

Unit Technical Manual

Forensic Biology

Archived Version

SAN DIEGO POLICE DEPARTMENT

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Unit Technical Manual

Forensic Biology

Method: **Use of the Crime-lite 80S Alternate Light Source**

Date: 1/12/05

Revision Date: 01/01/2007

Approved by: SAM

MATERIALS:

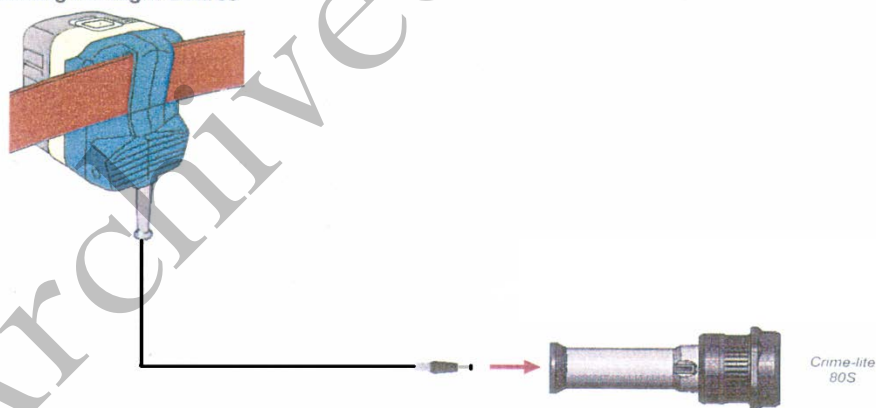
1. Crime-lite™ 80S Blue (450nm) or Crime-lite™ 80S Blue/Green (485nm)
2. Battery Adaptor with Makita® Rechargeable battery
3. Mains Adaptor
4. Orange goggles or filters

PROCEDURES:

Note: The Crime-lite™ 80S 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™ 80S with the awareness that both their own eyes and skin, and those in close proximity should be protected at all times. Even with protective goggles exposure time needs to be considered.

1. Connect the output plug holding the Makita® Rechargeable battery with battery adaptor to the Crime-lite™ 80S for portable use.

Connecting the Light Source



Connecting the Battery Adaptor to a Crime-lite 80L or 80S Forensic Light Source.

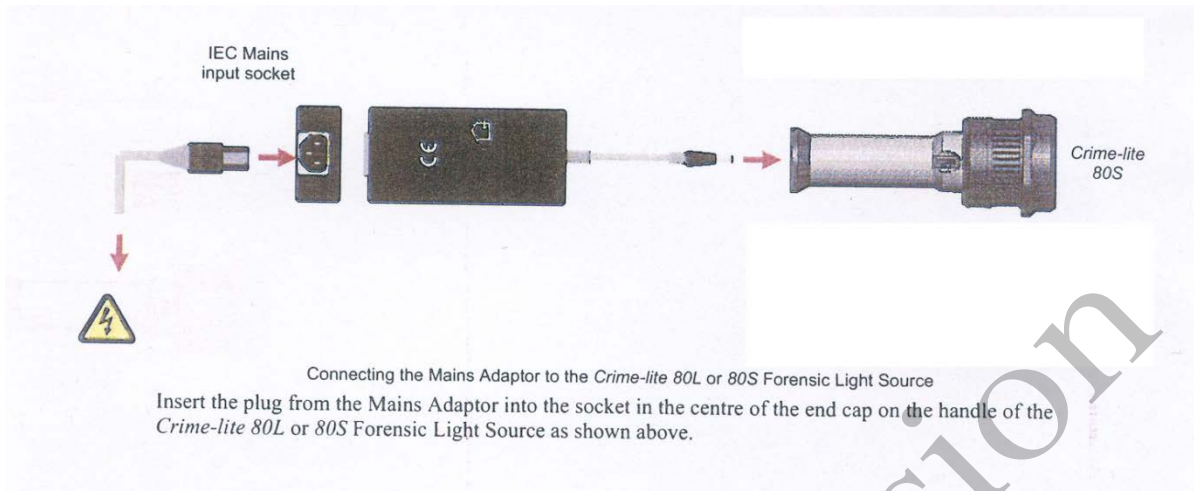
Insert the plug from the Battery Adaptor into the socket in the centre of the end cap on the handle of the Crime-lite 80L or 80S Forensic Light Source.

The light source is now ready for use.

