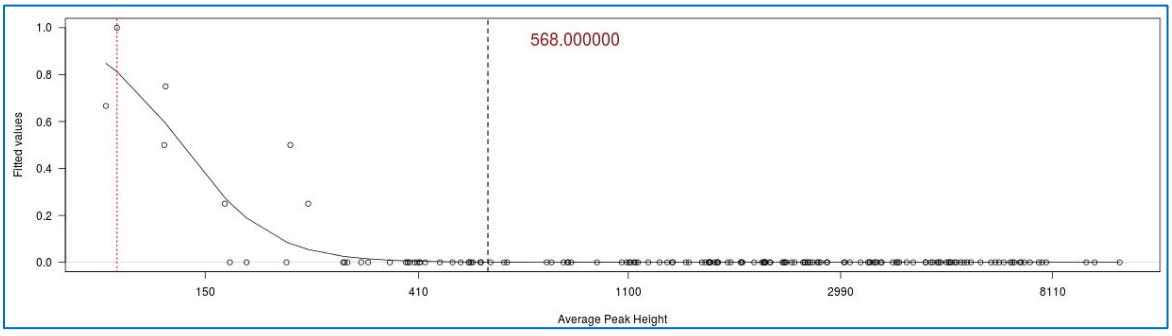
The Yfiler™ Plus amplification kit amplifies two duplicated loci on the Y-chromosome (DYS385a/b and DYF387S1a/b). The amplification products at these loci can be either homoallelic (the same allele present at both gene sites), or heteroallelic (different alleles present at each gene site). In general, homoallelic peaks are approximately twice the height of heteroallelic peaks as a direct result of allele dosage. When heteroallelic combinations occur, the result is analogous to heterozygous genotypes in autosomal loci in that peak height ratios can be determined and compared to peak height ratio expectations. As with heterozygous genotypes, heteroallelic genotypes generally have peak height ratios above 0.6 and tend towards larger imbalances as the heights of the peaks decrease toward the analytical threshold.



- Stochastic Threshold (probability of drop-out): There are two ways in which drop-out can be considered; either dropout has occurred for one allele of a duplicated locus, or dropout has occurred for a non-duplicated locus and so no information is seen. There is also the possibility that the absence of information at a locus is due to a silent allele. The comparison between the probability of drop-out and the probability of a silent allele for duplicated and non-duplicated loci yields two interpretational thresholds:
    - Non-duplicated loci: the peak heights of alleles across the profile must exceed 740 RFU to consider an allele at a particular locus to be silent rather than absent due to drop-out.




    - Duplicated loci: when a single peak is present, the height of that peak must exceed 570 RFU for the locus to be considered complete (ie. drop-out has not occurred and both alleles have the same number of repeats or the second allele is silent).



Discrimination Capacity (DC) using the Yfiler™ Plus kit.

The discrimination capacity (DC) of the Yfiler™ Plus kit is the measure of number of unique haplotypes in a population. The discrimination capacity of the Yfiler™ Plus amplification kit was measured at a probability 0.9979 that two individuals selected at random will have a different Yfiler™ Plus haplotypes [data from Applied Biosystems courtesy of Katherine Gettings at NIST].

REFERENCES:

SDPD Yfiler™ Plus Validation studies
Yfiler™ Plus User's Manual

METHOD: **STRmix™ Analysis**
Date: 10/05/2015 Revision Date: 06/09/2025 Approved by: ARD

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The following is a procedure for performing STRmix™ analysis after CE of samples on the 3500. Not all results will be suitable for STRmix™ analysis. Please refer to the Autosomal STR Interpretation Guidelines for additional information. The end of this protocol has a list of the various settings for STRmix™ analyses.

STRmix™ is a software program that applies a fully continuous approach to DNA profile interpretation. It standardizes the analysis of profiles in the laboratory by using estimates of variance of results derived from validation data. STRmix™ can be used to analyze samples with or without reference samples.

STRmix™ requires input data describing an electropherogram (epg) in order to run. Like all software, STRmix™ requires data to be formatted in a specific way for it to be entered. Non numeric values such as "OL" are not permitted within the STRmix™ input files. Unambiguous alleles including those that are rare should appear in the corresponding input file as their actual allelic size designation, for example D21 33.1. Where the designation of alleles is ambiguous, for example alleles more than two repeat units off the end of the allelic ladder, the alleles may be assigned an allelic value not observed in the population data (e.g., allele 60).

There is no function to accommodate somatic mutations or trisomy in STRmix™ calculations. If a profile has a triallelic STR pattern, STRmix™ can still use the input data if the locus is ignored. Sample names cannot contain commas (","). These issues must be corrected prior to exporting data from GeneMapper ID-X.

Sometimes the complexity of the profiles to be analyzed will be beyond the computing capacity of the computers within the Biology section. This will either cause STRmix™ to fail during analysis and/or presenting an out of memory

error. Alternatively, STRmix™ will run sufficiently slowly that an analysis will take weeks to reach completion. If an analysis does not complete due to an out of memory error, or is taking a very long time to complete, STRmix™ must be run in low memory mode such that the analysis is more likely to run to completion. Even with low memory mode, there are some mixtures that still may not run to completion. Results for samples that fail to complete in low memory mode are to be reported with the following statement:

*This sample is unsuitable for comparisons because the results exceed the memory capacity of the interpretational software.*

Additionally, sometimes evidence profiles may run, but a 99% lower HPD cannot be calculated because the Effective Sample Size returns NaN (not a number). This may be due to a missing stutter peak in the text file, and the data should be evaluated to see if a corrected file should be analyzed. If no obvious reason is apparent for the inability to calculate the 99% lower HPD, the sub-sub-source LR can be reported instead of the HPD value. This can be accomplished by deselecting Calculate HPD in the window in Figure 4. When necessary, the report should specifically reflect that the calculation presented is a point estimate of the LR.

**Exporting files from GeneMapper ID-X**

***Export Reference Files for STRmix™***

1. Choose Table Setting: **4 – STRmix™ reference export**

2. Select the REFERENCE samples that will be run through STRmix™, and **display plots.** Multiple reference samples can be exported at one time in one text file.

3. Choose to display **Genotypes Table**
   *Notes about this table: it should have "Sample File", and "Marker".*

4. **Export table**, and save to desired location.

5. Close these plots.

*Export Evidence Files for STRmix™*

1. Choose Table Setting: **3 – STRmix™ evidence export**

2. Select the EVIDENCE samples that will be run through STRmix™, and **display plots**

3. Choose to display **Genotypes Table**
   *Note: this table should have "Sample File", "Marker", "Allele" columns, corresponding "Size" columns, and corresponding "Height" columns across the top row. The number of columns no longer needs to be specified in STRmix, so the number can be increased to ensure that all the data is in the input file.*

4. **Export table**, and save to desired location.

5. Close these plots.

***Manual creation of files for STRmix™ input***

STRmix™ allows the user to calculate a likelihood ratio when the questioned sample and reference samples are analyzed in different autosomal profiling kits. LRs will only be provided for those loci in common between the two kits. For example, if you had a questioned sample analyzed with GlobalFiler and a reference analyzed in Identifiler™, or Identifiler Plus™, then you would be able to provide a LR using the DNA loci in common. For this analysis both the questioned sample and the reference sample have to input from appropriately formatted .txt files. This function also works with LR from Previous Analysis, provided .txt files are input. If an evidence and/or reference sample was previously analyzed with STRmix, the .csv file created in the Inputs folder for the previous run can be used instead of generating a new .txt file.

A Reference File Maker Excel workbook has been created to assist in the creation of the GlobalFiler reference files that were either: 1) not produced by the SDPD lab, or 2) not typed with the GlobalFiler typing kit. The format for the reference profile format must contain the *"Sample File", and "Marker"*.

If a STRmix™ deconvolution of a GlobalFiler evidence sample requires an assumed person, whose reference is in Identifiler format, it is recommended that the reference sample be re-profiled with GlobalFiler such that data from all loci will be used, perform the deconvolution without the assumption.

An additional option would be to use the Reference File Maker Excel workbook to make a reference file for the Identifiler reference data and perform the deconvolution conditioning using the "Ignore Locus" function for the loci without reference data; however, this will lower the discrimination power of the analysis.

### STRmix™

Start STRmix™ by double-clicking the STRmix™ icon on the desktop or select STRmix™ from Start > STRmix™. The STRmix™ main menu will display (see Figure 1).

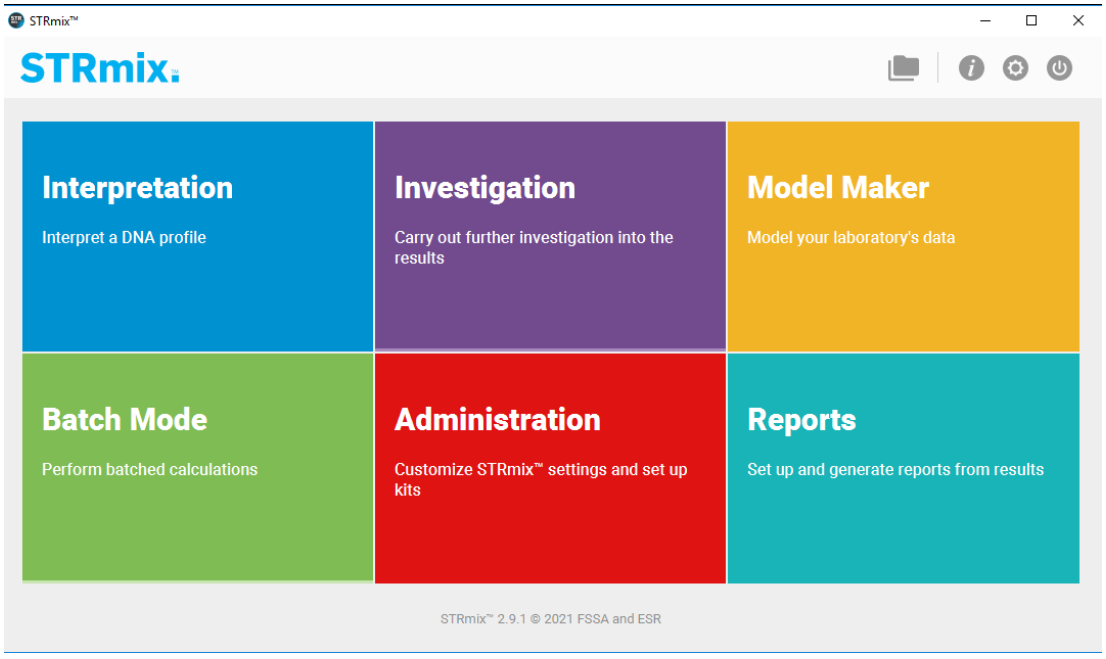



FIGURE 1

Each of the functions within STRmix™ can be accessed from the home screen.

"Interpretation" allows deconvolution of evidence samples.

"Investigation" evaluates previously deconvoluted evidence samples. It calculates LRs from reference samples, compares to a database of references, compares to a database of random samples (Hd True Tester), and can create batches of LR calculations and database searches. "Investigation" also has two functions, "Mix To Mix" and "Top Down Approach", which are currently not used by the SDPD crime Lab.

"Batch Mode" allows batching of every type of analysis and investigation.

"Model Maker" and "Administration" were both used during validation to create the analysis kits and settings used in casework at SDPD.

Reports are automatically generated with this version of STRmix, but the "Reports" button can generate additional or custom reports, if necessary.

The "Interpretation", "Investigation", and "Batch Mode" functions are key to evidence sample interpretation and reference comparison and are described in detail below.

To exit the software, click the top right-hand X button or circular exit button.

### Single Sample Analysis ("Interpretation")

1. Select "Interpretation" to enter the case details and initiate a deconvolution (see Figure 2). This initiates the process for the interpretation of single source and mixed DNA profiles.


FIGURE 2

Fill in the Case Number, Sample ID, any case notes (optional), and the number of contributors for the analysis.

Contributor Range: this is a Variable Number of Contributor (VarNOC) analysis. When running a VarNOC analysis, select the contributor range box, enter the minimum number of contributors, and the maximum number of contributors, and select "NIST_SDPD_All" as the Population for Range to use the combined NIST allele frequencies. Note that the difference between minimum and maximum number of contributors can only be 1. See the interpretation section for additional guidance on VarNOC. DNA Technical Manager approval is required to perform a VarNOC analysis. Approval must be documented in the technical record.

Additional settings can be accessed on this first screen under the "Run Settings" button (Figure 3). These settings include MCMC and burn-in accepts, Mx priors, Low Memory Mode, and setting the seed. The number of MCMC accepts and burn-in accepts should only be changed following documented consultation with the DNA Technical Manager if longer analyses are required to better resolve complex mixtures. All five person mixtures should be run in low memory mode, and Mx priors can be used when a

previous interpretation resulted in genotype weights that did not meet qualitative expectations.

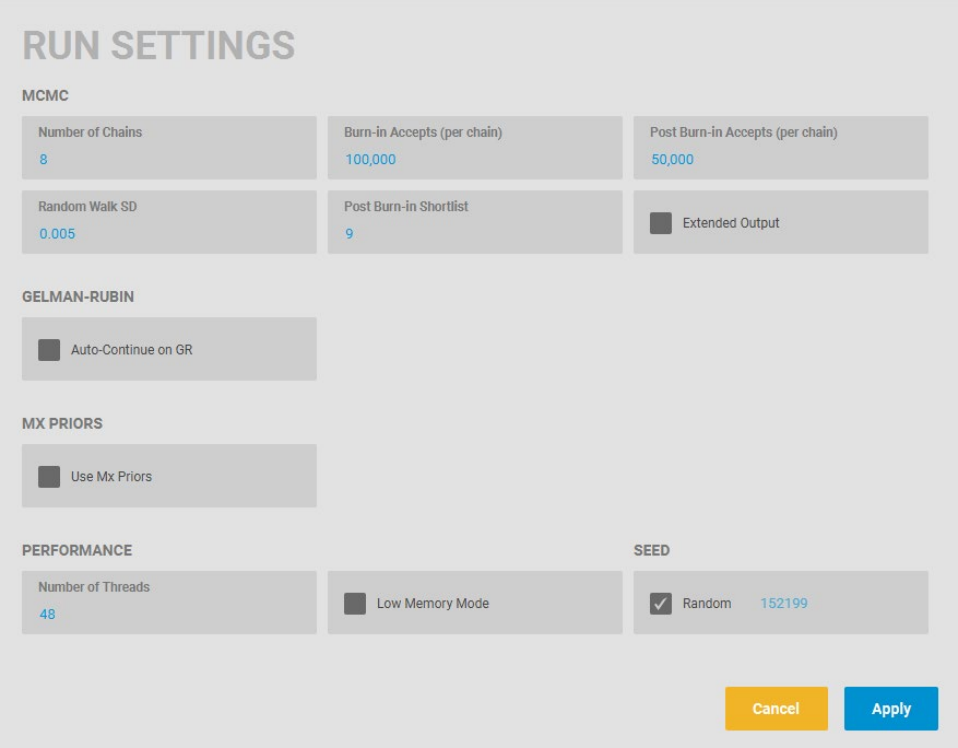



FIGURE 3

STRmix™ results folders are prefixed with the information entered into the Case Number field, followed by sample ID, then the date and time in the format yyyy-mm-dd-hh-mm-ss. The folder name should not be edited to remove the date and time information.

If any Run settings have been changed, select "Apply".

After case details have been entered and appropriate run settings chosen, select "Next".

2. The profiling kit can be selected and the input files entered on the following screen (Figure 4).

Select the appropriate DNA kit (SDPD GlobalFiler, SDPD 24s GlobalFiler, SDPD Identifiler Plus, or SDPD MiniFiler) for the analysis from the drop-down list in the upper left hand corner. For Identifiler Plus and MiniFiler reinterpretation, also consult the Forensic Biology Legacy Kit Interpretation Guidelines Manual.

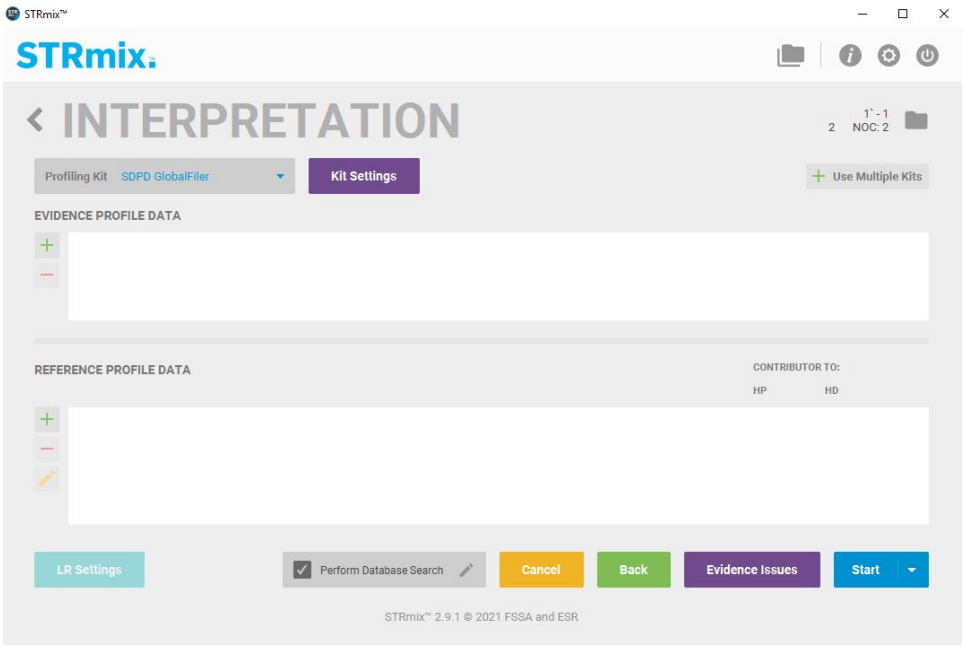



FIGURE 4

Kit settings can be accessed from this screen under the "Kit Settings" button (Figure 5). These settings include the variances, degradation max, the loci included in the kit, and stutter file information.

Kit Settings (e.g., ignore loci or degradation max) for an interpretation should only be changed if needed, following approval from the DNA Technical Manager. Approval must be documented in the technical record.

Select the evidence profile data – this can be done by dragging and dropping the input file (can contain multiple input files that you can subsequently

select) into the EVIDENCE PROFILE DATA window, or by clicking on the green + button on the left hand side of the window and navigating to the input file.

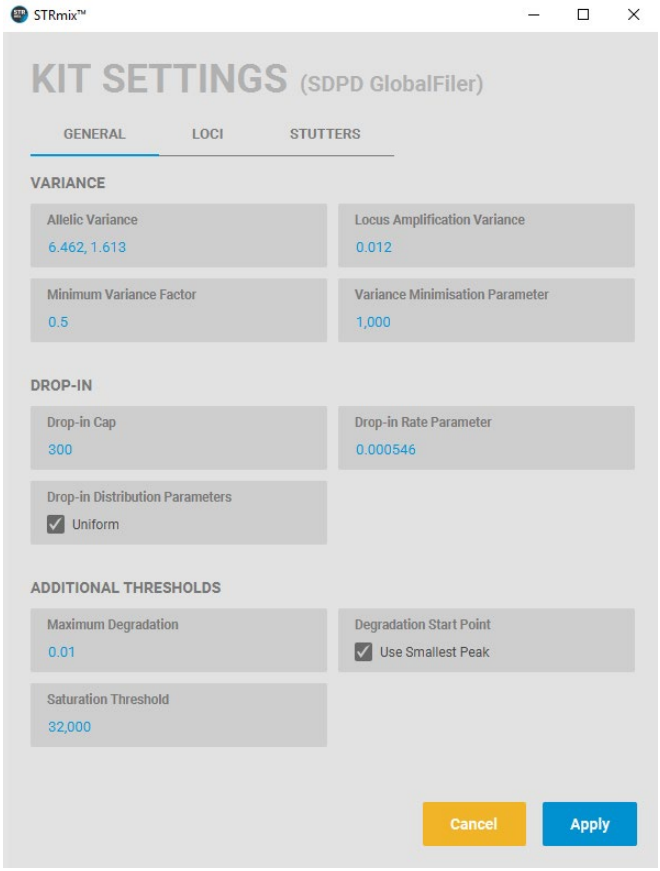



FIGURE 5

Multiple evidence profile files of replicate amplifications (from the same DNA extract) can be analyzed together by. This feature must not be used for samples that have been re-injected. The use of replicates within STRmix requires approval of the DNA Technical Manager. Approval must be documented in the technical record.

If any references can reasonably be assumed, they may be added at this step under "Reference Profile Data". Reference input files will automatically be

assigned as a known contributor under HP, so if a reference sample will be conditioned on, the "HD" box should be checked.

If loci need to be ignored (in the case of a tri-allele, or conditioning on a reference with incomplete data, etc.), or if any other kit settings need to be changed (e.g., max degradation), that can be done with the "Kit Settings" button under the appropriate tab – general, stutters, or loci.

On this screen, a database search can also be specified. A database search of the elimination DNA database profiles should be performed on each sample suitable for comparisons. If multiple deconvolutions are conducted on an evidence sample, it is not necessary to run a database search on each deconvolution; it is sufficient to only run a databse search on the deconvolution which will be reported.

When all sample files have been added and settings specified, the "Start" button can be selected to begin the deconvolution. Alternatively, "Queue" can be selected from the drop-down next to "Start" to add the deconvolution to the queue in Batch Mode.

Run time is dependent on mixture complexity and computing power. During the validation, single source mixtures and 2-person mixtures generally take <5 minutes. 3- and 4-person mixtures are typically under an hour. 5-person mixtures can take hours/days to complete.

**Calculation of a Likelihood Ratio in STRmix™ ("Investigation" > "LR from Previous")**

When a reference sample from a person of interest is available and has been analyzed, a comparison to a STRmix™ deconvolution can be performed. This comparison generally takes the form of a likelihood ratio, although manual comparisons for simple exclusions can be performed by an analyst. Comparisons are generally performed after the initial deconvolution of the sample, but may be done in conjunction with the initial deconvolution.

To calculate an LR after initial analysis, select "Investigation", and then "LR from Previous".

1. Choose a previous interpretation by dragging and dropping the deconvolution folder (or just the config.XML file from that folder) into the "Previous Interpretation" window or by selecting "Browse" and navigating to and selecting the appropriate config.XML file. Click "Next".

2. Input the appropriate reference file or files into the "Reference Profile Data" area by dragging and dropping the input file (can contain multiple references) or selecting with the green + button. This is similar to the screen as shown in Figure 4, but "profiling kit" and "evidence profile data" are disabled from making edits. Inputting multiple reference files at one time will create a combined LR calculation.

   Note: If any loci need to be ignored, this can be done using the "Kit Setting" button and selecting the "IGNORE?" box for each relevant locus under the "Loci" tab. The reason for ignoring loci for an LR must be documented in the technical record.

3. The "LR settings" button will open a window where the populations and other LR settings are located (Figure 6). This figure shows the settings that are used for SDPD LR calculations, with an HPD calculated using both MCMC and Allele Frequency Uncertainty, and four populations. This is a section in which populations can be added or removed, and LR settings can be changed if necessary. Click "Apply" to save any changes.

4. Select "Start" to start the calculation. A report will automatically be generated. Alternatively, "Queue" can be selected from the drop-down next to "Start" to add the LR to the queue in Batch Mode.

5. Results for this run will be located on the local computer in the STRmix™ Results folder. Transfer the folder to the FB network.

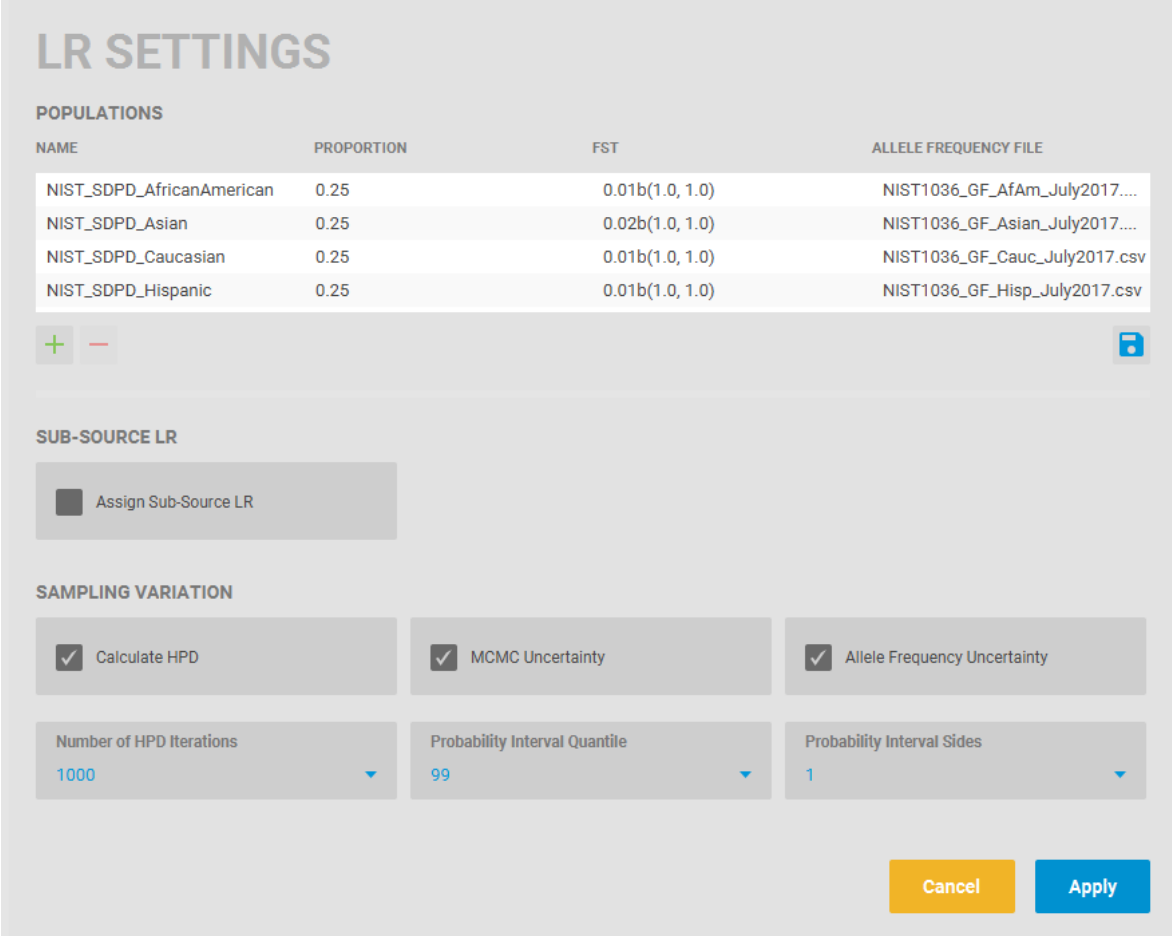



FIGURE 6

## LR Batches

This feature allows several LR calculations to be performed. Select "Investigation", and then "Investigation Batch" (see Figure 7). There are two portions to the screen: "INTERPRETATIONS", in which folders from previous deconvolution can be selected or dragged and dropped into the area, and "INVESTIGATIONS", from which "Add LR from Previous" can be selected.

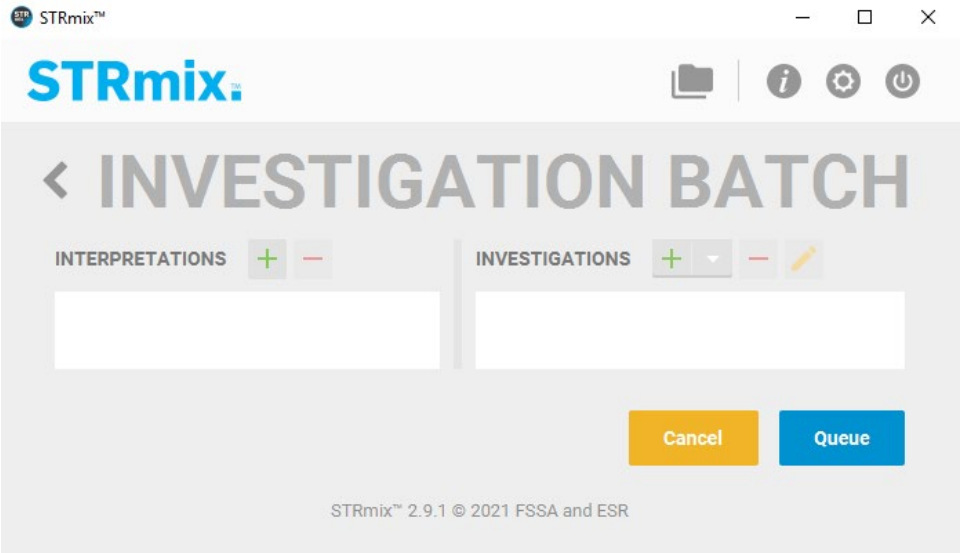



FIGURE 7

This opens a new "LR FROM PREVIOUS BATCH" window (see figure 8), into which reference input files can be selected or dragged and dropped into the "REFERENCE PROFILES" window.

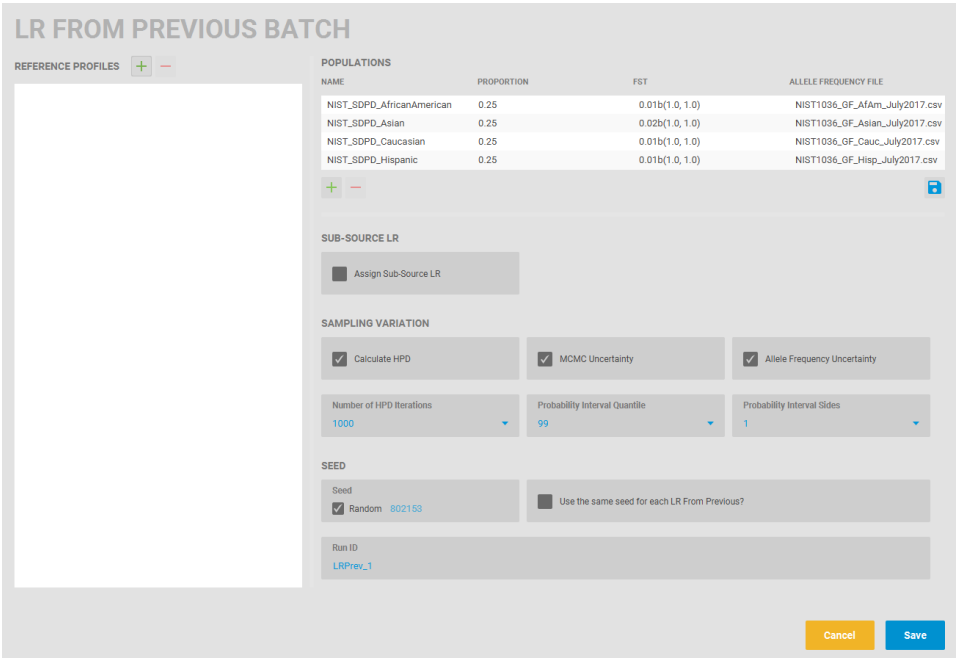



FIGURE 8

If one previous interpretation is added, and three reference profiles are added, three separate "LR from Previous" calculations (one for each reference) will be set up. Each LR batch setup is a comparison of a single reference profile to that investigation.

If two previous interpretations and three reference profiles are added, 6 "LR from Previous" calculations are set up – one for each reference to each interpretation.

Once the set of interpretations and references have been added, select "Queue" and you will be brought to "Batch Mode". Each of the calculations will be listed in Batch Mode (See Figure 10 below) to be queued for a run. At this point, there is the option of adding additional LR batches or other analyses (see the batch mode section below).

It is important that all the LR Default settings are correct because none of these settings can be edited on a run by run basis in the LR Batch interface.

**$H_d$-True Tester**

This feature gives information about the quality of the data and deconvolution. Select "Investigation", and then "Hd-True Tester".

Choose the previous investigation by dragging and dropping the deconvolution folder, select the number of reference to generate (i.e., 10,000), the population for sampling and LR (i.e., NIST_SDPD_All to use the combined NIST data), and Select "Use Importance Sampling" to use that feature. If left unselected, random profiles will be generated. The Test ID can be edited to change the name of the folder that the results will be saved to. Select Start.

At the end of this analysis, a PDF report of the results will be generated.

$H_d$-True Tester cannot be used on a VarNOC analysis.

## Database searching

STRmix™ has a database searching feature which offers the option of searching an deconvoluted evidence sample against a database of reference profiles stored in Excel format. Each of the individuals in the database are considered as a potential contributor in turn to the mixture under the following two hypotheses:

Hp: Database individual and N - 1 unknown contributors

Hd: N unknown contributors

where N is the number of contributors under consideration, as set by the analyst in the STRmix™ deconvolution analysis. The likelihood ratios in this feature incorporate $F_{ST}$.

The SDPD elimination DNA database has been created with the required Excel formatting. All mixture deconvolutions should be searched against the elimination DNA database. The STRmix database search function must be performed on any sample deconvoluted in STRmix that is suitable for comparisons. Only a single elimination database search is needed per sample and should be performed on the sample that provides the most discriminating deconvolution. A STRmix database search is more specific than CODIS searches due to the nature of how each program performs searches.

Individuals giving an LR above some list management value are investigated. Analysts must use a value no higher than 199 for database searches against the SDPD elimination database.

There are multiple options for performing a database search within STRmix™. Two will be discussed in the Batch Sample Analysis section below.

<u>Option 1</u>

1. From the STRmix™ main menu (Figure 1), select "Investigation" and then "Database Search" (see Figure 9).

2. Navigate to the previously analyzed sample folder of choice or drag and drop the relevant folder into the "Previous Interpretation" window.

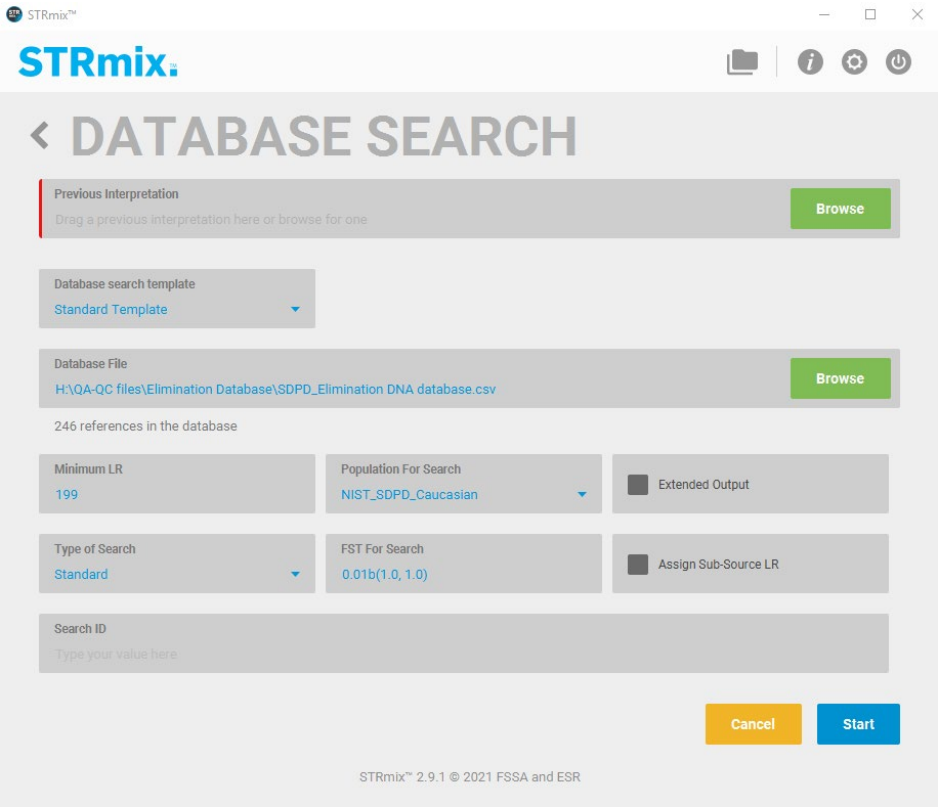



Figure 9

3. If the SDPD elimination database is not already selected in the "Database File" window or if you are performing a search of a different database, select the "Browse" button for the Database File to open a file chooser. Navigate to the database file and select Open. The number of profiles within the database appears in the window. Select the Minimum LR (i.e., 199) and the population for the search (NIST_SDPD_Caucasian). Check Standard to undertake a standard database search, and enter 0.01b(1.0,1.0) in FST for Search. Assign Sub-Source LR should be Unchecked.

4. Select "Start" to start the search or Cancel to return to the main menu. On completion of the search, a folder with results is created, including a PDF of the results. Database searches with no results need not be printed, but the database search files should be saved. If any matches to the elimination database are obtained, the PDF report or the DBSearchResults text file can be

printed. Refer to the Forensic Biology Policy Manual sections 4.5 and 4.6 for additional information on elimination database matches.

Option 2

1. During a deconvolution (either Single Sample Analysis or using Batch Mode), the "Perform Database Search" box can be checked at the bottom of the "INTERPRETATION" screen (Figure 4).

2. If database settings need to be confirmed or edited, select the pencil icon next to the "Perform Database Search Box". Select "Apply"

3. Follow steps 3 and 4 from Option 1.

**Batch Sample Analysis**

Batch Mode can now process all types of analyses and investigations all in one batch. This includes: Interpretations (deconvolutions), LR from Previous Calculations, Database Searches, $H_d$ True Tester, and LR Batches.

1. The process proceeds in the same way as Interpretations, LR from previous calculations, LR Batches, Database Searches, or $H_d$ True Tester analyses, but instead of immediately running, they will be added to a batch of analyses. After adding each analysis or investigation to the queue for the batch, the software will return you to the screen in Figure 10.

2. Additional STRmix™ analyses can be queued by choosing the appropriate analysis from the drop-down menu under "Add to Batch" and repeating the step above. Once all the samples for the batch have been added, selecting Start will commence the deconvolutions. The deconvolutions will proceed in the order that the user has added them. A batch counter is added that will tell

you the progress through the batch, the total batch time, and the time for that current analysis

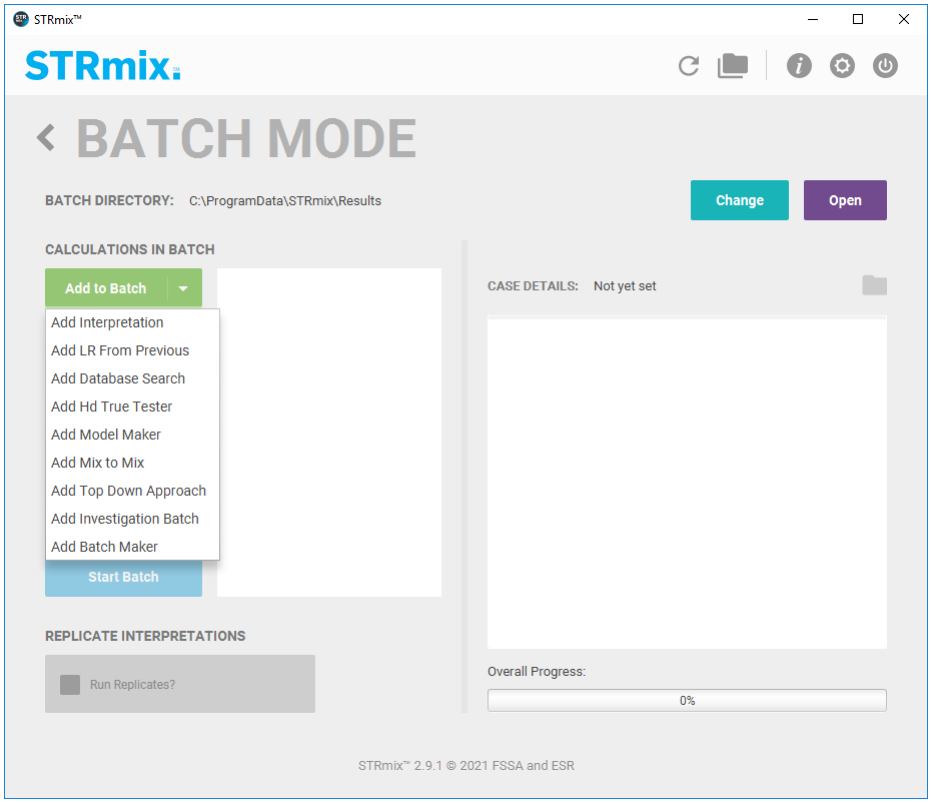



Figure 10

3. LR Batches can be added to the Batch Mode queue either by selecting "Investigation Batch" from the drop down in Batch Mode or by adding to the queue when performing an LR from Previous Batch from the Investigations Batch option within the Investigations Menu (see Figures 7 and 8 above).

4. A Database Batch can be performed from the Investigation Batch window by adding as many interpretations as desired to the left hand window in Figure 7 and selecting "Add Database Search" from the drop down next to "INVESTIGATIONS".

### Managing data files

The files produced by STRmix™ are discoverable and need to be maintained in such a manner that they can be located when needed. In addition, the technical review of a number of aspects of the STRmix™ data will be conducted electronically. As such, the following conventions need to be used when naming and saving files.

There is a folder called STRmix Data on the FB network and within it are subfolders for all casework analysts. STRmix™ results files should be saved within a folder with the appropriate case number in the STRmix Data folder.

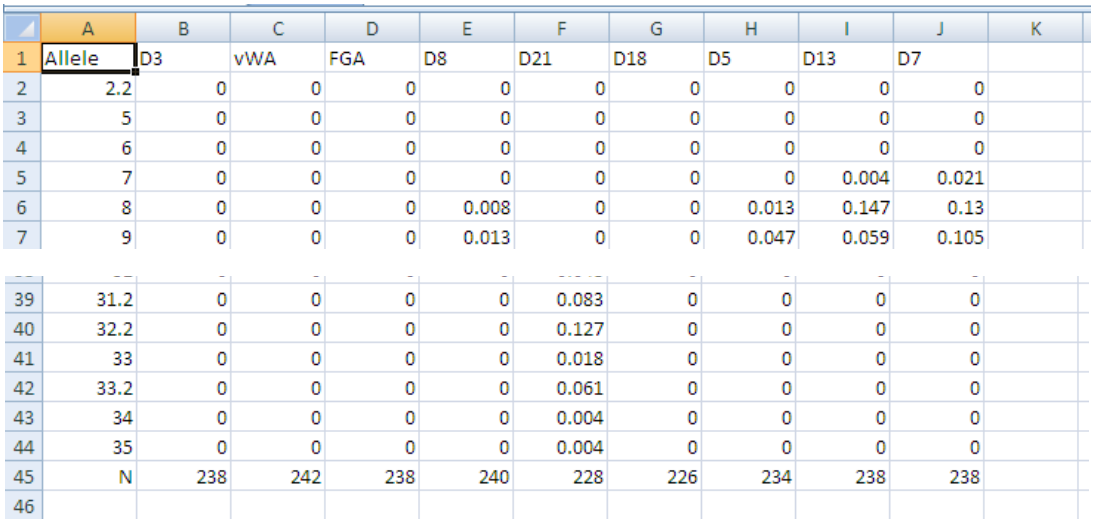
### Alternate Population Databases

If a different database is used for calculations than the population stated in section 4, it shall be included in the analytical record with the case file and noted in the report.

Alternate databases can be created for STRmix™ using the same format as the existing database files. An example appears in Figure 11. It is important that the last row has the total number of alleles observed for each locus. Save the file with the name of the population as a .csv file in the AlleleFreq folder of the STRmix™ folder within the Program Data folder. Once the allele frequency spreadsheet has been created it will need to be reviewed by a second scientist.

Once the new database has been reviewed it needs to be added to the STRmix™ software. From the STRmix™ main menu "Administration" (Figure 1) select "Populations". Add a new population by clicking the green + button. Enter the name of the Excel csv frequency file in the Pop Name field. Select Find File in the Allele freq file field to open a file chooser window. Select the frequency file for the alternate allele frequency database. Enter appropriate Pop proportion and default $F_{ST}$ values. Select Save Pop.