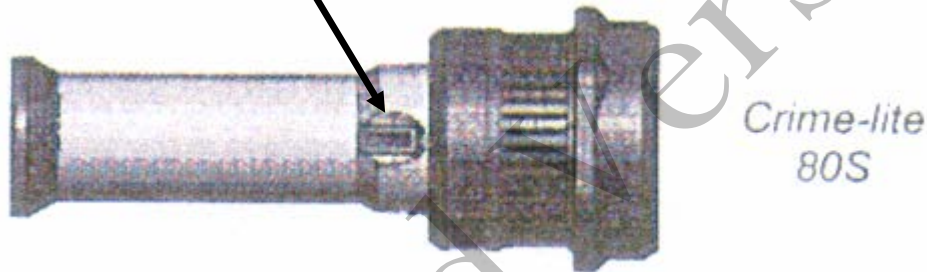
If the battery has a low charge or unavailable, attach the Mains Adaptor to the Crime-lite™ 80S and the nearest outlet.

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1. Turn on the power switch on the *Crime-lite™ 80S*.



2. Conduct a quality control check of the *Crime-Lite™* on known biological stains prior to examining evidence and record the results in the case notes:
4. Systematically pass the light over the entire item to be examined. A fluorescent area may indicate the presence of a body fluid stain. Note the area for further testing.
5. Once the examination is complete, turn off the power switch located on the handle. Caution: the light output head will become warm during use.
6. Secure the *Crime-lite™ 80S* by either placing it on a stable surface or in its carrying case (for long term storage) and removing the Mains Adaptor or Battery Adaptor.

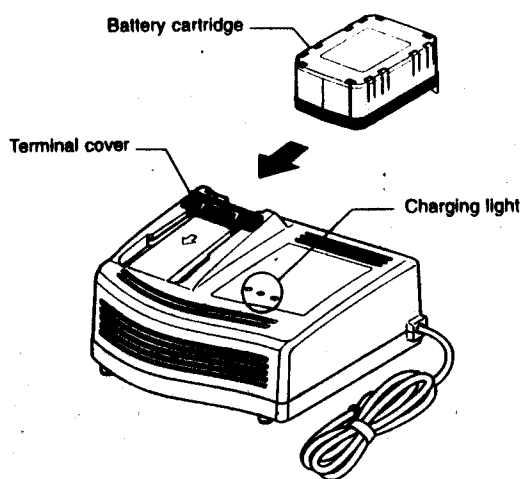
Charging the *Makita®* rechargeable battery:

The *Makita®* Rechargeable battery is a nickel-metal hydride 24V, DC that supplies a *Crime-lite™ 80S* sufficient power for up to 50 minutes at a fully charged capacity.

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Charging



1. Insert the battery cartridge into the charger until it stops adjusting to the guide of charger. Terminal cover of charger can be opened with inserting and closed with pulling out the battery cartridge.
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 two red ones indicates 80 – 99%.
4. With finish of charge, the charging lights will change from two red ones to two green ones.
5. The charging time is approximately 60 minutes for the *Makita*® Rechargeable battery.
6. If you leave the battery cartridge in the charger after the charging cycle is complete, the charger will switch into its “trickle charge (maintenance charge)” mode which will last approximately 24 hours.
7. After charging, unplug the charger from the power source.

Tips for maintaining maximum battery life:

1. Charge the battery cartridge before completely discharged. Always stop Crime-lite™ 80S operation and charge the battery cartridge when you notice less tool power.
2. Never recharge a fully charged battery cartridge. Overcharging shortens the battery service life.

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3. Charge the battery cartridge with room temperature at 10°C - 40°C. Let a hot battery cartridge cool down before charging it.

REFERENCES:

1. Auvdel MJ. Comparison of Laser and High-Intensity Quartz Arc Tubes in the Detection of Body Secretions. J Forensic Sci. 1988 Jul;33(4):929-45.
2. Foster and Freeman- Crime-lite™ Information sheet QCL/80S-B-BG-G. Issue 01 (September 2004)
3. Foster and Freeman- Crime-lite™ Information sheet QCL/83. Issue 01 (September 2004)
4. Foster and Freeman- Crime-lite™ Information sheet QCL/80. Issue 01 (September 2004)
5. Makita®- High Capacity Battery Charger Model DC24SA Instruction Manual.

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

Date: 08/15/2016

Approved by: SAM

MATERIALS:

1. Crime-lite® ML Blue (450-510nm) [CLBG2] or Crime-lite™ ML Blue/Green (430-470nm) [CLB2]
2. Orange goggles or OG550 filter [CLBG2]
3. Yellow goggles or GG595 filter [CLB2]

PROCEDURES:

Note: The Crime-lite® ML is a bench top 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® ML with the awareness that both their own eyes and skin, and those in close proximity should be protected at all times. Even with protective goggles exposure time needs to be considered.

1. Turn on the power switch on the Crime-lite™ 80S.



2. Conduct a quality control check of the Crime-Lite® ML on known biological stains prior to examining evidence and record the results in the case notes.
3. The Crime-Lite ML can be used with white light by pressing the arrow buttons (400-700nm) on the

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right of the magnifier window.

4. To screen for bodily fluids use the blue or blue/green light by pressing the 450-510nm or 430-470nm button (depending on whether using the CLBG2 or the CLB2 Crime-lites). The magnifier window can be fitted with the appropriate filter for viewing fluorescence, or orange goggles can be worn. The OG550 filter must be used in conjunction with the 450-510nm Crime-lite® ML [CLBG2] and the GG595 filter must be used with the 495nm Crime-lite® [CLB2].
5. Systematically pass the light over the entire item to be examined. A fluorescent area may indicate the presence of a body fluid stain. Note the area for further testing.
6. Once the examination is complete, turn off Crime-lite.

REFERENCES:

1. Auvdel MJ. Comparison of Laser and High-Intensity Quartz Arc Tubes in the Detection of Body Secretions. J Forensic Sci. 1988 Jul;33(4):929-45.
2. Foster and Freeman- Crime-lite™ ML Information sheet QCL/80S-B-BG-G. Issue 01 (September 2004)
3. Foster and Freeman- Crime-lite™ Information sheet QCL/83. Issue 01 (September 2004)
4. Foster and Freeman- Crime-lite™ Information sheet QCL/80. Issue 01 (September 2004)
5. Makita®- High Capacity Battery Charger Model DC24SA Instruction Manual.

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

Date: 4/2/98

Revision Date: 04/25/2016

Approved by: SAM

Note: This procedure has been specifically written for Analysts involved in screening evidence for the presence of semen but not involved in DNA testing.

1. Remove a very small portion of the putative semen stain and perform a test for the presence of acid phosphatase activity.
2. Remove approximately 1/4 of a swab or a suitable portion of the substrate and place in 1.5mL tube. Add 1mL of nanopure 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.
3. 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.
4. Microcentrifuge the sample extract at maximum speed for 3-5 minutes.
5. The supernatant may be used for serological tests for blood, semen, saliva, and/or feces. [Consult the specific procedure for details]. Remove an appropriate amount of the supernatant for any desired assays.
6. Carefully pipette off all but 50-100uL of the supernatant being careful not to disturb the pellet. The supernatant can be discarded. At least 50uL of supernatant should be left behind to ensure the cell pellet is not disturbed.
7. Resuspend the cell pellet in the remaining supernatant. Pipette 5-10uL of the sample on to the well of a slide (different amounts may be used). Place the slide in the incubator or on a heat block to dry.
8. Stain the slide with Xmas Tree Stain (refer to the Identification of Cellular Material using Xmas Tree Staining procedure).
9. 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.
10. Add back the substrate to what remains of the water extract and add 750uL of digest buffer and 25uL of

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proteinase K to the tube. Incubate the sample for at least one hour at 56°C.

11. Retain as much liquid from the substrate as possible and discard the substrate.
12. Microcentrifuge the sample at maximum speed for 3-5 minutes.
13. Carefully pipette off all but 50-100uL of the supernatant being careful not to disturb the pellet. The supernatant can be discarded. At least 50uL of supernatant should be left behind to ensure the cell pellet is not disturbed.
14. Add 1mL of water to the cell pellet and resuspend by lightly vortexing.
15. Microcentrifuge the sample at maximum speed for 3-5 minutes.
16. Carefully pipette off all but 50-100uL of the supernatant being careful not to disturb the pellet. The supernatant can be discarded. At least 50uL of the supernatant should be left behind to ensure the cell pellet is not disturbed.
17. 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:

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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, saliva, urine and feces. 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 distilled 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

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

Date: 09/13/2001

Revision Date: 02/06/2009

Approved by: SAM

REAGENTS:

1. 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.

2. 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.

3. 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 dH₂O. Adjust pH to 5. Good for nine months (refrigerated).

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 dH₂O then rub the sample and proceed as above with step 2 on the swab.

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MAPPING

1. Wet a piece of filter paper with dH₂O. 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, then the results are inconclusive.