Examples of Allele frequency spreadsheets created in Microsoft Excel:



| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Allele | D3 | vWA | FGA | D8 | D21 | D18 | D5 | D13 | D7 | |
| 2 | 2.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 3 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 4 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| 5 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.004 | 0.021 | |
| 6 | 8 | 0 | 0 | 0 | 0.008 | 0 | 0 | 0.013 | 0.147 | 0.13 | |
| 7 | 9 | 0 | 0 | 0 | 0.013 | 0 | 0 | 0.047 | 0.059 | 0.105 | |
| 39 | 31.2 | 0 | 0 | 0 | 0 | 0.083 | 0 | 0 | 0 | 0 | |
| 40 | 32.2 | 0 | 0 | 0 | 0 | 0.127 | 0 | 0 | 0 | 0 | |
| 41 | 33 | 0 | 0 | 0 | 0 | 0.018 | 0 | 0 | 0 | 0 | |
| 42 | 33.2 | 0 | 0 | 0 | 0 | 0.061 | 0 | 0 | 0 | 0 | |
| 43 | 34 | 0 | 0 | 0 | 0 | 0.004 | 0 | 0 | 0 | 0 | |
| 44 | 35 | 0 | 0 | 0 | 0 | 0.004 | 0 | 0 | 0 | 0 | |
| 45 | N | 238 | 242 | 238 | 240 | 228 | 226 | 234 | 238 | 238 | |
| 46 | | | | | | | | | | | |

FIGURE 11

Analysts are to include a copy of all non-standard database papers in their case file as a part of the analytical record. Reviewers don't need to re-check already validated databases but need to ensure that the correct population has been used.

### STRmix™ settings

Under "Administration" STRmix default settings, kit settings, populations, and report defaults can be set. The "administration" settings require a password to set and change, as these are the settings determined by validation.

### Profiling Kits

There are four profiling kits for SDPD analyses with STRmix. Two of these are used for current analysis (SDPD GlobalFiler and SDPD GlobalFiler 24s). Two of the kits are used for interpretation of legacy data (SDPD Indentifiler Plus and SDPD Minifiler). The settings are as follows:

| Kit Name | SDPD GlobalFiler |
|---|---|
| Size regression file | GlobalFiler_Size Regression.csv |
| Analytical Threshold | 100 |
| Allele Variance | 6.462, 1.613 |
| Locus Amplification Variance | 0.012 |
| Back Stutter Variance | 1.640, 11.023 |
| Maximum Back Stutter Ratio | 0.3 |
| Inversely Proportional To | Observed Height of Parent Allele |
| Back Stutter Regression File | SDPD_GF_Stutter.txt |
| Back Stutter Exceptions File | SDPD_GF_Stutter Exceptions.csv |
| Forward Stutter Variance | 3.065, 2.560 |
| Maximum Forward Stutter Ratio | 0.15 |
| Inversely Proportional To | Expected Height of Stutter Peak |
| Forward Stutter Regression File | SDPD_GF_ForwardStutter.txt |
| Double Back Stutter Variance | 2.807, 4.255 |
| Maximum Double Back Stutter Ratio | 0.05 |
| Inversely Proportional To | Expected Height of Stutter Peak |
| Double Back Stutter Regression File | SDPD_GF_DoubleBackStutter.txt |
| 2bp Back Stutter Variance | 5.464, 2.194 |
| Maximum 2bp Back Stutter Ratio | 0.07 |
| Inversely Proportional To | Expected Height of Stutter Peak |
| 2bp Back Stutter Regression File | SDPD_GF_2bpStutter.txt |
| Minimum Variance Factor | 0.5 |
| Variance Minimization Parameter | 1,000 |
| Drop-In Cap | 300 |
| Drop-In Frequency | 0.000546 (uniform) |
| Maximum Degradation | 0.01 |
| Degradation Start Point | Use Smallest Peak |
| Saturation Threshold | 32,000 |

| Kit Name | SDPD 24s GlobalFiler |
|---|---|
| Size regression file | GlobalFiler_Size Regression.csv |
| Analytical Threshold | 100 |
| Allele Variance | 9.356, 1.805 |
| Locus Amplification Variance | 0.013 |
| Back Stutter Variance | 2.558, 7.690 |
| Maximum Back Stutter Ratio | 0.3 |
| Inversely Proportional To | Observed Height of Parent Allele |
| Back Stutter Regression File | SDPD_GF_Stutter.txt |
| Back Stutter Exceptions File | SDPD_GF_Stutter Exceptions.csv |
| Forward Stutter Variance | 3.834, 4.355 |
| Maximum Forward Stutter Ratio | 0.15 |
| Inversely Proportional To | Expected Height of Stutter Peak |
| Forward Stutter Regression File | SDPD_GF_ForwardStutter.txt |
| Double Back Stutter Variance | 2.135, 8.147 |
| Maximum Double Back Stutter Ratio | 0.05 |
| Inversely Proportional To | Expected Height of Stutter Peak |
| Double Back Stutter Regression File | SDPD_GF_DoubleBackStutter.txt |
| 2bp Back Stutter Variance | 2.515, 8.637 |
| Maximum 2bp Back Stutter Ratio | 0.07 |
| Inversely Proportional To | Expected Height of Stutter Peak |
| 2bp Back Stutter Regression File | SDPD_GF_2bpStutter.txt |
| Minimum Variance Factor | 0.5 |
| Variance Minimization Parameter | 1,000 |
| Drop-In Cap | 510 |
| Drop-In Frequency | 0.000928 (uniform) |
| Maximum Degradation | 0.01 |
| Degradation Start Point | Use Smallest Peak |
| Saturation Threshold | 32,000 |

| Kit Name | SDPD Identifiler Plus |
|---|---|
| Size regression file | Identifiler_Size Regression.csv |
| Analytical Threshold | 50 |
| Allele Variance | 4.195, 0.923 |
| Locus Amplification Variance | 0.013 |
| Back Stutter Variance | 2.740, 1.854 |
| Maximum Back Stutter Ratio | 0.3 |
| Inversely Proportional To | Observed Height of Parent Allele |
| Back Stutter Regression File | SDPD_IdentStutter.txt |
| Back Stutter Exceptions File | SDPD_ID_stutter exceptions.csv |
| Forward Stutter Variance | 4.616, 1.522 |
| Maximum Forward Stutter Ratio | 0.07 |
| Inversely Proportional To | Expected Height of Stutter Peak |
| Forward Stutter Regression File | SDPD_Ident_FwdStutter.txt |
| Minimum Variance Factor | 0.5 |
| Variance Minimization Parameter | 1,000 |
| Drop-In Cap | 100 |
| Drop-In Frequency | 0.000365 (uniform) |
| Maximum Degradation | 0.01 |
| Degradation Start Point | Use Smallest Peak |
| Saturation Threshold | 10,000 |

| Kit Name | SDPD MiniFiler |
|---|---|
| Size regression file | MiniFiler_Size Regression.csv |
| Analytical Threshold | 100 |
| Allele Variance | 6.677, 3.921 |
| Locus Amplification Variance | 0.01996 |
| Back Stutter Variance | 2.639,10.041 |
| Maximum Back Stutter Ratio | 0.32 |
| Inversely Proportional To | Observed Height of Parent Allele |
| Stutter Regression File | SDPD_MF_Stutter.txt |
| Stutter Exceptions File | SDPD_MF_StutterExceptions.csv |
| Minimum Variance Factor | 0.5 |
| Variance Minimization Parameter | 1,000 |
| Drop-In Cap | 612 |
| Drop-In Frequency | 0.006 (uniform) |
| Maximum Degradation | 0.01 |
| Degradation Start Point | Use Smallest Peak |
| Saturation Threshold | 30,000 |

## Populations

STRmix uses the same population data for each kit. The allele frequencies used for the populations are the NIST 1036 allele frequencies. There is also one additional population created for use in VarNOC deconvolutions and database searches. For each of the populations, the Population proportion = 1 and Unrelated Proportion = 1, with no details about population size, number of children, or family relationships.

Below lists each population, the allele frequency file name, and the default FST. The allele frequency file has GF in the name to indicate that it contains all the markers in GlobalFiler, but can be used for other kits with fewer markers, as well. July 2017 indicates that the allele frequencies incorporate the corrections issued in the 2017 release of data.

NIST_SDPD_AfricanAmerican = NIST1036_GF_AfAm_July2017.csv, $F_{ST}$ = 0.01b(1.0,1.0)
NIST_SDPD_Asian = NIST1036_GF_Asian_July2017.csv, $F_{ST}$ = 0.02b(1.0,1.0)
NIST_SDPD_Caucasian = NIST1036_GF_Cauc_July2017.csv, $F_{ST}$ = 0.01b(1.0,1.0)
NIST_SDPD_Hispanic = NIST1036_GF_Hisp_July2017.csv, $F_{ST}$ = 0.01b(1.0,1.0)
NIST_SDPD_All = NIST1036_GF_All_July2017.csv, $F_{ST}$ = 0.01b(1.0,1.0)

## STRmix Defaults

There are general, likelihood ratio, and user interface default settings that can be set under administration. Several of them can be user defined, like what the default STRmix kit is, where results are saved, and whether or not Low Memory Mode is on. There are others that should be the same on every workstation, which are as follows:

General Settings
Number of chains = 8; Burn-in Accepts (per chain) = 100,000; Post burn-in Accepts (per chain) = 50,000; Random Walk SD = 0.005; Post Burn-in Shortlist = 9; Auto-Continue on GR = Unselected; Hyper-Rectangle Percent Accepts = 2.5; Number of Threads = 48
Likelihood Ratio Settings
Four default populations = NIST_SDPD_AfricanAmerican, NIST_SDPD_Asian, NIST_SDPD_Caucasian, and NIST_SDPD_Hispanic; Sub-source LR = Unselected (meaning that the sub-sub source LR is returned); for contributor range (VarNOC) analysis stratify LR = Unselected (MLE is returned for VarNOC

LR); and for Sampling Variation settings = Select Calculate HPD, Select MCMC Uncertainty, and Select Allele Frequency Uncertainty; Number of HPD iterations = 1000; Probability Interval Quantile = 99; Probability Interval Sides = 1.

User Interface Settings

Text File Renaming = Unselected.

METHOD: **Parentage Calculations using Popstats**

Date: 09/08/2020 Approved by: ARD

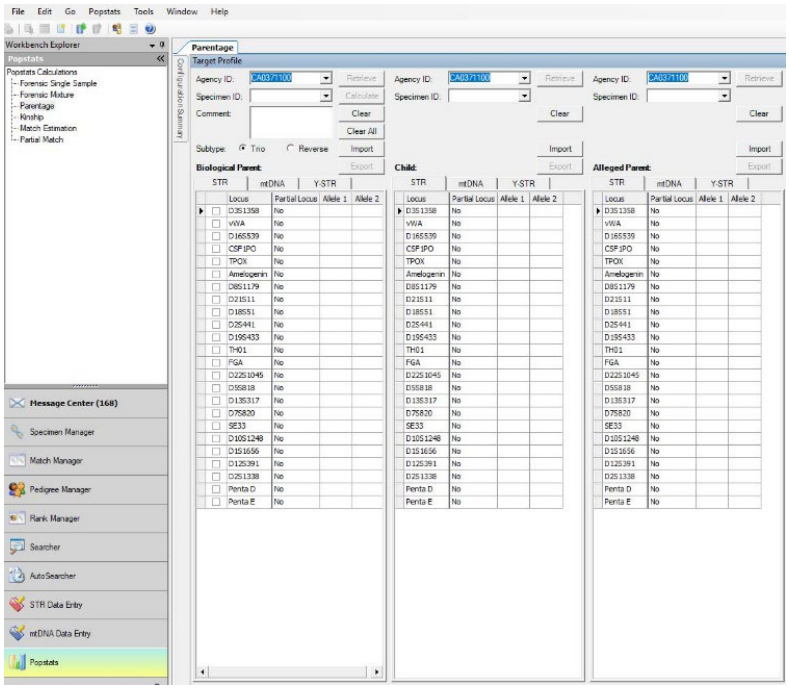
There are two types of paternity calculations that can be calculated within the Popstats software within the Analyst Workbench (i.e., CODIS software): the parentage trio calculation; and reverse parentage. For the Trio and Reverse cases, two known (Biological Parent and Child) STR DNA profiles are required.

The parentage trio calculation involves a known parent (usually the mother) with a known biological child. A third person who is not biologically related to the known parent is alleged to be the other biological parent to the child.

The reverse parentage calculation involves two known parents, and an alleged child of the two known parents.

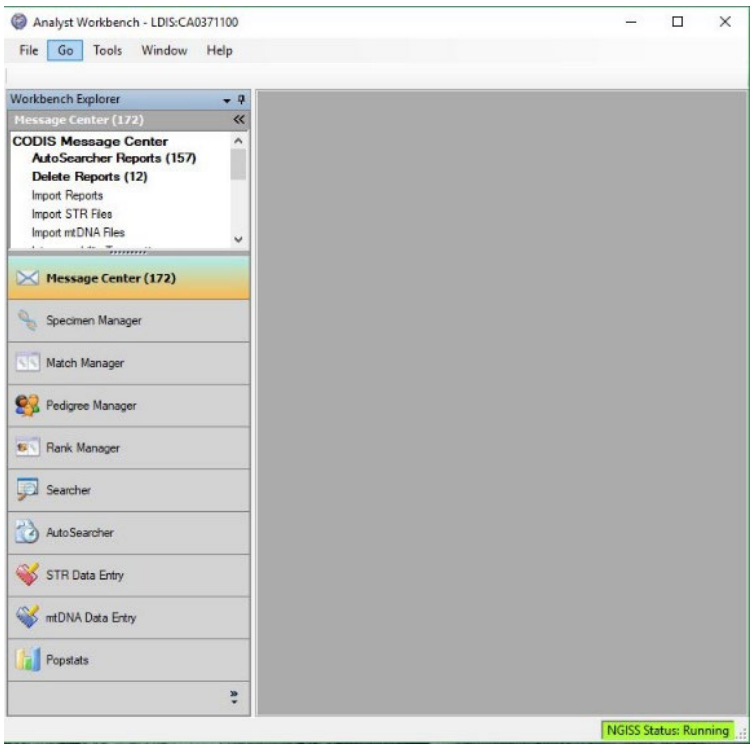
In both situations, the Parentage Index likelihood ratio will be reported.

1. Open Analyst Workbench (i.e., the CODIS software) and use the "Go" drop-down menu, or the navigation pane on the left to navigate to the Popstats application.



2. In the Popstats explorer pane, select "Parentage" and the following window will open:

3. Use the radio buttons, "Trio" or "Reverse", above the "Biological Parent" profile window to specify which type of calculation is being performed.




4. Enter the required specimen information:

   a. For a Parentage Trio, enter the Specimen IDs and DNA profiles of the Biological Parent, Child, and Alleged Parent into the appropriate windows then click "Calculate".

   b. For Reverse Parentage, enter the Specimen IDs and DNA profiles of the Biological Mother, Father, and Alleged Child into the appropriate windows then click "Calculate".

5. Calculations should be performed using the NIST 2017 allele frequencies taken from *U.S. population data for 29 autosomal STR loci, Forensic Science International:*

*Genetics 7 (2013) e82-83 and Corrigendum to "U.S. population data for 29 autosomal STR loci" [Forensic Science International: Genetics 7 (2013) e82-83], Forensic Science International: Genetics 31 (2017) e36-40* . If a different database is used for calculations, it shall be included in the technical record and disclosed in the report.

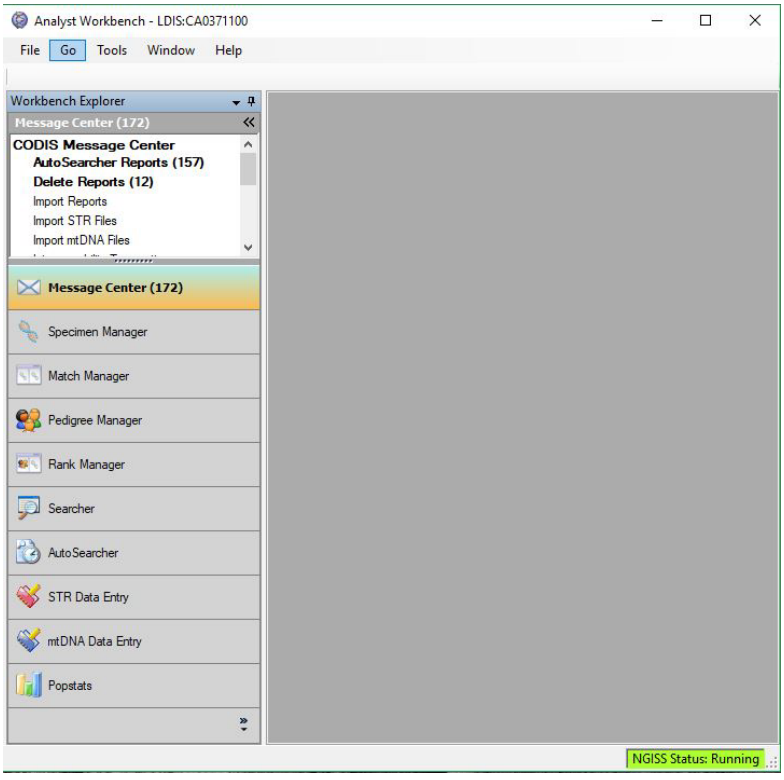
6. Print the calculation reports for the technical record. Only the calculation representing the ethnicity of the alleged parent needs to be reported. The others will be available in the technical record.

METHOD: **Kinship Calculations using Popstats**

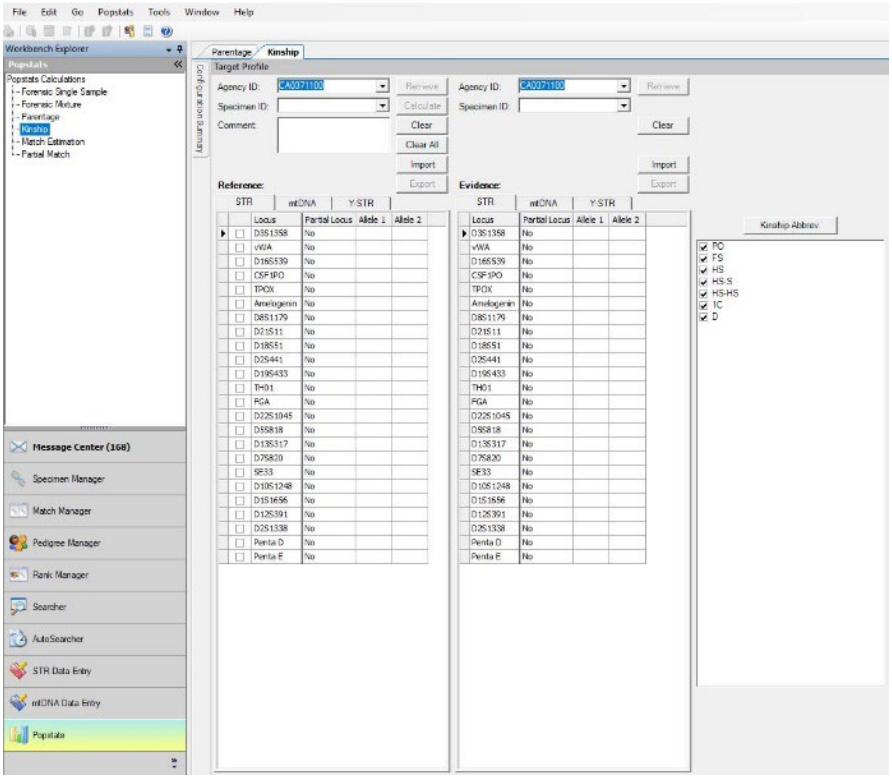
Date: 09/08/2020 Approved by: ARD

Kinship calculations evaluate the likelihood that the pair of given DNA profiles are associated by the specified kinship vs. by chance. This calculation also provides the single-parentage statistics for a pair of DNA profiles. The purpose of the Kinship module is to provide a tool for CODIS users to calculate the likelihood of a presumed kinship relationship between two specimens. It is also used to assess the likelihood of a specified kinship relationship with respect to other types of kinship when the relationship between two specimens is unknown.

1. Open Analyst Workbench (i.e., the CODIS software) and use the "Go" drop-down menu, or the navigation pane on the left to navigate to the Popstats application.



2. In the Popstats explorer pane, select "Kinship" and the following window will open:




3. Enter the Specimen IDs and DNA profiles of the relevant samples into the appropriate windows. IN general, the known profile is considered the "Reference" profile and the one for which the relationship is unknown is the Evidence; however, in practical terms is does not matter.

4. The putative relationships being tested can be selected in the pane on the right of the screen. Ensure the correct boxes are checked for the relationships being tested for the profile pair. Note: the "Kinship Abbrev" button can be used as a quick reference for which relationships each selection encompasses.

5. Click "Calculate".

6. Calculations should be performed using the NIST 2017 allele frequencies taken from U.S. population data for 29 autosomal STR loci, Forensic Science International: Genetics 7 (2013) e82-83 and Corrigendum to "U.S. population data for 29 autosomal STR loci" [Forensic Science International: Genetics 7 (2013) e82-83], Forensic Science International: Genetics 31 (2017) e36-40. If a different database is used for calculations, it shall be included in the technical record and disclosed in the report.

7. Print the calculation reports for the technical record. Only the calculation representing the ethnicity in question needs to be reported. The others will be available in the technical record.

METHOD: **Autosomal STR Interpretation Guidelines**

Date: 06/01/2004 **Revision Date: 06/09/2025** Approved by: ARD

## Introduction

The Scientific Working Group on DNA Analysis Methods (SWGDAM) states that:

> "The interpretation of DNA typing results for human identification purposes requires professional judgment and expertise. Additionally, laboratories that analyze DNA samples for forensic casework purposes are required by the Quality Assurance Standards for Forensic DNA Testing Laboratories (effective July 1, 2009) to establish and follow documented procedures for the interpretation of DNA typing results and reporting. Due to the multiplicity of forensic sample types and the potential complexity of DNA typing results, it is impractical and infeasible to cover every aspect of DNA interpretation by a preset rule. However, the laboratory should utilize written procedures for interpretation of analytical results with the understanding that specificity in the standard operating protocols will enable greater consistency and accuracy among analysts within a laboratory. It is recommended that standard operating procedures for the interpretation of DNA typing results be sufficiently detailed that other forensic DNA analysts can review, understand in full, and assess the laboratory's policies and practices. The laboratory's interpretation guidelines should be based upon validation studies, scientific literature, and experience."

Here we present a number of general interpretation guidelines for interpreting samples in forensic DNA casework. The rationale behind these guidelines is to establish a set of standards to ensure that conclusions are supported by reliable data, derived using a scientific approach, and that interpretation of results is as objective as possible and is consistent from one analyst to another.

The interpretation of DNA results should take into account the DNA results obtained, the nature of the sample, the condition of the profile, any forensically valid assumptions made, and should be scientifically justifiable and based on the collected experience and knowledge of the laboratory and the scientific

community. These guidelines are designed to provide a general foundation for interpretation.

See Appendix A for a glossary of terms.

DNA interpretation should take place in the following order:

Preliminary Evaluation of Data and Allele Designation (section 1)
Interpretation of DNA Typing Results (section 2)
STRmix™ analysis (section 3)
Statistical Analysis of DNA Typing Results (section 4)
Reporting of DNA Typing Results and Conclusions (section 5)

## Section 1 – Preliminary Evaluation of Data and Allele Designation

### 1.1 Detection of peaks

The detection of peaks in the data is performed by the GeneMapper ID-X software. The analytical thresholds (peak detection thresholds) for the autosomal STR kits are listed within documents specific to each individual kit.

### 1.2 Evaluation of internal standards

The internal size standard should be evaluated to ensure that for each sample it has produced the expected pattern of peaks. These peaks should generally be between 3000 and 10,000RFU for a 15 second injection and not show signs of poor resolution. If a sample has issues in the size standard that prevent proper sizing, or has signs of poor resolution, the sample should be re-injected or re-prepared.

For GlobalFiler analysis, GS600 LIZ peaks from 60-460nt are required. Alternate analysis ranges are possible if migration issues occur that prevent larger size standard peaks from being detected.

## 1.3 Evaluation of allelic ladders

The GeneMapper ID-X software evaluates the ladder injections in the run to determine that all expected ladder alleles are detected, the base pair spacing between any two ladder alleles are as expected, there is no off-scale (OS) fluorescent signal detected within each marker range, and the peak height ratio between the lowest and highest peak is equal to or greater than 15%.

GeneMapper ID-X analysis of at least one of the injected allelic ladders must produce the known allele peaks associated with the ladder in order for the software to type the other samples of the run. If a ladder injection passes the evaluation by the software, the ladder can be relied upon without further manual evaluation or reinjection.

## 1.4 Designation of alleles

Allele assignments are made by comparing the base pair size of the peaks in the amplified samples with the base pair size of the alleles within the allelic ladder(s). Allele assignments are performed automatically by GeneMapper ID-X. Analysts may on occasion need to manually edit "off-ladder" peaks to designate them as true alleles, or to redesignate peaks originally called as alleles by the software as artifacts.

1.4.1 Migration issues during the run may cause ***multiple allelic peaks*** to be ***improperly called as off-ladder alleles***. If this occurs, it may be necessary to only designate the sample category of a subset of the allelic ladder injections as "Allelic Ladders". Alternatively, separate projects may need to be created using samples and ladder combinations that eliminate off-ladder alleles. Another option would be to re-inject the entire run, or specific samples and allelic ladders.

1.4.2 Occasionally an ***allele*** will be detected that is ***outside the ladder range*** for a given locus. These peaks should be assigned an allelic value based on the fragment length as compared to the nearest allelic ladder peak. Care should be taken when assigning an allelic value to

these peaks as slight migration differences between the sample and ladder could make such determinations difficult.

Occasionally an ***allele*** will fall ***outside the ladder range between two loci***. An analyst should consider the size of the apparent allele and the expected zygosity in an attempt to determine to which locus the allele belongs. A list of variant alleles is maintained by NIST on the STRBase website (https://strbase.nist.gov). This list can be consulted to assist in determining which locus the allele belongs to. The allele will then be renamed with the allele designation based on a comparison to the nearest allelic ladder peak for the appropriate locus. Supporting documentation should be included in the analytical record for the case.

1.4.3 Off ladder alleles that occur within the ladder ranges (i.e., ***microvariants*** that contain an incomplete repeat motif) should be designated by the number of complete repeats and, separated by a decimal point, the number of base pairs in the incomplete repeat (e.g., D21S11 29.1 allele). ***Microvariants*** will be confirmed through a re-injection of the sample as a means of demonstrating the reproducibility of the retention time and sizing of the peak. If a microvariant is observed in multiple samples from the same case, re-injection for confirmation purposes is unnecessary. If a microvariant is observed that is consistent with being a stutter peak for an allele in a ladder bin or virtual bin, re-injection for confirmation purposes is unnecessary.

1.4.4 ***Tri-allelic patterns, partial null, and null alleles*** should be confirmed through re-amplification.

## 1.5 Designation of artifacts

Some data contained within the electropherograms may not represent actual alleles that originate in the sample. Non-allelic peaks such as stutter, non-template dependent nucleotide addition (minus-A), spikes, raised baseline, pull-up, or disassociated primer dyes should be identified prior to the comparisons of reference samples to the data. Non-allelic data such as stutter, non-template dependent nucleotide addition, disassociated dye, and incomplete spectral separation are

generally reproducible; however, spikes and raised baseline are generally not reproducible.

1.5.1 ***Off –scale data*** are often the result of over-amplification and can cause several other interpretational difficulties, such as: "minus-A" products, increased stutter, and pull-up (see below). If off-scale data are present in a sample, the sample may be re-injected with a decreased injection time, re-amplified using less input DNA, or the amplified product may be diluted for reinjection.

1.5.2 ***Pull-up*** results from poor spectral separation and is characterized by signal from a peak in one fluorescent dye color producing a smaller, artifactual peak in another, often adjacent, dye color. Amplification of less sample, injection of a sample for less time, dilution of amplified product, or performing electrophoresis on the samples after creating a new spectral for the instrument can reduce pull-up peaks. Pull-up above 4% of the peak causing it generally indicates that a new spectral is needed.

**1.5.3** ***Pull-down*** results from compromised spectral separation and is characterized by signal from a peak in one fluorescent dye color producing a smaller, artifactual peak(s) in another, often adjacent, dye color. This phenomenon can appear as raised (called) baseline between two peaks in an adjacent color.

1.5.4 ***Minus-A*** (-A) is a form of PCR product that does not possess an extra adenosine nucleotide at the 3' end. Over-amplification can result in prominent "minus-A" products. Re-amplifying using less input DNA will likely reduce minus-A peaks. Incubating amplified products at 60°C for an additional time period may also reduce minus A.

1.5.5 ***Stutter*** peaks are minor peaks appearing one repeat unit smaller (e.g., n-4nt in tetranucleotide loci) or larger (e.g., n+4nt in tetranucleotide loci), or sometimes one half repeat smaller (e.g., n-2nt in tetranucleotide loci) than a primary STR allele. The validation studies for the various kits used in the laboratory include an

assessment of the expected stutter at each locus within the kit. STRmix™ currently models n-1 and n+1 repeat stutter products, and evidence samples destined for STRmix™ analysis should have n-1 and n+1 repeat stutter peaks labeled. Additional stutter products (e.g., n-2 or n-1/2 repeat) should be filtered. Analysts can use the validation data for further information on stutter expectations.

For reference samples and evidence profiles, if a peak is suspected of being stutter, the validation study for that kit should be consulted to determine the expected stutter percentage for that allele.

In ***reference samples***, unfiltered stutter peaks that are less than the highest observed stutter ratio for an allele during the validation study can be interpreted as stutter peaks, provided that there are no additional indications that the sample is a mixture. An examination of the baseline may provide additional indication that the reference sample is single source. For rarer alleles that do not have enough data to establish a mean or meaningful standard deviation, the value of the putative stutter peak must be shown to be below the extrapolated regression line for three standard deviations to be considered as stutter. Care should be taken in assessing these instances as tri-allelic patterns, although rare, are known to occur.

Additional factors such as spectral overlap or the presence of a potential stutter peak between two true alleles (n+1/n-1 repeat position) may enhance stutter peaks above the expected values. Ultimately, re-amplification can help determine if a peak is truly stutter or if it may be DNA from a secondary source.

1.5.6 ***Spikes*** are artifacts of electrophoresis, which may resemble DNA peaks. Often spikes will be narrower than true DNA peaks and will be filtered by the GeneMapper ID-X software. Multi-color spikes can be identified by the presence of peaks occurring in several colors at the same data point location. Single-color spikes can be

identified by the presence of a sharp peak occurring in a single color in the raw data, with no spectral overlap into the other colors. Since spikes are generally random, reinjection of a sample should eliminate a questioned spike.

## 1.6 Evaluation of analytical controls

If the analytical controls are properly designated, the GeneMapper ID-X software will flag controls that fail to meet expectations. Controls that have passed GeneMapper ID-X verification can be relied upon without further evaluation.

1.6.1 The ***reagent blank*** is a check of the sample preparation reagents for possible contamination by extraneous DNA. If DNA types are detected above (or possible DNA peaks are observed below) the analytical threshold in the reagent blank this is an indication of a possible contamination event. Based on an evaluation of the DNA types in the reagent blank, the results of associated samples may be deemed inconclusive. The analyst and the DNA Technical Manager will confer on an appropriate action based on the particular circumstance.

Samples extracted in a batch where the reagent blank shows DNA types greater than the interpretation threshold requires the completion of a Quality Incident Summary. A review of the circumstances may require re-extraction of the associated samples. Not all contamination events will necessitate re-extraction and will be judged on a case-by-case basis.

In some cases, re-extraction of samples affected by a reagent blank containing detectable DNA may not be possible. Results for these samples may be reported if any possible concerns related to the reagent blank are adequately explained.

1.6.2 The ***negative amplification control*** (Amplification Blank, NAC, or No DNA Control) is a check of the amplification reagents for possible contamination. The appearance of DNA types in this control (or

possible DNA types below the analytical threshold) indicates that problems similar to those described for the reagent blank may have occurred during the amplification.

The interpretation of detectable DNA in the amplification blank should take into account the results obtained from any samples and reagent blanks amplified in the same batch. A batch where both the amplification blank and the reagent blank give the same typing result, or where only the amplification blank gives a result, may be caused by a problem limited to the amplification process. In this case, re-amplification of the samples may resolve the problem. If the reagent blank and amplification blank give different typing results, both the extraction and amplification processes are potentially suspect and may need to be repeated. Detection of types in a negative amplification control requires the completion of a Quality Incident Summary.

1.6.3 The ***positive amplification control*** (PAC) is a check for effectiveness of the amplification and the Short Tandem Repeat (STR) analytical processes. This control should always show interpretable results consistent with the known genotype published in the DNA kit literature. No other interpretable peaks should be present. Peaks that are determined to be artifacts (elevated stutter, pull-up, etc.) are not considered interpretable peaks.

Results from samples associated with a positive amplification control for which these criteria are NOT met require the completion of a Quality Incident Summary. The results must be evaluated by the DNA Technical Manager (or his/her designee) to determine the appropriate interpretation, taking the observed control results into account. If the observed control results bring the reliability of a sample result into question (as determined by the DNA Technical Manager), the sample result will not be interpreted or reported.

If the results from samples associated with the failed positive control are deemed usable, the failure of the positive control must be disclosed in the final report.

If the cause of the positive amplification control failure cannot be determined, the samples associated with the control are deemed to be non-reportable. Such samples should be re-amplified. Samples associated with the failed positive control that were consumed for the original amplification would require re-examination (re-extraction) of the original evidence. If no evidence remains, then the samples will be reported as inconclusive.

If the cause of the positive amplification control failure cannot be determined, the only time the results from samples associated with a failed positive amplification control that were consumed for the original amplification will be reported is when they form the basis of an exclusion. In this instance, troubleshooting of the problem must lead to the determination that the amplification for that sample is reliable and full disclosure of the unexpected result (failed positive amplification control) as well as the exclusion will be documented in the final report.

## 1.7 Evaluation of samples that have been re-injected, re-amplified, and/or re-extracted

1.7.1 Analysts should evaluate ***multiple data sets for an item amplified with the same autosomal STR kit*** using similar template amounts to determine if the results are consistent and if there are discrepancies between the data, which could be in the form of drastic differences in peak proportions between the injections/amplifications for the sample.

1.7.2 Analysts should evaluate data for an ***item amplified with multiple autosomal STR kits*** to verify that no unexplainable discordances exist between loci common to the kits. Possible reasons for discordances include; but are not limited to; degradation or inhibition affecting one of the amplifications to a greater degree than the other, variation in primer sequences, stochastic effects of low level samples, and the slight differences in peak proportions typically encountered during multiple amplifications of mixed samples.

## Section 2 – Interpretation of DNA Typing Results

In general, interpretation of DNA samples will be completed prior to comparison of reference samples. DNA interpretation will include the following steps:

Determine whether a sample is a mixture (section 2.1)
Determine the number of contributors to a sample and other assumptions (section 2.2)
Determine whether stochastic effects are likely (section 2.3)
Identify whether a sample is degraded and/or inhibited (section 2.4)
Identify uninterpretable samples (section 2.5)

These steps will be documented in the analytical record prior to comparison to reference samples.

### 2.1 Determination of whether a sample is a mixture

A mixed DNA profile possesses certain common attributes that will tend to indicate the presence of more than one contributor to the observed results. The attributes may include:

- Greater than two alleles present at a locus, unattributable to a mutation, or a tri-allelic pattern.
- Significant imbalance in peak height between peaks at a locus (expected peak height ratio studies should be consulted).
- Higher than expected stutter in multiple locations (expected stutter ratio data should be consulted).
- Generally, evidence of a mixed DNA sample exists at multiple loci.

To determine whether a mixed DNA profile exists, the DNA profile must be evaluated in its entirety. If a DNA mixture exists, a conclusion to that fact should be stated in the Forensic Biology report, and may be stated independently of the conclusion(s) surrounding potential individual contributors (see section 5).

## 2.2 Number of Contributors to a Sample and other Assumptions

Assumptions regarding DNA profiles may be made to assist the analyst in drawing conclusions and to put the evidence in the proper context. All assumptions regarding any interpretation will be presented in the notes and in the report (see section 5). While the assumed number of contributors is present on the STRmix documentation included in the technical record, analysts must indicate the assumed number of contributors on the electropherogram, and for mixtures, the locus/loci upon which the number of contributors determination is based must be also be included.

*Number of Contributors*

Generally, any estimate of the number of contributors is based on the locus that exhibits the greatest number of allelic peaks. This is accomplished by considering that each contributor would contribute at most two alleles to any locus (although tri-allelic patterns could be present, they are extremely rare). Additional information such as expected peak height ratios can be used to determine minimum number of contributors. The potential of peaks to be stutter should be considered. There is no conservative approach with which to bias estimates of number of contributors. The number of contributors chosen for the interpretation should be the most likely number required to reasonably explain the observed profile. Assumptions as to the number of contributors should be based on the most reasonable interpretation of the data.

In evidence samples, peaks in double back stutter (n-2 repeats), back (n-1 repeat), two nucleotide back stutter (n-½ repeat) [at the SE33 and D1S1656 loci only], and forward (n+1 repeat) stutter positions will not be filtered and should be evaluated to determine if they may be allelic for the purpose of determining the number of contributors. The validation data may be consulted to aid in the determination of the number of

contributors. Unfiltered peaks in n-1 repeat stutter positions that are more than three standard deviations from the mean observed for an allele during the validation study are more likely to be allelic. Data from the SDPD GlobalFiler Stutter Study have demonstrated that true stutter peaks, for both n-1 and n+1 above three standard deviations from the mean have been observed. Re-amplification can sometimes assist in determining whether a peak is allelic or stutter. In general, the results of the entire profile (including the Yindel, amelogenin, and DYS391) should be evaluated for determining the number of contributors. Any peaks at DYS391 that are not considered allelic (i.e., stutter or some other artifact) must be documented.

Assessing the number of contributors in mixtures of DNA becomes more difficult with increasing numbers of contributors and analysts should use additional caution when evaluating such mixtures. In these situations, where possible and appropriate, it is recommended that the deconvolution be conditioned on reference samples from relevant individuals (for example the donor of an intimate sample), as it will reduce the complexity of the interpretation. However, conditioning these mixtures is not required.

Sometimes the number of contributors cannot be determined. This could be because the profile is complex and may contain putative indications of additional contributors, or because case circumstance suggests that the possibility of further contributors exists. Analysts may take into account sub-analytical threshold peaks and high stutter if these appear genuine.

In circumstances when the number of contributors cannot be estimated, the profile should be reported as “uninterpretable” and no analyses should be conducted. Sometimes doing a second amplification may help in determining the number of contributors.

*Assumed Contributors*

In certain circumstances, it may be reasonable to assume the presence of a contributor in a DNA mixture. Examples may be, but are not limited to, intimate samples, consensual partners, routinely handled objects, or steering wheels. In these circumstances the DNA profile from the assumed contributor(s) can be used to assist interpretation of the additional contributor(s) DNA profile (section 3.3). The assumption of any contributor to a mixture must be supported by the data.

The assumption of the presence of one or more contributors may require the analyst to change the assumption as to the number of contributors to a mixture. For example, if a homozygous individual is assumed to be a contributor to a locus with four alleles, three contributors would be required to account for all alleles instead of two contributors.

STRmix™ requires an estimate to the number of DNA contributors in a sample. Assumptions as to the number of contributors should be based on the most reasonable interpretation of the data. See section 3.2 for additional information.

Multiple STRmix™ analyses under different number of contributor assumptions may be necessary in some circumstances. This can be performed using the Variable Number of Contributors function within STRmix (see section 3.4 for additional information). A VarNOC analysis in STRmix requires approval of the DNA Technical Manager. This approval must be documented in the technical record.

## 2.3 Determination of whether stochastic effects are likely

Stochastic effects manifest themselves in increasing imbalance between heterozygote peaks. These effects generally increase as the level of detected peaks decreases. STRmix™ incorporates assessments of stochastic imbalance into the analysis process.

Analysts can consult the individual kit thresholds document for information on when increased stochastic effects may be expected. Altered injection times will affect stochastic expectations.

## 2.4 Determination of whether inhibition and/or degradation is present

2.4.1 ***Inhibition*** of the PCR process can be caused by a variety of substances and is generally described as a reduction in efficiency of the PCR. GlobalFiler kits have been optimized to reduce the effects of PCR inhibitors. The effects of inhibition can manifest differently depending on the severity of the inhibition. PCR inhibition can alter the amplification efficiency of various loci inconsistent with expectations, cause a profile to appear degraded (see 2.4.2), or not amplify at all. In general, inhibition will affect all contributors to a DNA mixture equally and could raise the concern of actually detecting all alleles that should be present in the mixture.

Evaluation of the IPC in the quantification assays can be helpful to determine whether inhibitors may be present. Because a larger amount of DNA may be amplified than used for quantitation, a sample with an IPC result within expectations may still show evidence of inhibition when amplified with an STR kit.

Several options exist to reduce inhibition. Purifying the sample using the QIAGEN BioRobot EZ1 reduces some inhibitors as compared to organic purification. Reducing the amount of template DNA amplified can also reduce the amount of co-purified inhibitors introduced into the PCR process.

2.4.2 DNA is generally stable if stored properly; however, ***degradation*** of DNA can occur over time, through exposure to the elements, exposure to certain chemicals, or through the action of bacteria. Degradation can be identified by a classic pattern in samples where rather robust results are obtained at the smaller (base pair) loci while little to no results are obtained at the larger (base pair) loci.

The overall results of degradation have been modeled on an exponential curve with a negative slope.

Degradation may not affect all contributors equally (***differential degradation***) and mixtures containing degraded DNA can pose a challenge to the analyst attempting to interpret them. Analysts are encouraged to exercise caution when interpreting a mixture that shows evidence of severely degraded DNA. STRmix™ incorporates assessments of degradation into the analysis process.

## 2.5 *Uninterpretable samples*

Samples (or loci) with poor or limited quality DNA results (e.g., data with OL peaks that are undetermined in nature or data where poor resolution has affected genotyping)

Samples where no assumptions as to the number of contributors can be reasonably made (see also section 2.2) are considered uninterpretable (e.g., if the number of detected DNA types does not appear reflective of the number of contributors (up to two DNA types detected at any marker when the data indicate that three or more contributors may be present)).

## 2.6 *Samples that are unsuitable for comparisons*

2.6.1 Mixtures of 6 people or more are considered unsuitable for comparisons.

2.6.2 Samples amplified with GlobalFiler that have data at five autosomal markers or fewer are unsuitable for comparisons.

2.6.3 Mixture samples with more than one unknown contributor that have been analyzed in STRmix where each contributor has an estimated template amount of ≤200 RFU are unsuitable for comparisons.

2.6.3.1 If a search of the elimination database results in a potential association or if the case scenario suggests that it is reasonable to assume an individual is a contributor to a mixture, it is acceptable to compare a reference from the individual to a mixture with an estimated template of ≤200 RFU to evaluate whether the preliminary LR provides sufficient statistical support to condition the interpretation on the individual.

2.6.3.1.1 If the LR for the individual described above provides sufficient statistical support (e.g., greater than $10^4$), an MCMC conditioned on the individual will be performed. If one or more contributors to the conditioned MCMC are >200 RFU and/or if there is only one unknown contributor in the conditioned MCMC, the sample will be suitable for comparisons.

2.6.3.1.2 If the LR is too low (e.g., less than $10^4$) to support conditioning on the individual, the sample will be reported as unsuitable for comparisons and the LR will not be reported.

2.6.3.1.3 If the MCMC conditioned on the individual described above has more than one unknown contributor and each contributor has an estimated template amount of ≤200 RFU the sample is unsuitable for comparisons and the LR will not be reported.

2.6.3.2 This policy was initiated to minimize analyst subjectivity in determining which evidence profiles are suitable for analysis, not because STRmix was determined to have any issues with data at this level.

2.6.3.3 The exception to this will be analyses performed using the variable number of contributors function. If any analyses under the range of contributors assessed has multiple unknowns with all estimated template levels ≤200 RFU, comparisons can still be made to the deconvolutions.

## Section 3 – STRmix™

STRmix™ is a software program that is used to attach a statistical weighting to comparisons of reference profile(s) to evidence sample profile(s). STRmix™ at the SDPD can be used to process up to five person profiles and can also deconvolute (break down into individual contributors/components) a mixture in the absence of any references. See the STRmix™ protocol for information on using the STRmix™ software.

All mixtures, and at least one of a matching set of single source profiles, need to be deconvoluted and reported (i.e., including multiple PCRs and any assumed contributors). The exceptions to this are:

- If the number of contributors cannot be estimated in the evidence profile
- If there are more than five contributors to the profile
- If samples amplified with GlobalFiler have data at five autosomal markers or fewer
- If the number of detected DNA types does not appear reflective of the number of contributors (e.g., up to two DNA types detected at any marker when the data indicate that three contributors may be present).

If a single source profile does not provide probative information in the context of the case and matches a reference sample (e.g., non-sperm fraction of an intimate sample that matches the source of the sample), there is no need to use STRmix™.

This section outlines workflow and result presentation for STRmix™ use at the SDPD.