The color change observed after adding ortho-dianisidine should occur within 45 seconds to be considered a positive reaction. A strong positive reaction will produce an intense color change within a few seconds. A color change within 10 seconds that lacks intensity will be considered a positive reaction. Any color change that occurs after 10 seconds is considered a weak positive reaction, regardless of color intensity. Any weak reactions should be noted in the case notes. 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

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

Date: 01/11/2001

Revision Date: 02/06/2008

Approved by: SAM

REAGENTS:

1. .26g SERI AP Spot Test (SERI Catalog #R558)
2. 10mL Distilled/Deionized water

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

MATERIALS/EQUIPMENT:

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.

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The color change observed after adding ortho-dianisidine should occur within 45 seconds to be considered a positive reaction. A strong positive reaction will produce an intense color change within a few seconds. A color change within 10 seconds that lacks intensity will be considered a positive reaction. Any color change that occurs after 10 seconds is considered a weak positive reaction, regardless of color intensity. Any weak reactions should be noted in the case notes. 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

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

Date: 01/20/2000

Revision Date: 01/01/2007

Approved by: SAM

REAGENTS:

1. NUCLEAR FAST RED STAIN

Dissolve 5.0g of aluminum sulfate in 100mL of hot distilled 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.

Stain can be ordered pre-made from Seri, catalog #R540.

2. PICROINDIGOCARMINE STAIN

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.

Stain can be ordered pre-made from Seri, catalog #R540.

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 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

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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.

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Method: p30 Detection Using the Abacus p30 Diagnostic Card

Date: 3/31/00

Revision Date: 05-13-2013

Approved by: SAM

REAGENT:

Sterile Nanopure 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. If one swab or less is tested, add 250µL nanopure water to saturate the material. If 1¾ swabs is used, then 300-350uL nanopure water should be enough 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 nanopure 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.
4. Remove the spin basket containing the substrate and retain if necessary. 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.

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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 20ul of the supernatant to a sterile 1.5mL tube containing 200ul of water.
6. Load 200µL of the supernatant from Step 4 onto the sample loading “S” area of the test card. If the supernatant was diluted, load the entire contents of tube created in step 5 onto 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. The test results can be documented by using a document scanner or digital camera. Once a hard copy of the test cards is obtained 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.

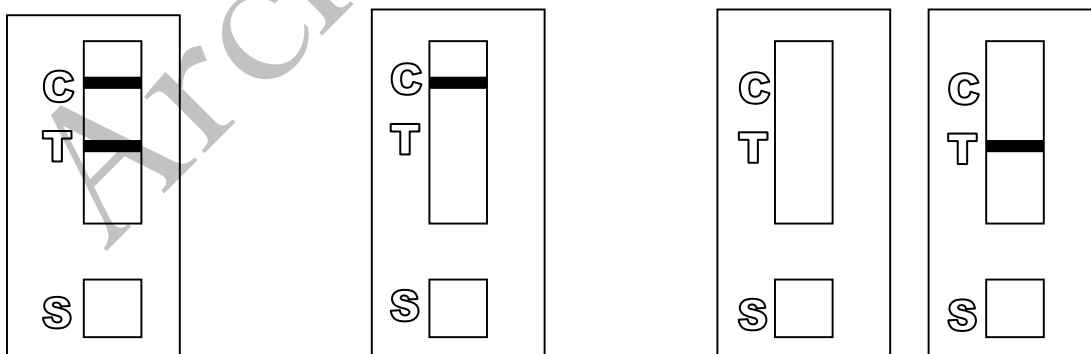
8. If a positive result for the p30 protein is obtained for a given sample it may be appropriate to also test a substrate control for that sample. For positive results from a questioned stain it could prove useful to test additional serial dilutions of the sample to provide information on how concentrated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



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POSITIVE

NEGATIVE

INVALID

REMARKS:

The principle behind the Abacus p30 Diagnostic Test Card is that p30 protein present in a sample will react with a mobile monoclonal antihuman p30 antibody creating a mobile antigen-antibody complex. This mobile antigen-antibody complex migrates through the absorbent test device towards the test area. In the test area "T", a polyclonal antihuman p30 antibody is immobilized. This immobilized antibody captures the above complex creating an antibody-antigen-antibody sandwich. The conjugated pink dye particles concentrate in a narrow zone on the membrane. When the p30 concentration in the samples exceeds 4ng/mL the pink dye particles will form a pink colored band in the test area "T" indicating a positive result. An internal positive control in area (C) consists of an immobilized anti-immunoglobulin antibody that binds all p30 antibody-dye conjugates that are unable to bind to the antibody in the test area (T). The captured dye particles will form a pink band at test area "C" indicating that the test has worked properly.