### *3.1. Acceptable genotypes for comparisons*

In general, the STRmix output provides the list of possible genotypes for the autosomal loci included in the deconvolution. If STRmix is not used to develop the possible genotype combinations of the contributors, analysts must document a list of acceptable genotypes to be used for any subsequent comparisons.

### 3.2. *Samples amplified with an autosomal STR kit other than GlobalFiler*

There may be instances where further work may be required in a case where data were originally generated using another autosomal STR kit and/or electrophoresis instrument (such as Identifiler on a 3130). The Forensic Biology Legacy Kit Interpretation Guidelines Manual should be consulted for any reinterpretation of legacy data (reevaluation of any of the allele calls or genotype calls [to include potential allelic drop-out], removal of alleles (or entire loci) from statistical estimates, or a change in the assumptions (including number of contributors).

In evaluating samples with legacy data, there are four options:

- If the sample(s) can be re-extracted and/or if DNA extract remains, the sample(s) can be re-amplified and run with our current kit on our current instrument.
- If the interpretation of the original sample(s) included a documentation of the acceptable genotypes for comparison, and reinterpretation of the sample(s) is not required, comparisons of the data from the original interpretation to additional reference samples can be performed by an analyst previously qualified in the STR typing kit or currently authorized to interpret legacy data for that kit.
- The previously reported results remain as they are, and only results generated with current kits on the 3500 are analyzed and reported using STRmix™.
- If the legacy data have not been previously reported and now requires reporting (e.g., through a database link to an old case) and/or if reinterpretation of the sample(s) is required and there is no DNA extract left for analysis, then reinterpretation will need to be carried out in accordance with the Forensic Biology policy manual and the Forensic Biology Legacy Kit Interpretation Guidelines Manual.

## 3.3. Using assumptions

*Number of Contributors*

STRmix™ requires an estimate to the number of DNA contributors in a sample for analysis. Assumptions as to the number of contributors should be based on the most reasonable interpretation of the data. Multiple STRmix™ analyses under different number of contributor assumptions may be necessary in some circumstances.

Generally, an estimate of the number of contributors is based on the locus that exhibits the greatest number of allelic peaks and the fact that each contributor would contribute at most two alleles to the locus (although tri-allelic patterns could be present, but are extremely rare). Additional information at specific markers like Amelogenin, the Yindel, DYS391, or expected peak height ratios at other loci can be used to determine minimum number of contributors. The potential of peaks to be stutter should be considered. There is no conservative approach with which to bias estimates of number of contributors.

Mixtures of DNA with four or more contributors should be interpreted with care as the possibility of misinterpreting the actual number of contributors increases with each additional contributor. In these situations, where possible and appropriate, it may be beneficial to condition the deconvolution on reference samples from relevant individuals, as it will reduce the complexity of the interpretation. However, conditioning these mixtures is not required

Sometimes the number of contributors may be unclear. This could be because the profile is complex and may contain putative indications of additional contributors, or because case circumstance suggests that the possibility of further contributors exists. Analysts are to use their professional judgment when assessing the number of contributors, and may take into account sub-analytical threshold peaks and high stutter if these appear genuine.

In circumstances when the number of contributors cannot be determined, the profile is reported as "Inconclusive number of contributors – cannot be analyzed" and no analyses should be conducted. Sometimes doing a second PCR may help in determining the number of contributors.

Additional assumptions regarding DNA profiles may be made to assist the analyst in drawing conclusions and to put the evidence in the proper context. All assumptions regarding any interpretation will be presented in the notes and in the report.

*Assumed Contributors*

In certain circumstances, it may be reasonable to assume the presence of a contributor in a DNA mixture. Examples may be, but are not limited to, intimate samples, consensual partners, or routinely handled objects, such as steering wheels. In these circumstances the DNA profile from the assumed contributor(s) can be used to assist interpretation of the additional contributor(s) DNA profile. The assumption of any contributor to a mixture must be supported by the data.

The assumption of the presence of one or more specific contributors may require the analyst to adjust a previously assumed number of contributors to the mixture.

An assumption that someone has contributed DNA to an evidence profile is sometimes reasonable and that information may be used for the STRmix™ deconvolution. Assuming that someone is present in both Hp and Hd fixes their genotype during the deconvolution process (essentially forces an LR of 1 at every locus for their portion of the genotype set).

There are two main reasons for assuming that someone has contributed DNA to an evidence profile: for conditioning an MCMC process on a DNA profile that can reasonably be expected to be part of a DNA result, or to

refine contributor genotypes in a mixture for the purposes of searching CODIS.

For conditioning the MCMC process and calculating LRs:

3.3.1. The assumption has to be valid (e.g., intimate swab) and supported by the data. If an analyst determines that the data clearly support the presence of an assumed contributor, this should be documented and can be used as a basis for using an assumption.

A visual evaluation for the purposes of determining whether to assume the contribution of an individual to a sample should take into account peak height ratio expectations, apparent mixture ratios, and stutter ratios in the evidence. The expectations for these elements are available in the validation data for the specific kits. In addition, information from associated fractions (i.e., sperm and non-sperm fractions) of the same sample can be used in this evaluation.

Alternatively, a deconvolution can be performed using STRmix™ and the component interpretation used to justify the assumption that a specific individual is a contributor to the mixture.

3.3.2. If the case scenario suggests that an assumption of a contributor is reasonable, but the component deconvolution does not allow for a clear assessment that the assumed individual is present, an LR supporting the assumption should be performed and included in the case packet. The LRs must be strong enough to support the assumption. The value of the LR will depend on the amount and quality of the data in the sample, but as a guideline the LR should be $10^4$ or greater to justify the use of the assumption.

Refining contributor genotypes for searching purposes:

It is possible to assume that an interpreted profile from a POI or an unidentified person is a contributor to a sample in order to resolve additional unidentified contributors for the purposes of searching the CODIS databases. For example, if the component interpretation yields a single genotype with a weight greater than 99% at all or most loci, one may condition the MCMC on the genotypes for this contributor to refine the genotypes for the other contributor(s). For component genotypes with weights less than 99%, consultation should be sought with the CODIS Administrator or DNA Technical Manager prior to conditioning on a possible unidentified contributor. This is for CODIS upload purposes only. Since this is for searching purposes only, the report should state that subsets of the results are being searched in the CODIS databases. The case notes will detail what was done to generate the searchable profile.

For this to be done the analyst must manually create a reference file for the unidentified person and can then use it as an assumed contributor in a regular STRmix™ deconvolution (see STRmix™ protocol for details on creating this file).

3.3.3. Any reference or interpreted single contributor in a case can be used as an assumed contributor to an evidence profile for the purposes of interpreting another profile for searching CODIS.

A printout of the deconvolution including the component interpretation supporting the assumption should be included in the case file if it is an unambiguous inclusion.

3.3.4. If this is done for a mixture where the component deconvolution is not clear, an LR supporting the assumption should be performed and included in the case packet. The LRs must be strong enough to support the assumption.

3.3.5. The deconvolution should be clearly labeled that it is for upload purposes only. This deconvolution should be searched against the staff database prior to upload.

## 3.4. Variable Number of Contributors Analysis.

3.4.1. The variable number of contributors option in STRmix allows for the analysis of a sample across a range of contributor numbers. The range can only differ by a single contributor (i.e., N and N+1).

3.4.2. The results of a variable number of contributors option produces a probability of the number of contributors given the observed data for each number of contributors in the range tested.

3.4.2.1. Care should be taken when evaluating these diagnostics as STRmix does not assess things like observable peaks below the analytical threshold to calculate these probabilities.

3.4.3. The variable number of contributors option within STRmix uses slightly different propositions from a normal STRmix MCMC analysis. These propositions are;

3.4.3.1. the POI and n to n' unrelated individuals are the sources of DNA considered against (n+1) to (n'+1) individuals, unrelated to the POI, are the sources of DNA.

3.4.4. Using the variable number of contributors option within STRmix requires approval of the DNA Technical Manager. This approval must be documented in the technical record.

## 3.5. Setting up of hypotheses for calculating the likelihood ratio (LR)

Analysis should be set up with the first hypothesis (named Hp in STRmix™) comprising the person, or persons, of interest and known or unknown individuals to fill up to the number of contributors (e.g., for a three-person mixture, Hp could be a POI and two unknowns). The second

hypothesis (named Hd in STRmix™) should consist of all unknown individuals up to the number of contributors (e.g., for a three-person mixture Hd could be three unknowns).

The exception to this is when an individual can be assumed to be a contributor under both hypotheses. In this case the hypotheses would be:

Hp = Assumed contributor + POI + unknown individuals
Hd = Assumed contributor + unknown individuals

As an example, for a three-person mixture:

Hp = Assumed contributor + POI + 1 unknown individuals
Hd = Assumed contributor + 2 unknown individuals

Numerous hypotheses are possible and they depend on the case circumstances, number of contributors, and the number of references available for comparison. Please see section 4.3 for additional information.

## 3.6. Multiple STRmix™ MCMC analyses

Each time a profile is analyzed using STRmix™, the results will vary slightly. To avoid bias, each analysis should only ever be run once and the result reported. The exception to this is when an analysis has produced a result that requires further investigation and hence further analyses (see next section). If an analysis has been carried out more than once then the result with the more appropriate diagnostics should be reported. If more than one analysis has been carried out under the same conditions, then the lowest of the LR results should be reported.

If multiple MCMC analyses have been carried out, only the STRmix™ summary output results from the most appropriate analysis should be included in the case file (e.g., the higher iteration run). The results from other STRmix™ results will be stored electronically. It is advisable to

indicate in the case file that multiple analyses were run under the same conditions and only the lowest is being included in the case file and reported.

## 3.7. Evaluation of the STRmix™ results

STRmix™ performs deconvolutions of samples based on biological phenomena that have been well characterized. STRmix™ models template amount and degradation for each contributor, locus amp efficiencies across the DNA profile, as well as stutter. STRmix™ also takes into account sampling variation when assessing whether a possible answer could be true. These factors are fundamental concepts and; therefore, the results of the deconvolution should be intuitive, in that the results should conform to qualitative expectations given the data obtained from the instrument.

A review of the weights by the analyst remains the primary diagnostic during the interpretation of a STRmix™ output of a mixture deconvolution. Therefore, it is important that the STRmix™ results be assessed by examining the weightings of various genotypes and the DNA profile(s) observed.

There may be instances when the results obtained do not conform to qualitative expectations for the observed data. Examples of this may be:

- Genotype weights or genotypes that are outside of expectations
- Mixture proportions not reflective of the observed data
- Degradation value not reflective of the observed data
- Large LRs (greater than 1) are obtained for each locus, except one where the LR is 0, and the POI reference appears consistent with being a contributor to the observed profile

Causes for these issues include:

- The MCMC has not run for enough iterations.
- The number of contributors has not been correctly interpreted.
- The PCR has been affected (e.g., inhibition or significant degradation).
- An artifact has not been omitted from the results imported into STRmix™.

There are potential solutions for these issues:

- Report the profile as "inconclusive number of contributors – cannot be analyzed".
- Perform further work on the sample (e.g., re-amplify or re-extract).
- Run STRmix™ at an increased number of iterations.
- Correct the STRmix™ input file.
- Use Mx Prior (i.e., informed priors) analysis

Additional diagnostics information within the summary output of STRmix™ may indicate that a STRmix™ deconvolution has not converged on the best sample space. There are six additional diagnostics that could be used to provide information of profile quality or MCMC performance, all of which appear at the top of the PARAMETERS section of the deconvolution raw output. Each is discussed in turn below.

### 3.7.1. Total iterations

The value displayed in this section indicates the total number of post burn-in iterations that the MCMC ran during its analysis. 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.

For example, in an analysis carried out with 100,000 burn-in accepts and 500,000 total accepts in 4.5 million iterations. The total iterations were required to produce the 400,000 post burn-in

accepts. If we divide the post burn-in accepts by the total iterations, we get the acceptance rate: 400,000 / 4,502,052 = 0.0888 or 1 in 11.25

While this value does not provide a diagnostic as to whether the MCMC has converged, it can inform the user as to how clustered the parameter values had to be about a specific point in order to be accepted. A very low acceptance rate (e.g., 1 in thousands to millions) may, in combination with the other diagnostics, indicate the analysis needs to be run for 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.

### 3.7.2. Effective Sample Size

Effective sample size (ESS) 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. This potential will be taken into account during HPD interval generation in any LR calculations (unless the genotype sets are completely resolved on a single combination, in which case there will be no effect of ESS on the HPD interval). A low ESS on its own is not an indication that rework is required.

### 3.7.3. Average log(likelihood)

This value shows the average $\log_{10}$(likelihood) for the entire post burn-in MCMC. This 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 number of contributors is wrong and there are forced stochastic events in the STRmix™ run as a result (e.g., large heterozygote peak imbalances or variation in mixture proportions across the profile)
- Data have been removed that was real, particularly stutter peaks, and must now be described in STRmix™ by dropout
- Artifactual peaks have been left labeled and must now be accounted for in STRmix™ by drop-in.

A low or negative value for the average log10(likelihood) may indicate to users that the analysis requires additional scrutiny. To note, good quality mixed DNA profiles are likely to give higher average log10(likelihood) values than good quality single source profiles. So low average log10(likelihood) values alone are not necessarily an indicator of an issue.

### 3.7.4. Gelman-Rubin convergence diagnostic

This diagnostic informs the user whether the MCMC analysis has likely converged. STRmix™ uses multiple chains to carry out the MCMC analysis and ideally each chain will be sampling in the same space after burn-in. If the chains spend their time in different spaces, then it is likely that the analysis has not run for long enough. 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 can be used to calculate the variance of what is called the “stationary distribution”. If all chains have sampled the same

space, the within chain variance and the variance of the stationary distribution will be approximately equal. If chains have spent time in different spaces, the variation between the chains is likely to be larger than the variation within the chains. This fact is reflected in the Gelman-Rubin convergence diagnostic (GR), which is a ratio of the stationary distribution and within-chain variances. For a converged analysis the GR will be 1.

In earlier versions of STRmix, if the GR was above 1.2, there exists the possibility that the analysis hasn't converged. In newer versions of STRmix, the GR value may be above 1.2 due to the way the software runs the independent chains. Higher values of GR still indicate the results of the analysis should be scrutinized, but are less likely to be definitive of actual issue in the analysis. Running the analysis for a larger number of iterations (definitely burn-in accept number but also likely total as well) will sometimes reduce the GR in these instances to below 1.2.

### 3.7.5. Allele Variance and Stutter Variance constants

Both of these values are the average value for variance and stutter variance constants across the entire post burn-in MCMC analysis. These values can be used as a guide as to the level of stochastic variation in peak heights that is present in the profile.

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.

Used in conjunction with the average log10(likelihood), a large variance or stutter variance constant can indicate poor PCR.

If the sample is simply low level, this should result in a low average log10(likelihood) and an average variance constant.

If some data have been omitted, left on or misinterpreted this should result in a low average $\log_{10}$(likelihood) and high variances.

### 3.7.6. Troubleshooting

Should the weights and/or the diagnostics suggest to the user that further scrutiny is required then a number of re-work options are available, if required. For example, a review of the proposed number of contributors could be considered. Further analysis such as a re-run, a re-amplification or a clean up to strengthen the number of contributors assumption or assist with allele designation/sub optimal PCR performance. Total iterations may be increased, for example, if the acceptance rate is low, the ESS is low and/or the GR value is above 1.2.

## 3.8. Dealing with uncharacterized variants, allelic and chromosomal abnormalities

STRmix™ is based on the idea that given a set of mass parameters (DNA amount, degradation, amplification efficiency) the expected height of a peak of a certain size, at a given locus can be determined. There are biological reasons for the expected height to be incorrect such as:

- Primer binding site mutations
- Trisomy
- Somatic mutations
- Null alleles

STRmix™ is unable to handle loci with these effects and an "ignore locus" function has been built in to the software to deal with these phenomena.

All deconvolutions in all evidence profiles should be run omitting the locus with the biological anomaly. These deconvolutions should be used for the comparison to all reference samples provided in the case. The only exception would be a single source profile matching the reference of an individual who did not have the anomaly.

If the fit to Hp is poor, then the profile should be deconvoluted still omitting the locus and be reported as "POI excluded". The locus should be omitted for all reference profile comparisons, not just the reference sample of the individual with anomaly.

The following caveats are available for inclusion in the reports should these circumstances arise:
*The reference DNA profile of <name> has a possible genetic anomaly at the <locus name> marker. As a result, this marker has been omitted from the statistical calculations.

*The reference DNA profile of <name> is tri-allelic at the <locus name> marker. This means that instead of two DNA types at this marker, as expected, there are three DNA types. This is a recognized biological occurrence. As a result, this marker has been omitted from the statistical calculations.

If reporting individuals as excluded due to a poor fit to Hp, the following caveat can be used in the report:

To compensate for the omission of locus FGA in the STRmix™ analysis, I have compared the FGA alleles in the mixed evidence profiles to the alleles in the reference profiles provided in this case. Where the alleles in the evidence profile cannot be explained by the reference profile, that person has been excluded as a possible contributor to the recovered DNA. Where the complexity of the allele pattern at locus X in the evidence profile did

not permit me to clearly exclude a person, I have reported the LR calculated by STRmix™.

## 3.9. Quality Assurance Check of STRmix Results

3.9.1. The quality assurance check performed for every STRmix™ deconvolution is a comparison of the deconvolution against the elimination DNA database. This check is designed to identify potential DNA contamination by checking deconvoluted samples against elimination DNA profiles.

3.9.2. The QA checks are carried out using the "Search Database" function of STRmix™. The checks should be conducted prior to the submission of a file for technical review.

3.9.3. The elimination database file exists on the FB network (H:\QA-QC files\Elimination Database).

3.9.4. QA checks should be performed as follows:

All samples must be checked against the elimination database. The exception is samples deemed unsuitable for comparison.

The QA check can be performed retrospectively:

- Select the config.xml file that contains the deconvolution of interest
- Ensure STRmix™ is navigating to the correct database file; C:\Program Data\STRmix\Databases\SDPD Elimination DNA database.csv
- Ensure the "minimum match" level is set to 199.
- DB Search calculations will be performed using an $F_{ST}$ value of 0.01b(1.0,1.0).
- Ensure "NIST SDPD Caucasian" is the default population.

- Click search.
- A folder will be created in the results directory of STRmix™ with the name:
- "CaseNumber – SampleName – DBSearch – date".
- Move the DBSearch folder with the deconvolution and the LR calculation folders for this sample to the appropriate STRmix™ case folder.
- All possible links to elimination profiles in the database with database LR values greater than 199 must be brought to the attention of the DNA Technical Manager and a Quality Incident Summary initiated.

## 3.10. $H_d$-True Tester

3.10.1. The $H_d$-True Tester tool within STRmix™ will be used to help provide context for any likelihood ratios greater than 2.

3.10.2. Please refer to the $H_d$-True Tester section in the STRmix™ procedure for instruction and specifications on using this feature.

3.10.3. The 2nd page of the $H_d$-True Tester report must be included in the technical record accompanying the first relevant association (i.e., associations to individuals not reasonably expected to have DNA on an item) to any sample. Any subsequent associations can use the initial $H_d$-True Tester information to provide context.

## 3.11. Reporting Likelihood Ratios

When reporting any inclusionary LR calculations, the *lowest* value of the 99% lower HPD interval for all population groups will be reported. The 99% lower HPD incorporates a measure of allele and MCMC variance. For uninformative and exclusionary LRs, value of the LR need not be reported.

In certain circumstances the 99% lower HPD will be dramatically different from the sub-sub-source LR calculation from the MCMC (e.g., approximately $10^6$-fold difference). This may occur when one or more loci

favor exclusion, but the vast majority of loci favor inclusion. In this scenario, the lowest sub-sub-source LR will be reported. Analysts should evaluate the data when this occurs to determine if the underlying reason for the large difference between the sub-sub-source LR and HPD can be identified and resolved (e.g., mixture results require an additional contributor to explain the data or the presence of an unresolved allele).

### 3.12. STRmix™ analysis unable to be completed due to limitations in computing capacity

Situations may arise when the complexity of the profiles to be analyzed will be beyond the computing capacity of the computers within the Biology section. This will either cause STRmix™ to fail during analysis and/or presenting an out of memory error. Alternatively, STRmix™ will run sufficiently slowly that an analysis will take days to reach completion. In the latter case, an analysis must be run for a minimum of 4 calendar days if time permits, but can be terminated after that time. Results are to be reported with a caveat indicating the sample was beyond current computing capability.

## Section 4 – Statistical Analysis of DNA Typing Results

*All calculations will be generated for the Caucasian, African American, Hispanic, and Asian populations using the DNA allele frequency data taken from U.S. population data for 29 autosomal STR loci, Forensic Science International: Genetics 7 (2013) e82-83 and Corrigendum to "U.S. population data for 29 autosomal STR loci"[Forensic Science International: Genetics 7 (2013) e82-83], Forensic Science International: Genetics 31 (2017) e36-40. If a different database is used for calculations, it shall be included in the technical record and disclosed in the report.*

With the incorporation of STRmix™, the statistical model employed is able to simultaneously measure strength of evidence that could favor the defense hypothesis, as well as the prosecution hypothesis. The traditional analysis is a

two-step consecutive process: a) Is there a match? b) What is the strength of evidence if there is a match? With STRmix™ the requirement to decide a definitive "match" based on subjective criteria of cases are therefore avoided.

Each DNA association must be clearly and properly qualified in a test report by either 1) a statistic; or 2) a qualitative statement.

If the inclusion has no probative value a calculation may not be necessary, and a qualitative statement may be used. A qualitative statement must provide sufficient information to clearly express the significance of the association. For example, a qualitative statement could be that a single source sample obtained from the victim matches the victim at 21 markers. Qualitative statements not based on a statistical calculation should be limited to situations in which the presence of an individual's DNA on an item is reasonably expected.

If an individual is included as a possible contributor to the DNA from multiple items in a case, a single statement may be used. It is possible to group multiple samples together in a statement, but the strength of each should be clearly defined. For example, if several results have been found to match a person of interest, the result providing the highest discrimination can be used, while conveying the range of the other results in the report.

Associations for multiple items or profiles may be qualified with a single statement if the sub-sub-source LRs are identical.

Calculations provided by STRmix™ are likelihood ratio calculations of the form:

$$LR_C = \frac{\sum_j w_j \Pr(S_j \mid H_1)}{\sum_u w_u \Pr(S_u \mid H_2)},$$

These LR calculations use the Balding and Nichols formula for allele frequencies (NRCII Recommendation 4.2):

$$p_A \rightarrow \frac{m_A\theta + (1 - \theta)p_A}{m\theta + (1 - \theta)}$$

The allele frequencies used by STRmix are adjusted to posterior mean frequencies prior to the Balding and Nichols adjustment using the formula below:

$$p(A) = \frac{x_a + \frac{1}{k+1}}{N+1}$$

There is not a set minimum allele frequency for use in STRmix; the effective minimum allele frequency for use in STRmix for alleles not observed in the population frequency data is the case of $x_a$ = 0 in the posterior mean frequency formula above. For calculations using Popstats, the minimum allele frequency of 5/2N is used.

For typical likelihood ratio calculations, the numerator is the probability of obtaining the evidence under the hypothesis that a person of interest is one of the contributors to the DNA mixture along with additional known or unknown people. The calculation in the denominator is the probability of obtaining the evidence under the hypothesis that a randomly selected individual (other than the person of interest) is contributing to the DNA mixture along with additional known (generally the same as the numerator) or unknown contributors. If the result of the ratio is a number greater than one, the DNA results are more probable if the person of interest is a contributor to the mixture. If the result of the ratio is less than one, the DNA results are more probable if the evidence is a mixture of DNA from randomly selected individuals.

Comparisons of additional reference samples to legacy data for which the interpretation of the original sample(s) included documentation of the acceptable genotypes for comparison (including documentations such as "predominant DNA types") can be performed by an analyst previously or currently authorized to interpret legacy data. The conclusions and statistical

methods for these comparisons can be found in the relevant section(s) of the Forensic Biology Legacy Kit Interpretation Guidelines Manual.

## 4.1. Hypotheses for Likelihood Ratio Calculations in STRmix

Analysis should be set up with the first hypothesis (named Hp in STRmix™) comprising the person of interest and known or unknown individuals to fill up to the number of contributors (e.g., for a three-person mixture Hp could be POI and two unknowns).

The second hypothesis (named Hd in STRmix™) should consist of all unknown individuals up to the number of contributors (e.g., for a three-person mixture Hd would generally be three unknowns).

The exception to this is when an individual can be assumed to be a contributor under both hypotheses. In this case the hypotheses would be:

Hp = Assumed contributor + POI + unknown individuals
Hd = Assumed contributor + unknown individuals

As an example, for a three-person mixture:
Hp = Assumed contributor + POI + 1 unknown individuals
Hd = Assumed contributor + 2 unknown individuals

## 4.2. Defining LR results

4.2.1. Support for inclusion: LR≥2.

4.2.2. Support for exclusion: LR $10^{-2}$–0.5 (excluding LR of exactly 0.5).

4.2.3. Exclusion. LR<$10^{-2}$ or LR = 0. Additionally, any comparison that would clearly result in an LR of 0 without a STRmix™ calculation can be reported as an exclusion.

4.2.4. Uninformative. LR 0.5-2 (excluding LR of exactly 2).

4.2.5. Inconclusive. In certain circumstances, a person's association to an item cannot be determined due to inconsistencies between the LR value and qualitative expectations of a comparison, despite the LR value appearing to support inclusion. In these instances, with documented DNA Technical Manager approval, the potential association may be deemed inconclusive.

4.2.6. Uninterpretable. Mixtures with six or more contributors or where no assumptions can be reasonably made to the number of contributors will be deemed uninterpretable. An analyst may determine that due to a lack of information in the results that no comparisons can be made to the profile.

If the positive amplification control does not provide the expected results, or if DNA types are detected in the negative amplification control or the relevant reagent blank, the sample may not be useful for comparison (see section 1.6) and would thus be uninterpretable.

## 4.3. Comparisons to persons of interest

For cases with multiple POIs, each reference will need to be compared separately. If multiple POI comparisons result in LRs of 2 or greater, the analyst shall also perform comparisons that combine multiple POIs in Hp. Various combinations may be needed in order to determine the possibility of inclusion.

For example, if two POIs have individually been compared to a 3-person mixture and both receive LRs of 2 or greater, it is expected that the analyst will also run a comparison using an Hp that includes both POIs (i.e., Hp = POI1 + POI2 +1 unknown). Hd in this situation will be 3 unknown individuals.

If a deconvolution is such that there are only a few genotype possibilities for the contributors, comparison of a POI would not require the calculation of a STRmix™ LR if the POI is clearly excluded. Such exclusions could be documented in the notes and reported.

When STRmix™ performs an LR for a person of interest, the information regarding contributor order is determined within STRmix™ (this is the component the POI had the highest LR for). This information is available immediately after an LR is run in the results window and is available in the STRmix Report.

When comparisons to an evidentiary profile within STRmix result in LRs supporting inclusion for any reference sample, analysts are required to document that their qualitative assessment of the genotypes/haplotypes at Amelogenin, Yindel, and DYS391 also support the inclusion.

## 4.4 Parentage

In cases involving disputed parentage, a parentage calculation will be provided using the formulae integral in the Popstats software. If manual calculations are necessary because the parents and child are from a racial group for which Popstats does not have relevant population data, the formulae can be found in the "Help" section of Popstats. Calculations for three parentage scenarios are performed: when one parent is alleged and the other is known (section 4.4.1), when one parent is alleged and the other is unknown (section 4.4.2), and when both parents are known and the child is alleged (section 4.4.3).

4.4.1 The scenario when an allegation of parentage exists and DNA types from the child, the known parent, and the alleged parent exist is referred to as a ***parentage trio***. In this circumstance, a Parentage Index (PI) is calculated. The PI is the Likelihood Ratio of the probability of the genetic evidence given that the alleged parent is the biological parent of the child versus the probability of the genetic evidence given that a randomly selected, unrelated

individual is the biological parent of the child. See the complete document for information on considering mutations.

4.4.2 In cases where one parent is alleged and the other parent is either unknown or for which DNA results are unavailable, a ***single parent case*** exists. These are often referred to as a "motherless paternity" or "fatherless maternity" cases. Single parent-offspring calculations are within the kinship calculations in Popstats. In setting up the calculation, the profile for the (alleged) child should be in the "Reference" (left hand) side of the calculation and the (alleged) parent should be in the "Evidence" (right hand) side of the calculation.

4.4.3 When the child of two parents is disputed, a ***Reverse Parentage Index (RPI)*** will be calculated. This is the Likelihood Ratio of the probability of the genetic evidence given that the alleged child is the biological child of the parents versus the probability of the genetic evidence given that the alleged child is not the biological child of the parents.

4.4.4 If the race of the known individuals in the parentage calculation is unknown, the race providing the lowest Parentage Index (or Reverse Parentage Index) will be reported.

4.4.5 For parentage calculations involving a male child and a known or alleged father, the sex determining loci should be evaluated to qualitatively assess if they are consistent with paternity.

## 4.5 Kinship

When the biological relationship, or kinship, of two individuals is disputed, a ***Kinship Index (KI)*** will be calculated. This is the Likelihood Ratio of the probability that the two individuals' genotypes are associated by kinship, versus the probability that the two individuals' genotypes are associated by chance. In cases involving disputed kinship, a kinship calculation will be provided using the formulae integral in the Popstats software. If the race of

the individuals in the kinship calculation is unknown, the race providing the lowest kinship calculation will be reported.

## Section 5 – Reporting of DNA Typing Results and Conclusions

The following are meant as guidelines for reporting conclusions for DNA testing in Forensic Biology Unit reports. There are many ways to state a conclusion in a DNA report and the suggestions listed below do not cover every possible scenario. Alternative wording may be used to express a conclusion so long as the conclusion is correct, supported by the data, and is appropriate based on the statistical probability limits employed by the Unit.

### 5.1 Verbal Scale

The following represents the SWGDAM recommended verbal scale used to convey the strength of the likelihood ratio calculation for evidence samples.

| Likelihood Ratio | Verbal Wording |
|---|---|
| ≥1,000,000 | Very strong support for inclusion |
| 10,000-999,999 | Strong support for inclusion |
| 100-9,999 | Moderate support for inclusion |
| 2-99 | Limited support for inclusion |
| 0.5-<2 | Uninformative |
| 0.01 - <0.5 | Limited support for exclusion |
| 0 - $<10^{-2}$ | Excluded |

### 5.2 Reporting of conclusions

5.2.1 The likelihood ratio in STRmix™ is calculating the probability of obtaining the evidence given the person of interest is a contributor against the probability of obtaining the evidence if the person of interest is not a contributor. The calculation result reflects the probability as it relates to the mixture as a whole.

5.2.2 Conclusions for each item should be present in the report. It is acceptable to group similar conclusions for multiple items together.

5.2.3 If a case involves multiple persons of interest and multiple likelihood ratio calculations have been performed for an item of evidence, the individual likelihood ratio calculations as well as the likelihood ratio calculation(s) considering the multiple persons of interest in combination will be reported. If likelihood ratios are performed to determine the potential inclusions of non-probative individuals (e.g., victims or consensual partners) for the purposes of conditioning a STRmix analysis, the individual likelihood ratio calculations do not need to be reported, but must be available in the notes.

5.2.3.1 As examples, if two POIs have likelihood ratios calculated individually that both support their inclusion and a likelihood ratio is calculated for the two people together that also support their inclusion together, the calculation of them together in the mixture will be performed and all three calculations should be reported. If conversely, two individual likelihood ratios support inclusion, but the likelihood ratio calculated for their presence together in the mixture suggests that cannot occur (LR=0), the two individual LRs should be reported with the information that they both could not be contributing DNA together to the mixture. Different situations may require different reporting strategies, and in general, what is reported should be the most informative for the case.

5.2.4 Conclusions should indicate if the sample is not suitable for STRmix™ analysis. A brief description of the reason that it is not suitable should be included.

5.2.4.1 It may in some circumstances be relevant to indicate whether male is present in the sample even if no STRmix™ analysis can be conducted on the sample.

5.2.5 If suitable for STRmix™ analysis, conclusions should state:

5.2.5.1 The assumed number of contributors used in the analysis and the estimated percent contribution of each contributor (if not single source).

5.2.5.2 Any assumptions made regarding the comparisons and/or the statistical calculations must be listed in the conclusion.

5.2.5.3 If the sample is determined to be unsuitable for comparisons after STRmix, the reason should be indicated in the report.

5.2.5.4 Whether the persons of interest are included or excluded, if comparisons are performed.

5.2.5.5 The contributor order the POI best fits with will be reported.

5.2.5.6 If an inclusion is made, and if the inclusion warrants statistical weight (see section 4), the applicable HPD LR value must be stated.

5.2.5.6.1 Reported likelihood ratios should be truncated to three significant digits (e.g., 2.0199 should be reported as 2.01)

5.2.5.6.1.1 Likelihood ratios ≥1000 should be reported in scientific notation (e.g., $8.90 \times 10^{23}$).

5.2.5.6.2 If the difference between the sub-sub-source LR and the 99% lower HPD LR has a difference $10^6$-fold or greater, the sub-sub-source LR will be reported.

5.2.5.7 The verbal scale indicated by the reported LR will be presented in the report.

5.2.5.8 If an evidence profile results in a component (or components) that are searchable in, and uploadable to CODIS (see the CODIS section of the policy manual for additional information), a statement as to whether DNA types or a subset

of the DNA types from an item will be searched in CODIS and what level of CODIS they are being searched at must be included.

5.2.6 If a reference sample produces an incomplete DNA profile, even after troubleshooting, the report will indicate that a partial profile was obtained and that comparisons could only be performed where complete information from the reference sample was obtained.

5.2.6.1 For reference samples with incomplete DNA profiles: A partial DNA profile was recovered from the reference sample from John/Jane Doe; therefore, comparisons were made to the evidence excluding the missing [incomplete] data.

5.2.7 Statements when samples are uninterpretable:

No DNA types were detected in this sample.

This sample is uninterpretable because the number of contributors could not be determined.

5.2.8 Statements when samples are unsuitable for comparison:

This sample is unsuitable for comparisons because the results lack sufficient information.

This sample is unsuitable for comparisons because the results exceed the memory capacity of the interpretational software.
This sample is unsuitable for comparisons because it has more than five contributors.

5.2.9 Examples of inclusions in common scenarios:

5.2.9.1 Example of an inclusion as a contributor to a mixture when the number of contributors is assumed (likelihood ratio scenario)

It is X.XX x $10^{YY}$ times more likely to obtain the DNA results if <POI> is a contributor than if this person is not a contributor.

5.2.9.2 Example of an inclusion as a contributor to a mixture when an assumption is made regarding the number of contributors and with one or more assumed contributors

[Assuming <Name> is a contributor to the mixture, } it/It is X.XX x $10^{YY}$ times more likely to obtain the DNA results if <POI> is a contributor than if this person is not a contributor. (the bracketed portion is not required)

5.2.9.3 Example of an inclusion in a paternity case

It is X.XX x $10^{YY}$ times more likely to obtain the DNA results if John Doe is the biological father of Baby Doe than if a random (ethnicity) man was the father. The probability of John Doe being the biological father is greater than 99.9999%.

5.2.9.4 Inclusions to complete single source profiles on non-probative samples (22 markers for female, 24 for male)

A qualitative statement must provide sufficient information to clearly express the significance of the association:

A full DNA profile was detected (22 markers). The results are consistent with Jane Victim's DNA types.

5.2.9.5 Inclusions to partial single source profiles on non-probative samples (fewer than 22 markers for female or 24 for male)

A qualitative statement must provide sufficient information to clearly express the significance of the association:

A partial DNA profile was detected at ## markers. The results are consistent with Jane Victim's DNA types at these detected markers.

5.2.9.6 Non-probative inclusions to mixtures (22 markers for female, 24 for male)

DNA types were detected at 24 markers. The results are consistent with a mixture John Victim's and Jane Victim's DNA types at these detected markers.

5.2.10 Exclusions (if applicable) will be listed out in each conclusion.

5.2.10.1 Individuals whose LRs provide limited support for exclusion will be listed separately from those individuals whose LRs more strongly support exclusion.

5.2.11 Reporting Staff Matches

When an evidence DNA profile is found to include a staff member of the laboratory the analyst should refer to the section 3 of the Laboratory Quality Manual and section 4.6 of the FB Unit Policy Manual.

5.2.11.1 Example of wording for staff matches

It is X.XX x $10^{YY}$ times more likely to obtain the DNA results if Staff Sample ZZ is a contributor than if this person is not a contributor.

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METHOD: **COSTaR Suite**

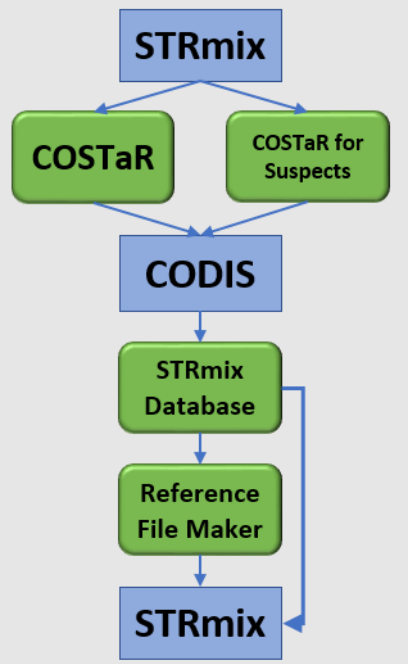
Date: 09/08/2020 Approved by: ARD

COSTaR Suite is an in-house created Excel workbook that uses macros to launch various other Excel workbooks to assist in accomplishing various CODIS-related functions. Instruction for using COSTaR suite are followed by instructions on each component that can be launched from the COSTaR Suite workbook.

COSTaR Suite

The COSTaR Suite Map is a single Excel workbook that enables an analyst to navigate to the various COSTaR workbook applications that have been created to facilitate CODIS tasks related to STRmix.

1. Launch the COSTaR Suite Map workbook located in G:\Misc DATA\CODIS\Excel. The file will open to the "Map" tab.



2. Click on the button for the desired COSTaR application to open the associated Excel workbook then see the instructions below for the relevant application.

3. Close the COSTaR Suite Map when finished.

COSTaR

COSTaR was designed primarily as a tool for analyzing STRmix output data and developing profiles for entry into CODIS. It consists of five main sheets, the STRmix Data sheet, where data from STRmix are added; the Contributors sheet; the Allele Weights Summary sheet, where the summed allele weights for each allele in the profile are shown; the MME sheet, where MME calculations are performed; and the CODIS sheet.

1. The latest version of COSTaR can be opened by clicking on the COSTaR button in the COSTaR Suite Map, or can be found in H:\Worksheets\CODIS Worksheets.

2. On the "STRmix Data" tab, press the Import Data and Create CODIS sheet button.


3. Navigate to the STRmix results file for the deconvolution to be evaluated for CODIS suitability and hit "Open" in the file selection window. COSTaR will retrieve the deconvolution information from the selected MCMC and insert the data into the "Contributors" tab then automatically assess the data for each contributor based on the thresholds in the "Settings" tab and create Excel workbooks for each contributor. Files are located in the same folder as the MCMC file the results folder was selected from.

4. The individual component files made by COSTaR can be evaluated by opening each and examining the CODIS, MME, and Allele Weights tabs. In general, analysts will not be required to alter any of the decisions made for which alleles to include in a search (if applicable). The one exception may be if the MCMC assessed by the COSTaR macros was conditioned on a known DNA profile.

    4.1. If a conditioning DNA profile was used in the MCMC, then the CODIS search profiles must be evaluated for alleles possessed by the assumed contributor that were not detected in the evidence data.

    4.2. If any alleles that were not detected in the data are contained within the alleles to be searched, they should be removed from the list of alleles for that locus on the MME tab. This will affect the risk threshold for that locus (those loci), alter the MME of the search, and may require removal of the entire locus from the search.

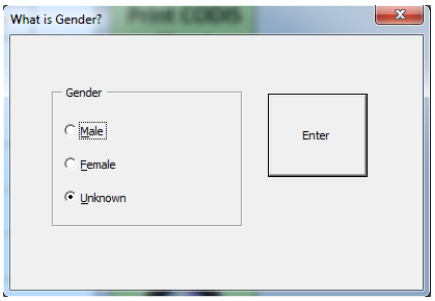
    4.3. Any changes to the COSTaR-mediated CODIS search should be documented within the COSTaR component file.

    4.4. If any questions arise, please consult the CODIS Administrator or the DNA Technical Manager.

5. On the CODIS tab, ensure that the Specimen Category, Source ID, and Partial cells are filled in appropriately based on the sample and component to be searched. Click Save.

6. Print the CODIS sheet tab by clicking on the "Print CODIS Sheet" button.

7. A window will open asking for the gender information of the current profile. Select the appropriate radio button in the window and click "Enter".




8. Include the printed CODIS Worksheet(s) for the searchable components of the MCMC as a numbered page(s) in the technical record. Please note that case circumstances and NDIS procedures may render a component, or an entire sample ineligible for CODIS searching. Please consult the CODIS section of the Forensic Biology Policy Manual, the CODIS Administrator, or DNA Technical Manager as needed.

9. Save and close the COSTaR workbook(s).

After the technical record has been reviewed by a qualified analyst and the profiles have been deemed eligible and suitable for CODIS searching.

10. Open the COSTaR workbook relevant sample component and navigate to the CODIS tab.

11. Click on the "Export" button to export a .cmf file for the component to the following location: H:\Misc DATA\CODIS Import Files.


12. Save and close the COSTaR Excel workbook and repeat steps 10 through 12 as needed.

COSTaR for Suspects

COSTaR for Suspects is an Excel workbook that uses macros to create CODIS worksheets for eligible suspect DNA profiles from the STRmix input files for those samples. Please refer to the Forensic Biology Policy Manual and the relevant SDIS procedures to determine which reference samples qualify for inclusion in the local or State database.

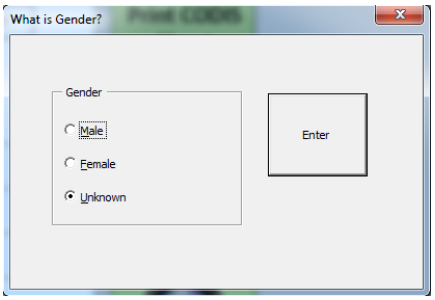
1. The latest version of COSTaR for Suspects can be opened by clicking on the "COSTaR for Suspects" button in the COSTaR Suite Map, or can be found in H:\Worksheets\CODIS Worksheets.


2. On the "STRmix Data" tab, press the Import Data and Create CODIS sheet button.


3. Navigate to the STRmix REF input file used for any comparisons to MCMC data and hit "Open" in the file selection window. COSTaR will retrieve the reference file and place the data into the appropriate fields on the CODIS Sheet.

4. Print the CODIS sheet tab by clicking on the "Print CODIS Sheet" button.

5. A window will open asking for the gender information of the current profile. Select the appropriate radio button in the window and click "Enter".




6. Include the printed CODIS Worksheet(s) for the searchable components of the MCMC as a numbered page(s) in the technical record.

7. Select “Save As” and name the file appropriately.

8. Close the COSTaR workbook(s).

After the technical record has been reviewed by a qualified analyst and the reference DNA profiles have been deemed eligible and suitable for entering into CODIS.

9. Open the COSTaR workbook relevant reference sample and navigate to the CODIS tab.

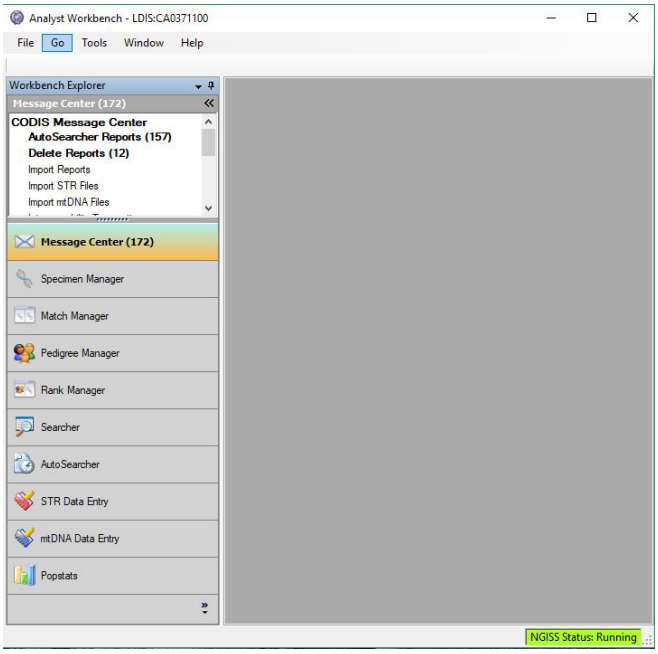
10. Click on the “Export” button to export a .cmf file for the component to the following location: H:\Misc DATA\CODIS Import Files.