During the initial validation work on the Abacus p30 Test Card performed at the San Diego Police Department, a single concentrated male urine sample gave a positive test result with the test card. However, the small amount of p30 protein present in male urine was not detected in dried urine stains prepared from urine that had been diluted with water (1:5). Three controls were previously prepared for use with Abacus p30 Test card consisting of an extract prepared from a swatch with undiluted semen, a 1:20 dilution of the semen extract, and an extract prepared from a swatch with undiluted male urine. Additional experiments demonstrated that dried male urine did not produce positive results with the test cards. As such, the undiluted male urine control is unnecessary and the only controls required are the neat and 1:20 diluted semen controls. These controls should be prepared alongside extracts from the questioned samples. The neat semen and 1:20 dilution of semen should yield a positive result with the test card. A reagent blank (negative control) should also be run with the test card.

Analysts should be aware that the p30 protein is used as a diagnostic marker for prostate cancer where it is significantly over expressed. In males with prostate cancer the p30 protein can be found in very high levels in both urine and in blood.

REFERENCES:

1. Abacus Diagnostics p30 Test Card Product Insert "One step ABACard p30 Test for the Forensic Identification of Semen".
2. Armbruster, D.A. P30: biochemistry, analytical methods, and clinical application. *Clinical Chemistry*. v39(2), p 181-95, 1993.
3. Benton, K.A., Donahue, J.A., and Valdez, Jr., Manuel. Analysis of the ABACard OneStep p30 Test for use in the forensic laboratory. Presented at the Spring Meeting of SWAFS. 1998.
4. Engelmann UH, Schramek, P., Tomamichel, G., Deindl, F., Senge, T.H. Vasectomy reversal in central Europe: results of a questionnaire of urologists in Austria, Germany, and Switzerland. *Journal of Urol*. v143(1), p64-67, 1990.

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5. Graves, H.C.B. et al. Postcoital detection of a male-specific semen protein. Application to the investigation of rape. *New England Journal of Medicine*. v312(6), p338-343, 1985.
6. Hochmeister, M., Rudin, O., Borer, U.V., Gehrig, C., Kratzer, A., Dirnhofer, R. Evaluation of prostate-specific antigen (PSA) membrane tests for the forensic identification of semen. *Journal of Forensic Science*. v44, p1057-1060, 1999.
7. Jimenez, Verdejo A., Osana, E. et al. Study of the enzymatic activity of GGT, LDH, PAP, and p30 in semen stains: application to age calculation. *Forensic Science Int*. v68(1), p7-15, 1994.
8. Sensabaugh, G. Isolation and characterization of a semen-specific protein from human seminal plasma: a potential new marker for semen identification. *Journal of Forensic Science*. v23, p106-115, 1978.
9. Sokoll, L.J., Chan, D.W. P30: Its discovery and biochemical characteristics. *Urologic Clinics of North America*. v24(2), p253-9, 1997.
10. Stamey, T.A. et al. Identity of p30 purified from seminal fluid by different methods: comparison by amino acid analysis and assigned extinction coefficients. *Prostate*. v27(4), p198-203, 1995.
11. Stowell LI et al. An enzyme-linked immunabsorbent assay (ELISA) for p30. *Forensic Science Int*. v50(1), p125-38, 1991.
12. Willot, G.M. Frequency of azoospermia. *Forensic Science Int*. v20(1), p9-10, 1982.

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Method: **Leucomalachite Green (LMG) Presumptive Test for Blood**

Date: 4/1/99

Revision Date: 08/14/2009

Approved by: SAM

REAGENTS:

1. LEUCOMALACHITE GREEN (LMG) SOLUTION

0.1g LMG (p,p-benzylidene-bis-N,N-dimethylaniline)
66mL glacial acetic acid
33mL distilled H₂O

Alternative amounts of the above reagents (in the same proportions) may be used depending on the amount of desired reagent. 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.

2. HYDROGEN PEROXIDE 3%

Prepare by 1:10 dilution of 30% stock H₂O₂ or use a commercial 3% solution.

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 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.

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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. Analysts may denote a reaction as weak if they can articulate their interpretation (the underlying reason for calling a reaction weak - color change that lacks intensity or a delayed color change not to exceed 30 seconds – should be added to the notes).

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.