11. Save and close the COSTaR Excel workbook and repeat steps 9 through 11 as needed.

STRmix Database

STRmix Database is an Excel workbook designed to simplify the process of comparing possible hits within the CODIS software using the Database search function of the STRmix software.

1. Open the latest version of Analyst Workbench (i.e., the CODIS software) then use the "Go" drop-down menu or the navigation pane to navigate to "Match Manager".




2. Set the "Target" column lab to CA371100. Highlight the relevant matches to create a DB file for.

3. "Print Preview" the short version of either the Match Details Report or the Specimen Details Report. Note: the STRmix Database workbook has a maximum capacity of 1000 matches.

4. Export the results as a .csv file and save it to an appropriate folder on the network. Suggested location is G:\Misc DATA\CODIS\CODIS Matches.

5. Open the latest version of the STRmix Database tool by clicking on the "STRmix Database" button in the COSTaR Suite Map, or from H:\Worksheets\CODIS Worksheets.


6. On the "Database" tab, press the Import Data and Create CODIS sheet button.


7. Navigate to and select the .csv folder saved in step 4. Click "Open".

8. The STRmix Database tool will automatically create a STRmix compatible database file from the specimens selected in Match Manager. Click on the STRmixDB sheet Excel and save the sheet as a .csv file into an appropriate folder on the network.

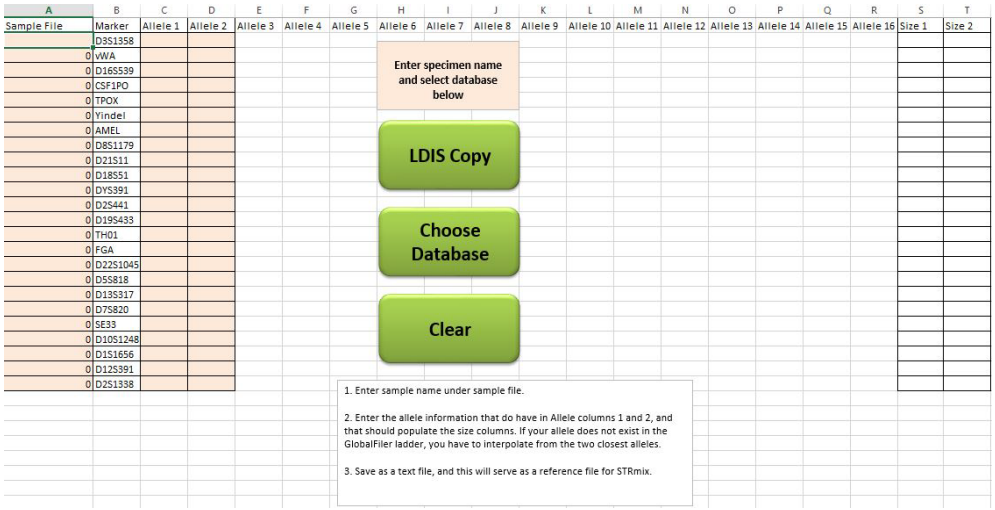
9. This file can be used as the target DB with the Database Search function within STRmix. See the STRmix protocol for additional information on this feature of STRmix.

Reference File Maker

The Reference File Maker is an Excel workbook that will create reference files compatible with the LR from Previous STRmix function from a STRmix database file, or from a reference sample not within GMID-X.

1. Open the latest version of the Reference File Maker tool by clicking on the "Reference File Maker" button in the COSTaR Suite Map, or from H:\Worksheets\CODIS Worksheets.


2. In Sample File column cell A2, enter the identifier of the sample the STRmix compatible reference file is required for. If that sample is within the current version of the exported local CODIS database, the profile information will automatically populate.




3. If the profile information does not automatically populate (i.e., the sample is either not in the local database, or the local database file was created prior to that sample being uploaded), manually enter the DNA profile information for the reference sample appropriately into cells C2:D25. The allele size will automatically populate for most alleles.

4. Save the sheet as a .txt file into an appropriate location on the network. This file will be compatible with the LR from Previous function in STRmix. See the STRmix protocol for additional information on this feature.

METHOD: **Y-STR Interpretation Guidelines** Date:

06/01/2004 **Revision Date: 06/09/2025** Approved by: ARD

## Introduction

The Scientific Working Group on DNA Analysis Methods (SWGDAM) states in their Interpretation Guidelines for Y-Chromosome STR Typing document, that:

> "The interpretation of DNA typing results, including the results for Y-STR testing, for human identification purposes requires professional judgment and expertise. Additionally, laboratories that analyze DNA samples for forensic casework purposes are required by the Quality Assurance Standards for Forensic DNA Testing Laboratories to establish and follow documented procedures for the interpretation and reporting of DNA typing results. Due to the multiplicity of forensic sample types and the potential complexity of DNA typing results, it is impractical and infeasible to cover every aspect of DNA interpretation by a preset rule. However, the laboratory should utilize written procedures for interpretation of analytical results with the understanding that specificity in the standard operating procedures will enable greater consistency and accuracy among analysts within a laboratory. It is recommended that standard operating procedures for the interpretation of Y-STR typing results, to include those from single source and mixed samples, be sufficiently detailed that other forensic DNA analysts can review, understand in full, and assess the laboratory's policies and practices. The laboratory's interpretation guidelines should be based upon validation studies, scientific literature, and experience."

Here we present a number of general interpretation guidelines for interpreting samples in forensic Y-STR casework. The rationale behind these guidelines is to establish a set of standards to ensure that conclusions are supported by reliable data, derived using a scientific approach, and that interpretation of results is as objective as possible and is consistent from one analyst to another.

The interpretation of Y-STR results should take into account the DNA results obtained, the nature of the sample, the condition of the profile, any forensically valid assumptions made, and should be scientifically justifiable and based on the

collected experience and knowledge of the laboratory and the scientific community. These guidelines are designed to provide a general foundation for interpretation.

DNA interpretation should take place in the following order:

Preliminary Evaluation of Data and Allele Designation (section 1)
Interpretation of DNA Typing Results (section 2)
Comparison of DNA Typing Results (section 3)
Statistical Analysis of DNA Typing Results (section 4)
Reporting of DNA Typing Results and Conclusions (section 5)

This document will reference the SWGDAM Interpretation Guidelines for Y-Chromosome STR Typing document and the SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories where appropriate.

## Section 1 – Preliminary Evaluation of Data and Allele Designation

Please refer to section 1 of the Autosomal STR Interpretation Guidelines document as all the information therein is relevant to Y-STR Interpretation Thresholds.

## Section 2 – Interpretation of DNA Typing Results

In general, interpretation of Y-STR data will be completed prior to comparison of reference samples. All interpretation of samples will be performed using the actual electrophoresis data. DNA interpretation will include the following steps:

Determine whether a sample is a mixture (section 2.1)
Estimate the number of contributors to a sample (section 2.2)
Identify whether a sample is degraded and/or inhibited (section 2.3)
Document relevant assumptions (section 2.4)
Non-detection of one or more Y-STR loci (section 2.5)
Haplotype designation (section 2.6)
Unresolvable Y-STR components (section 2.7)
Samples unsuitable for comparison (section 2.8)

2.1 Determination of whether a sample is a mixture

A mixed Y-STR profile possesses certain common attributes that will tend to indicate the presence of more than one contributor to the observed results. The attributes may include:

- More than one allele is present at a locus other than the duplicated loci (DYS385 and DYF387S1) where both homoallelic and heteroallelic genotypes are possible. Note that gene duplications and even triplications have been observed in Y-STR loci [Butler. Advanced Topics in Forensic DNA Typing: Interpretation: Academic Press 2015].
- Significant imbalance in peak height between peaks at DYS385 or DYF387S1.
- Generally, evidence of a mixed DNA sample exists at multiple loci.

To determine whether a mixed Y-STR profile exists, the DNA profile must be evaluated in its entirety. If a DNA mixture exists, a conclusion to that fact should be stated in the Forensic Biology report, and may be stated independently of the conclusion(s) surrounding potential individual contributors (see section 5).

2.2 Estimation of the number of contributors to a sample

Generally, any estimate of the number of contributors is based on the locus that exhibits the greatest number of allelic peaks. This is accomplished by considering that each contributor would contribute at most one alleles to any non-duplicated locus (although locus duplication and triplication could be present, they are extremely rare). Additional information such as expected peak height ratios at the duplicated loci can be used to determine minimum number of contributors. The potential of peaks to be stutter should be considered. There is no conservative approach with which to bias estimates of number of contributors. The number of contributors chosen for the interpretation should be the most likely number required to reasonably explain the observed profile. Assumptions as to the number of contributors should be based on the most reasonable interpretation of the data. It may be necessary after examining the number of contributors to a Y-STR profile to reassess whether the results of the Y-STR analysis necessitate a re-evaluation of

the autosomal results under a different number of contributors. It is recommended that this number of contributor assessment be documented if it could lead to a re-evaluation of the autosomal results.

In evidence samples, alleles in stutter positions may be filtered and all stutter peaks should be evaluated to determine if they may be allelic for the purpose of determining the number of contributors as well as for interpretation. The validation data may be consulted to aid in the number of contributor determination. Peaks in stutter positions that are more than three standard deviations from the mean observed for an allele during the validation study are more likely to be allelic. Data from the SDPD Yfiler™ Plus Stutter Study have demonstrated that true stutter peaks above three standard deviations from the mean have been observed. Re-amplification can sometimes assist in determining whether a peak is allelic or stutter. In general, the results of the entire profile should be evaluated for determining the number of contributors. Any peaks at any locus that are not considered allelic (e.g., stutter or some other artifact) must be documented.

Assessing the number of contributors in mixed Y-STR samples becomes more difficult with increasing numbers of contributors and analysts should use additional caution when evaluating such mixtures. In these situations, where possible and appropriate, it is recommended that the interpretation be conditioned on reference samples from relevant individuals (for example, the donor of an intimate sample or a consensual partner), as it will reduce the complexity of the interpretation. However, conditioning these mixtures is not required.

Sometimes the number of contributors may be unclear. This could be because the profile is complex and may contain putative indications of additional contributors, or because case circumstance suggests that the possibility of further contributors exists. Analysts may take into account sub-analytical threshold peaks and high stutter if these appear genuine.

In circumstances when the number of contributors cannot be estimated, the profile should be reported as “This sample is uninterpretable because the number of contributors could not be determined.” and no analyses

should be conducted. Sometimes doing a second amplification may help in determining the number of contributors.

Many of the loci amplified in the Yfiler™ Plus amplification kit are very close in proximity on the Y chromosome. If loci containing multiple alleles are located near one another on the Y-chromosome using relative Y-STR position information such as found in the Figure 4 below (e.g., <1 Mb), then the entire section of the Y-chromosome may have been duplicated at some time in the past and now possess divergent alleles. The further the putative duplicated loci are apart on the Y-chromosome (e.g., DYS19 and DYS438 which are on different arms of the Y-chromosome), the more likely a sample containing multiple alleles at multiple loci is a mixture.

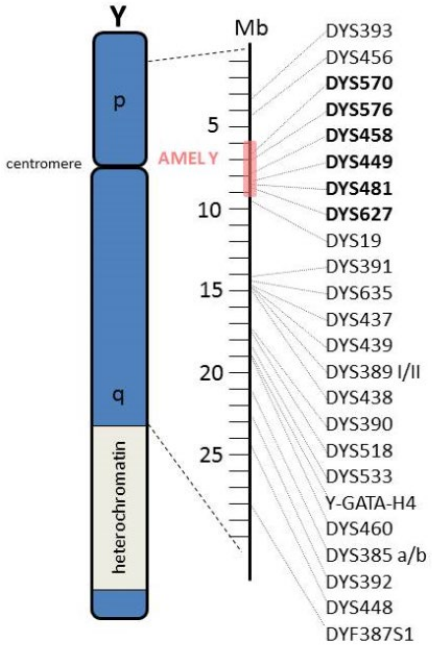



FIGURE 4 - Y-CHROMOSOME MAP OF RELATIVE LOCUS POSITIONS

2.3 Determination of whether inhibition and/or degradation is present

Please refer to section 2.4 of the Autosomal STR Interpretation Guidelines document for more information on inhibition and degradation. The SDPD Yfiler™ Plus validation does have some examples of inhibited Y-STR profiles, but no examples of degraded Y-STR profiles; however, the same basic tenets apply to both autosomal and Y-chromosome analysis when degradation is considered.

2.4 Documentation of Assumptions

For interpretation of mixed Y-STR profiles, ***assumptions regarding the number of contributors*** must be made to assist the analyst in determining major versus minor allele designations. All assumptions regarding the number of contributors will be presented in the report and must also be in the notes (see section 5).

In certain circumstances ***assumptions regarding the presence of a contributor*** in a DNA mixture is permissible (e.g., intimate samples). In these circumstances the DNA profile from the assumed contributor(s) can be used to assist interpretation of the additional contributor(s) DNA profile/types.

Prior to making comparisons, the profile should be evaluated to ***determine what loci and/or combinations of DNA types are acceptable to be assigned to a particular contributor (e.g., major versus minor contributors)***. Please see section 2.6 for additional information.

2.5 Non-detection of one or more Y-STR loci not due to stochastic effects.

Non-detection of a Y-STR allele can occur through many of the same mechanisms that null or partial null alleles can arise in autosomal STR loci. These generally occur through chromosomal rearrangements, primer binding site mutations, or stochastic effects (see the Yfiler™ Plus Interpretation Thresholds for more information on stochastic thresholds). One cause of non-detection of alleles that also occurs in autosomal STRs is the deletion of a section of chromosome containing either the primer binding site or the entire locus. In autosomal STRs, these deletions events

tend to affect single loci due to the loci being on separate chromosomes, or separated by several megabases. In Y-STRs, these deletions may also affect more than one locus due to the juxtaposition of the Y-STR loci on the Y-chromosome. If the average peak height of a contributor is above the stochastic threshold for non-duplicated loci and one or more alleles are missing, it may be due to a deletion of a section of the Y-chromosome affecting multiple loci. In these instances, Figure 4 can be referenced to determine the locations on the chromosome of the affected loci to better inform any conclusions. As with duplications and triplications, these deletions are inheritable from fathers to sons.

## 2.6 Haplotype designation

If ***any stutter peaks*** (filtered or unfiltered) occur at the same relative peak height to the average peak height of a lower level contributor to the sample (e.g., a minor contributor), the analyst must evaluate it as potentially allelic if any allele determinations for the lower level contributors will be made. This is especially true in the instance where a single higher level allele is detected at a marker. In instances where the consideration of such a peak in a stutter position as being allelic (e.g., when the peak in a stutter position would be a third potentially allelic peak in a non-duplicated marker in an assumed 2-person mixture), and the peak height of the putative allelic peak is consistent with the stutter ratio expectations, it is unnecessary to consider the stutter peak as allelic. After the evaluation of the stutter peaks, it may be necessary to reassess the assumed number of DNA contributors to the sample.

***Single haplotypes***: if a Y-STR profile is determined to originate from a single source, the haplotype used for comparisons must be documented in the case notes. This may be accomplished by documenting the allelic peaks in the haplotype, or alternatively by designating any unfiltered stutter or artifact peaks as non-allelic. For duplicated loci, the combination of alleles (homoallelic or heteroallelic genotype) from the contributor must be documented. The stochastic thresholds and peak height ratio expectations must be used when determining the acceptable haplotype for comparison.

*For any low level partial YSTR results that appear single source, consult the DNA Technical Manager to determine whether the sample is sufficient for interpretation as a single source profile.*

***Mixed haplotypes***: Interpretation thresholds for major and minor contributors

- For a single source component to be interpreted from a mixture of two or three individuals, the thresholds derived from the equations outlined in **Figures 1, 2, or 4 through 6** must be met or exceeded.
- For the minor component of a mixture to be interpreted, the minor allele must be detected, or if potentially masked, the minor contributor average peak height must be above the stochastic threshold, and the locus must surpass the threshold derived from the equation outlined in **Figure 3.**
- In addition to the peak height thresholds being met, the observed mixture proportion needs to be within ±0.2 of the expected ratio. For example, for a 10:1 mixture ratio, the expected proportions of the components are 0.9 (major) and 0.1 (minor). To interpret a major component, its proportion must exceed 0.7. For a minor component to be interpreted, its proportion cannot exceed 0.3.
- If the locus is duplicated, then the average peak height of the proposed pair must be above the thresholds in the first two bullet points. In addition, the peak pairs must be within the balance thresholds given in the Yfiler™ Plus Interpretation Thresholds (page 140).
- Applying the guidelines in the previous three bullets there can be only one peak (or peak pair) that meets all criteria.
- If the criteria in the previous 4 bullets are met, a component must possess a mixture proportion of at least 0.2 greater than the closest minor component to be considered a major component.

  When applying the interpretational thresholds in the figures below, an appropriate mixture ratio for the profile in question should be used. The mixture ratio should ideally be the average ratio across the profile rather than the ratio at an individual locus. Caution should be used when evaluating a mixture where the contributors are contributing DNA at levels below the stochastic threshold. In the event that there appears to be a likely DNA type (or likely DNA types) just below the

detection threshold, the analyst should use a value of 99 RFU for that (or those) peaks in the calculation of the mixture ratio. The inclusion of the 99 RFU value allows for the average mixture ratio to be based on more loci and generally results in a more accurate estimation. It is not advised to use the duplicated loci for determination of the mixture ratio.

Not all possible mixture ratios are accounted for in **Figure 4 through 6.** When using **Figures 4 through 6** the most appropriate ratio is that between the apparent major peaks and the peaks of the most intense second contributor (e.g., if the mixture appears to be 10:5:1, the peaks of the more intense minor component are half that of the major peaks so a 2:1:1 ratio would be used in the equations in **Figures 4 through 6**.

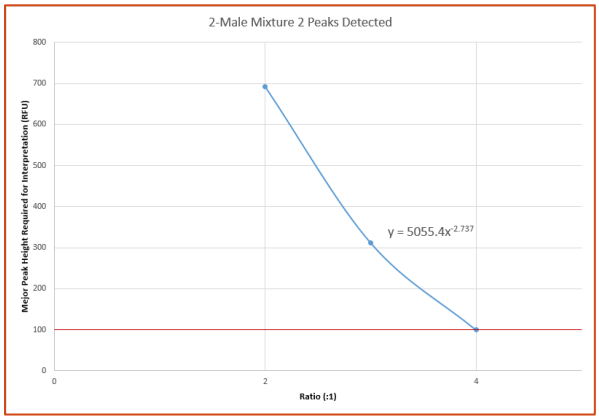
Thresholds for the interpretation of a minor component from a 3 person mixed profile are not provided. Such an interpretation should be carried out with extreme caution and a communication with the DNA Technical Manager must be documented and included in the case file.

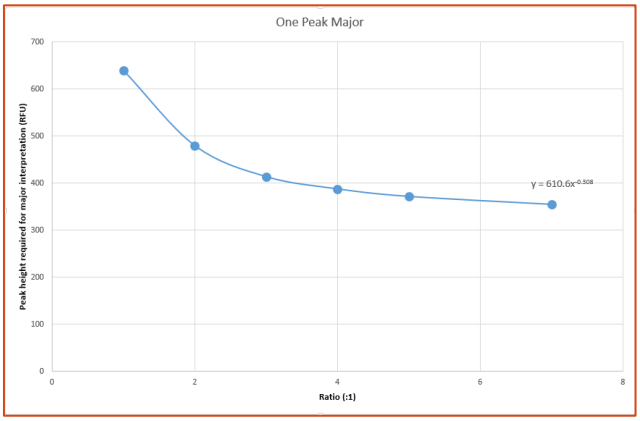
**From the above guidelines, there are 3 possible outcomes:**

1. **The thresholds are met at all loci.** In this situation a complete single source component can be interpreted.

2. **The thresholds are met at some loci.** In this situation an incomplete component can be interpreted. If the comparison of an incomplete interpreted component to a reference profile results in a non-exclusion, care must be taken to ensure that an exclusion does not exist at any of the non-interpreted loci.

3. **The thresholds are not met at any of the loci.** In this situation the profile is deemed to be an unresolvable mixture (see section 2.7).




FIGURES 1 AND 2 – EQUATIONS FOR INTERPRETATION OF MAJOR COMPONENTS IN 2-PERSON MIXTURES

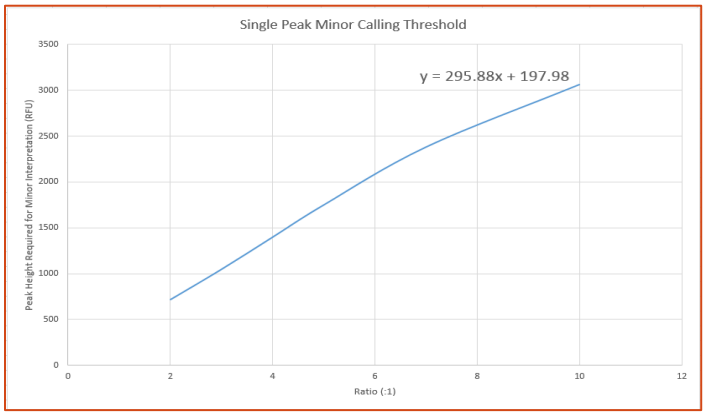



FIGURE 3 – EQUATION FOR INTERPRETATION OF A MINOR COMPONENT IN A 2 PERSON MIXTURES WHEN 1 PEAKS IS DETECTED

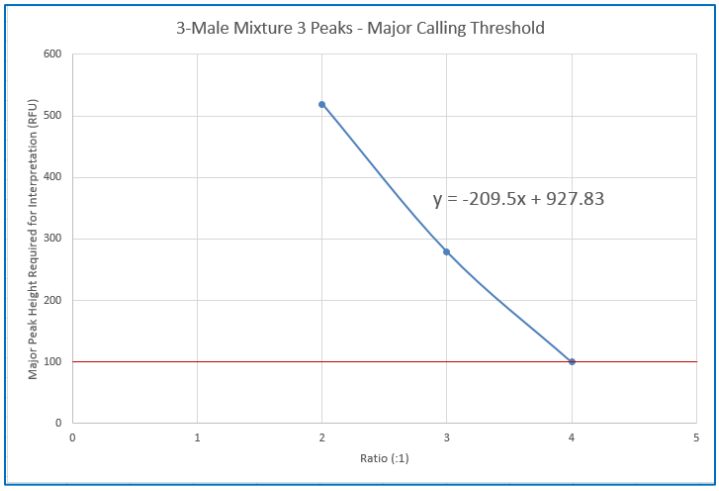



FIGURE 4 – EQUATION FOR INTERPRETATION OF A MAJOR COMPONENT IN A 3 PERSON MIXTURES WHEN 3 PEAKS ARE DETECTED

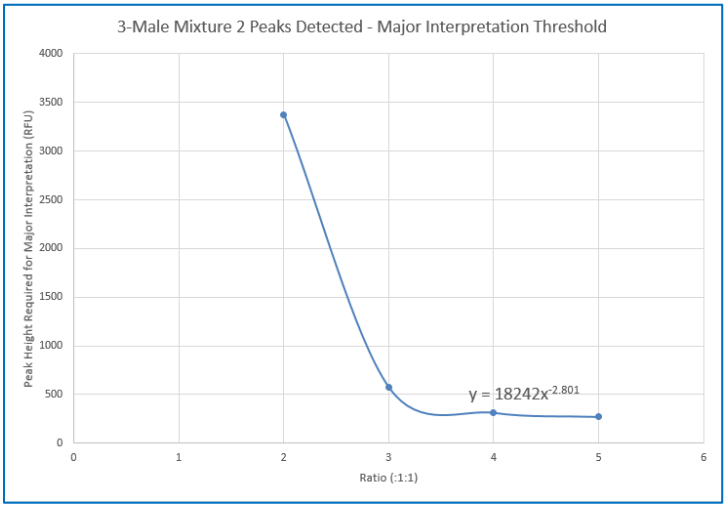



FIGURE 5 - EQUATION FOR INTERPRETATION OF A MAJOR COMPONENT IN A 3 PERSON MIXTURES WHEN 2 PEAKS ARE DETECTED

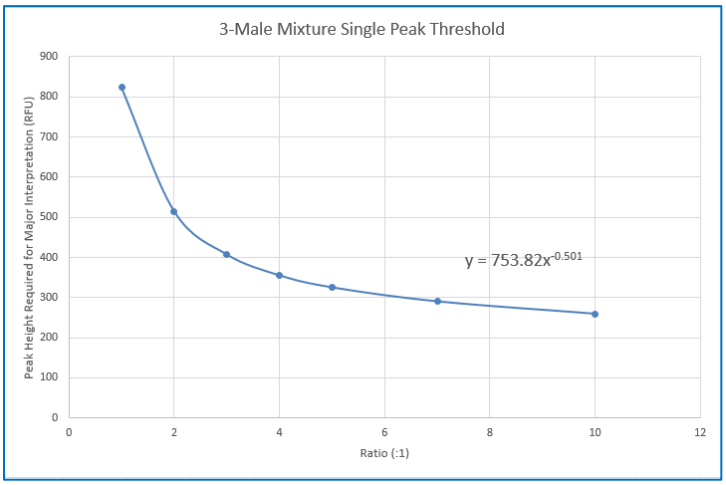



FIGURE 6 - EQUATION FOR INTERPRETATION OF A MAJOR COMPONENT IN A 3 PERSON MIXTURES WHEN A SINGLE PEAK IS DETECTED

## 2.7 Unresolvable Y-STR components

When the mixture proportion for 2 contributors is within 0.2 (mixture ratio <2:1), the mixture is determined to be unresolvable. In the event that a mixture is determined to be unresolvable, the average peak heights for loci exhibiting the same number of peaks as the assumed number of contributors (e.g., loci with two alleles in a 2-person mixture) across the

non-duplicated loci should be evaluated. If the average peak height is above the 740 RFU stochastic threshold, then all information can be considered present and all types can be used for comparison. The significance of any inclusion must be calculated using the CA-DOJ Mixture Tool.

Depending on the mixture it may be possible in 3-person mixtures to isolate a partial "major mixture" based on average peak heights between the contributors. In these instances, the major mixture component must possess a mixture proportion of at least 0.2 greater than the closest minor component.

2.8 Samples unsuitable for comparison

Y-STR profiles with 4 or more contributors are generally considered unsuitable for comparisons. In the instance that there are obvious major or minor components, an interpretation can be conducted on the provision that permission from the DNA Technical Manager is obtained for the interpretation and that the approval is documented in the case file. The possibility of a major component being the result of the additive effect of allele sharing must be ruled out.

## Section 3 - Comparison of DNA Typing Results

3.1 ***Inclusion.*** If a person of interest has no unexplainable differences from the alleles or allele combinations determined to be useful for statistical support of inclusions, and thus comparison, they are included as a source of the DNA or possible contributor to the DNA mixture.

3.2 ***Exclusion.*** If a person of interest has unexplainable differences from the alleles or allele combinations determined to be useful for comparisons, they are excluded as a source of the DNA or possible contributor to the DNA mixture.

3.3 ***Inconclusive.*** Some mixtures or components of such mixtures may not be suitable for comparison. Comparisons to these mixtures will be considered inconclusive.

An analyst may determine that due to a combination of variables including (but not limited to) the number of DNA contributors as well as a lack of information in the results that no comparisons can be made to the profile (or comparison for possible minor contributors).

## Section 4 - Statistical Analysis of DNA Typing Results

Y-STR loci are linked on the Y-chromosome and do not undergo linkage disequilibrium. Therefore, neither Recommendation 4.1 (NRC 1996) nor the Product Rule can be applied to Y-STR haplotypes. The significance of a haplotype match in casework must be based upon the observed frequency of the particular haplotype in one of the databases described below (the counting method). In general, the count in the combined, or total number of samples will be reported, unless case specific circumstances require reporting a single population.

For the purposes of expressing the significance of a Y-STR haplotype match, all alleles for loci deemed acceptable for comparison will be searched in the database. More DNA profiles in the available databases have a subset of the Yfiler™ loci than have the full complement of the Yfiler™ Plus loci. As a result, an analyst may elect to search a subset of the Y-STR results in the database for the purposes of obtaining a more discriminating search of the database.

The 95% upper confidence interval will be reported for Y-STR statistics. The 95% upper confidence interval is a conservative measure to account for possible sampling variation in the databases. The 95% upper confidence provides an estimate of the population frequency, where, if the process was repeated, 95% of the upper confidence intervals obtained would be equal to or less common than the population frequency, 5% would be more common. All the databases described below provide the 95% upper confidence interval.

If an inclusion has no probative value, a calculation may not be necessary, and a qualitative statement may be used. A qualitative statement must provide sufficient information to clearly express the significance of the association. For example, a qualitative statement could be that a Y-STR profile from a swab of

the suspect matches the suspect at 25 markers. Qualitative statements not based on a statistical calculation should be limited to situations in which the presence of an individual's DNA on an item is reasonably expected.

## 4.1. Available Haplotype Databases

Y-STR haplotype frequencies can be calculated from a number of established databases. The laboratory has incorporated the CADOJ Y-Mix version 4.2 R60(US)1 for the calculation of Y-STR haplotype frequencies. This haplotype calculator can be found on the forensic biology computer network:

H:\Worksheets\Statistical Tool\Y-Mix - version 4.2 R60(US)1

This tool will not only allow for the frequency estimates of single source haplotypes, but will also allow for calculations to be done on Y-STR mixture profiles. Instructions are located within the Excel spreadsheet on the "Instructions" tab. The database that this spreadsheet uses for its haplotype and Y-STR mixture calculations is release 60 of the Y-chromosome Haplotype Reference Database (www.yhrd.org).

To search for a haplotype, enter the alleles of interest for each marker by using the drop-down menu, or typing the alleles into each appropriate cell in the worksheet (only one allele per cell). To specify a value not listed in the drop-down menu, enter the variant allele into the appropriate new variant cell. www.yhrd.org is a useful resource for Y-STR information. If mutation rates are required for any Y-STR calculations, analysts must use the mutation rates listed on this website.

### 4.1.1. Using Y-Mix version 4.2 R60(US)1

Open the Y-Mix tool. Entered haplotype information for all available loci in alpha-numerical order with each individual DNA type listed in a single cell within the appropriate locus column. The Y-Mix tool allows for haplotype mixtures to be searched.

The Y-Mix tool has an option to either limit the search to only database samples with the same set of data as the entered haplotype or to search against all samples within the database. Selecting "Yes" for *Limit database to samples with all the loci entered above?* results in searching against only samples within the database than contain all the loci entered. Selecting "No" results in a search against all haplotypes in the database. Selecting "No" will always result in a more discriminating search and should be the default search parameter when searching haplotypes.

Y-Mix Filter Tool v4.2 also has tool allowing drop-out to be considered at the duplicated markers during the database search of single source samples. When "Yes" is selected for *Treat this profile as a single source sample?*, an option appears: *Allow for dropped alleles at multicopy loci?*. Selecting "No" searches for the exact haplotype entered. When only one type is entered at a duplicated locus (i.e., DYS385 and DYF387S1), selecting "Yes" allows for any haplotypes with the entered type as a homoallele, and the entered allele paired with any other type (heteroallelic haplotype) to be returned in the search. This feature is useful when single types below 570RFU are present at either of the duplicated markers.

The search and results should be saved to an analyst folder as a separate Excel file.

4.1.2. YHRD may be used on occasions when a search of a broader spectrum of the haplotypes are required to be searched. YHRD contains data from over 340,000 haplotypes from around the world. All samples searched against YHRD are only searched against samples with the same complement of loci that are entered for the haplotype to be searched. YHRD does not accommodate searching mixtures.

To use the YHRD, access www.yhrd.org, then click on the *Estimate Frequency* tab. Click on the *Manually enter haplotype(s)* button (alternatively, the YSTR haplotype may be uploaded via an Excel spreadsheet, .csv file, or GeneMapper ID-X export file). Select *Yfiler Plus*, enter the all available loci as appropriate, and click the "Search" button. Click on the *Add feature to the report* and select "National Database (with subpopulations, 2014 SWGDAM – compliant)". Ensure the drop down lists *United States* to obtain the data for the US population dataset. The statistical calculations of interest will be listed under the *Observed* title.

To achieve more discriminating searches, users may alternately reduce the number of loci by selected the YFiler, or Minimal, buttons to reduce the number of loci searched (without having to re-enter the haplotype).

Searches using YHRD can be printed, but cannot be saved.

## Section 5 - Reporting of DNA Typing Results and Conclusions

The following are meant as guidelines for reporting conclusions for DNA testing in Forensic Biology Unit reports. There are many ways to state a conclusion in a DNA report and the suggestions listed below do not cover every possible scenario. Alternative wording may be used to express a conclusion so long as the conclusion is correct, supported by the data, and is appropriate based on the statistical probability limits employed by the Unit.

### 5.1 Reporting of DNA typing results

Please refer to the Autosomal STR Interpretation Guidelines document for additional information.

### 5.2 Reporting of Conclusions

Conclusions for each item should be present in the conclusions section of the report. It is acceptable to group similar conclusions for multiple items together.

5.2.1 In general, conclusions should state the assumed number of contributors (if a determination can be made). The conclusion should indicate if the sample is not suitable for comparison purposes. A brief description of the reason that it is not suitable should be included. In the case of a mixture that is only suitable for comparison to possible major contributors, an indication should be included that the sample is not suitable for comparison to possible minor contributors.

If the sample is suitable for comparison and if comparisons are made, the conclusion must indicate whether the persons of interest are included or excluded. If the comparison is determined to be inconclusive, a statement giving reasons leading to the inconclusive determination.

If an inclusion is made, and if the inclusion warrants a probability calculation (see section 4), the applicable probabilities should be listed.

Any assumptions made regarding the number of contributors, or regarding comparisons and/or the probability calculations will be listed in the conclusion.

If the inclusion of an individual requires a larger number of contributors than the minimum based on allele number and peak height ratios, a statement to that effect should be included.

5.2.2 Example of an inclusion to an apparent single source Y-STR haplotype

It is estimated that 1 in X males would be included as a contributor to the results.

5.2.3 Example of an inclusion to a Y-STR mixture

It is estimated that 1 in X males would be included as a <major/minor> contributor to the results.

5.2.4 Example of a Y-STR sample that is unsuitable for comparison

The minor male DNA types are not suitable for comparison. [May be added to a mixture conclusion if applicable.]

Insufficient information was obtained during male specific Y-STR analysis of the evidence. This sample is unsuitable for comparisons.

This sample is uninterpretable because the number of contributors could not be determined.

REFERENCES:

1. Yfiler™ Plus PCR Amplification Kit User's Manual
2. SDPD AmpF*l*STR Yfiler™ Plus validation studies
3. SWGDAM Interpretation Guidelines for Y-Chromosome STR Typing (2016 Revision)
4. www.usystrdatabase.org
5. www.yhrd.org
6. SDPD verification of the CADOJY-Mix Tool
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## Appendix A: Glossary of terms (adapted from SWGDAM)

**Allelic dropout**: failure to detect an allele within a sample or failure to amplify an allele during PCR.

**Analytical threshold**: the minimum height requirement at and above which detected peaks can be reliably distinguished from background noise; peaks above this threshold are generally not considered noise and are either artifacts or true alleles.

**Artifact**: a non-allelic product of the amplification process (e.g., stutter, non-template nucleotide addition, or other non-specific product), an anomaly of the detection process (e.g., pull-up or spike), or a by-product of primer synthesis (e.g., "dye blob").

**Composite profile**: a DNA profile generated by combining typing results from different loci obtained from multiple injections of the same amplified sample and/or multiple amplifications of the same DNA extract. When separate extracts from different locations on a given evidentiary item are combined prior to amplification, the resultant DNA profile is not considered a composite profile.

**Conditional**: an interpretation category that incorporates assumption(s) as to the number of contributors.

**Conditioning**: The assumption of a particular contributor's presence in a DNA mixture in both the numerator and denominator of a likelihood ratio. This fixes the genotype of the assumed contributor(s) within the STRmix™ analysis. A person may be assumed as a contributor if it reasonable to assume the presence of their DNA given case scenario and the results.

**Deconvolution**: separation of contributors to a mixed DNA profile based on quantitative peak height information and any underlying assumptions.

**Deduced**: inference of an unknown contributor's DNA profile after taking into consideration the contribution of a known/assumed contributor's DNA profile based on quantitative peak height information.

**Differential Degradation**: a DNA typing result in which contributors to a DNA mixture are subject to different levels of degradation (e.g., due to time of deposition), thereby impacting the mixture ratios across the entire profile.

**Evidence sample**: also known as Questioned sample.

**Exclusion**: a conclusion that eliminates an individual as a potential contributor of DNA obtained from an evidentiary item based on the comparison of known and questioned DNA profiles (or multiple questioned DNA profiles to each other).

**Guidelines**: a set of general principles used to provide directions and parameters for decision making.

**Heteroallelic:** in Y-chromosome analysis where gene duplication has occurred, the state of having different alleles at each of the gene locations.

**Heterozygote**: an individual having different alleles at a particular locus; usually manifested as two distinct peaks for a locus in an electropherogram.

**Homoallelic:** in Y-chromosome analysis where gene duplication has occurred, the state of having the same allele at each of the gene locations.

**Homozygote**: an individual having the same (or indistinguishable) alleles at a particular locus; manifested as a single peak for a locus in an electropherogram.

**Inclusion**: a conclusion for which an individual cannot be excluded as a potential contributor of DNA obtained from an evidentiary item based on the comparison of known and questioned DNA profiles (or multiple questioned DNA profiles to each other).

**Inconclusive**: an interpretation or conclusion in which an association to an item cannot be determined. Inconclusive determinations require DNA Technical Manager approval.

**Intimate sample**: a biological sample from an evidence item that is obtained directly from an individual's body; or one where it is not unexpected to detect that individual's allele(s) in the DNA typing results.

**Known sample**: biological material for which the identity of the donor is established and used for comparison purposes (referred to as a "K").

**Legacy data:** data generated by a typing test kit, platform, or technology that is no longer in use by the laboratory.

**Likelihood ratio (LR)**: the ratio of two probabilities of the same event under different hypotheses; typically, the numerator contains the prosecution's hypothesis and the denominator the defense's hypothesis.

**Major Mixture:** in Y-STR testing, the state of having two major contributors in a mixture that are unresolvable from each other. The major mixture portion may be resolvable from a third minor contributor at all or some DNA markers.

**Masked allele**: an allele of the minor contributor that may not be readily distinguishable from the alleles of the major contributor or an artifact.

**MCMC:** Markov Chain Monte Carlo. This is a class of algorithm that is used in statistics to sample from probability distributions. The Markov Chain Monte Carlo is the basis for the deconvolutions performed in the STRmix™ software.

**Mixture**: a DNA typing result originating from two or more individuals.

**Mixture ratio**: the relative ratio of the DNA contributions of multiple individuals to a mixed DNA typing result, as determined by the use of quantitative peak height information; may also be expressed as a percentage.

**Noise**: background signal detected by a data collection instrument.

**No results**: no allelic peaks detected above the analytical threshold.

**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.

**Partial profile:** a DNA profile for which typing results are not obtained at all tested loci expected to produce results (e.g., a female reference sample would not be expected to produce results at Yindel or DYS391; therefore, would not be

considered partial if no data were present at those markers). The partial profile may be due to DNA degradation, inhibition of amplification and/or low-quantity template.

**Peak height ratio (PHR)**: the relative ratio of two alleles at a given locus, as determined by dividing the peak height of an allele with a lower relative fluorescence unit (RFU) value by the peak height of an allele with a higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage; used as an indication of which alleles may be heterozygous pairs and also in mixture deconvolution.

**Platform:** the type of analytical system utilized to generate DNA profiles, such as capillary electrophoresis, real-time gel and end-point gel instruments or systems.

**Questioned sample**: biological sample recovered from a crime scene or collected from persons or objects associated with a crime (referred to as a "Q").

**Reference sample**: also known as Known sample.

**Signal-to-noise ratio**: an assessment used to establish an analytical threshold to distinguish allelic peaks (signal) from background/instrumental noise.

**Single-source profile**: DNA typing results determined to originate from one individual based on peak height ratio assessments and the number of alleles at given loci.

**Stochastic effects**: the observation of intra-locus peak imbalance and/or allele drop-out resulting from random, disproportionate amplification of alleles in low-quantity template samples.

**Stochastic threshold**: the peak height value above which it is reasonable to assume that, at a given locus, allelic dropout of a sister allele has not occurred.

**Stutter**: a minor peak typically observed one repeat unit smaller than a primary STR allele resulting from strand slippage during amplification.

**Technology:** the type of forensic DNA analysis performed in the laboratory, such as STR, YSTR, SNP, or mitochondrial DNA.

**Test kit**: a preassembled set of reagents (or laboratory assembled equivalent) that allows the user to conduct a specific DNA extraction, quantification, or amplification method.

**Uninterpretable**: an interpretation or conclusion in which the DNA typing results are insufficient, as defined by the laboratory, for interpretation due to the low quality of the data, or where no assumptions to the number of contributors can be reasonably made.

**Unsuitable for comparisons**: an interpretation or conclusion in which the DNA typing results are not suitable for comparisons. This may be due to being a mixture of 6 or more individuals, insufficient data obtained from the sample, or samples with more than a single unknown contributor where the estimated template levels are ≤200 RFU for all contributors

**Appendix B: List of common abbreviations used in the forensic biology unit.**

| | | | |
|---|---|---|---|
| + | Positive | CNTNG | Containing |
| - | Negative | CODIS | Combined DNA Index System |
| ~ | Approximately | CPT | Clear plastic tube |
| ALS | Alternate light source | Crim | Criminalist |
| AP | Acid Phosphatase | CSS | Crime Scene Specialist |
| AT or A/T | Analytical Threshold | CSU | Crime Scene Unit |
| AUSA | Assistant US Attorney | DDA | Deputy District Attorney |
| BC or B/C | Barcode | DE or Diff | Differential extraction |
| BPB | Brown paper bag | DEIF | Deionized formamide |
| BP | Base pair(s) | Det. | Detective |
| BPS | Brown paper sack | DNE | Do not enter (into CODIS) |
| CD or cond | Conditioned | DNU | Do not use (for CODIS) |
| CE | Capillary Electrophoresis | DTM | DNA Technical Manager |

| | |
|---|---|
| e-gram/epg | Electropherogram |
| FB | Forensic Biology |
| Form | Formamide |
| HemaTrace | Test for the confirmation of blood |
| ILS | Internal lane standard (see also ISS) |
| Imb | Imbalance |
| Inc # | Incident Number (event number) |
| ISS | Internal size standard (see also ILS) |
| LMG | Leuco Malachite Green |
| LR | Likelihood Ratio |
| LSE | Limited support for exclusion |
| LSI | Limited support for inclusion |
| MCMC | Markov chain Monte Carlo (STRmix deconvolution) |
| MSI | Moderate support for inclusion |
| NA or N/A | Not analyzed or not applicable |
| ND | Not detected |
| NE | Nucleated epithelial cells |
| Neg | Negative |
| NOC | Number of Contributors |
| NS | Non-sperm fraction |
| nt | nucleotide |
| p30 | seminal protein p30 (PSA) |
| Pheno | Phenolphthalin |
| PHI | Peak Height Imbalance |
| Pk | Peak |
| PR or P/R | Property Room |
| PSA | Prostate Specific Antigen |
| PT or P/T | Property Tag |
| R/b | Red-brown |
| RB | Reagent blank |
| RFU | Relative fluorescence units |
| RMS | Reference Mouth Swabs |
| RMSCK | Reference Mouth Swab Collection Kit |
| RT or R/T | Room Temperature |
| (s) | Suspect |
| S, SF, or SP | Sperm fraction |
| SAEK | Sexual Assault Evidence Kit |
| SAFE | Sexual Assault Forensic Evidence (collection kit) |
| SAO | Same as original |
| SART | Sexual Assault Response Team (evidence kit) |
| SDPD | San Diego Police Department |
| SDSD/SDSO | San Diego Sheriff Department |
| SSI | Strong Support for inclusion |
| TL | (DNA) Technical Leader |
| TM | (DNA) Technical Manager |
| TMTC | Too many to count. |
| TS or T/S | Tape sealed; sealed with evidence tape, dated and initialed |
| Undet. | Undetermined (no quantifiable DNA present) |
| Unk or unk. | Unknown |
| (v) | Victim |
| VSI or VSSI | Very strong support for inclusion |
| WBC | White blood cells |