Unit Technical Manual

Forensic Biology

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

Date: 4/1/99

Revision Date:08/14/2009

Approved by: SAM

REAGENTS:

1. PHENOLPHTHALIN STOCK SOLUTION

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

10g phenolphthalein powder
100g potassium hydroxide
100g zinc shavings
500mL distilled H₂O

Place the flask in a heating mantle and attach a reflux unit. 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.

2. 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.

3. HYDROGEN PEROXIDE 3%

Prepare by 1:10 dilution of 30% stock H₂O₂ or use a commercial 3% solution.

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.

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2. Moisten a cotton swab or filter paper (the test substrate) with distilled 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. Analysts may denote a reaction as weak if they can articulate their interpretation (the underlying reason for calling a reaction weak - color change that lacks intensity or a delayed color change not to exceed 30 seconds – should be added to the notes).

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.

Unit Technical Manual

Forensic Biology

Method: **Luminol Detection of Latent Bloodstains**

Date: 01/02/2001

Revision Date: 01/01/2007

Approved by: SAM

PURPOSE:

The Luminol test is a presumptive test for the detection of latent bloodstains not visible to the naked eye. The test is based on the peroxidase-like activity of the hemoglobin molecule. Hemoglobin derivatives greatly enhance the chemiluminescence exhibited by luminol when it is oxidized in an alkaline solution.

The luminol test is very well suited for, but not limited to, crime scene applications, especially where there is a suspicion of possible clean-up of bloodstained areas. This test may also be employed to help in the visualization of certain bloodstain patterns that may be only partially visible to the naked eye (i.e. drag marks, shoe impressions, etc.).

POLICY:

This test is not to be used as a substitute for careful visual examination for blood. The area of interest should be searched in daylight or with the aid of high intensity light prior to the use of luminol.

A Criminalist will be called to the scene to take part in the luminol procedure and to interpret any patterns that may develop.

REAGENTS AND MATERIALS:

1. Luminol powder (3-aminophthalhydrazide)
2. Sodium Perborate
3. Sodium Carbonate
4. Distilled water
5. Plastic hand pump spray bottle
6. 1L plastic bottle

CONTROLS:

Positive Control: Known blood (or bloodstained area) or a copper penny.

Negative Control: Area void of any suspected bloodstains.

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PROCEDURE:

1. The following proportions of reagents should be employed for 100mL of luminol spray:

0.1g	luminol powder
0.7g	sodium perborate
5.0g	sodium carbonate
100mL	distilled water

2. Weigh out the appropriate amount of the reagents and place them into a plastic 50mL tube. If the reagents are not to be used immediately it is best to store the sodium perborate in a separate 50mL tube. The luminol and the sodium carbonate may be stored together. Once the reagents are mixed together they have a limited shelf life (approximately 24 hours).
3. Measure the appropriate amount of water and place it in a plastic bottle. Immediately prior to luminol application the reagents may be added to the water in the plastic bottle and thoroughly mixed. Once mixed, the luminol reagent should be decanted into the plastic hand pump spray bottle. Undissolved powder may have a tendency to clog the pump mechanism.
4. Prior to the application of the luminol spray, a copper penny should be placed in the general vicinity of the area to be tested. Set up any camera equipment required for documentation of the chemiluminescence at this point, if required.
5. Darken the area suspected of containing latent bloodstains to near or total darkness. It is advisable to allow time for eyes to adjust to darkness.
6. The nozzle of the plastic hand pump spray bottle should be set to the finest mist setting. Lightly spray the area of interest, as well as the copper penny. If applicable lightly spray an area known to contain bloodstains, as well as a known negative area.

INTERPRETATION:

A strong bluish chemiluminescence (positive reaction) should be exhibited by the penny when sprayed with the luminol mixture. This glow results from the penny because the luminol mixture is known to react with certain metal halides of copper and iron. The positive reaction from the penny can be used as a quality control of the luminol mixture.

A positive reaction in the area of interest is also identified by the presence of a strong chemiluminescent “glow”, which lasts several seconds. Caution must be exercised in interpreting faint, or weak, reactions as well as “flashes” which could result from the interaction of luminol with metallic halides. Generally, “flashes” will fade to total darkness immediately after spraying, while a positive bloodstained area will exhibit a longer lasting continuous reaction.

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The chemiluminescence exhibited from the oxidation of luminol by a bloodstained area is bluish-white in nature and best visualized under total darkness.

Positive reactions with the luminol reagent should be judged on the color of the reaction, the intensity of the reaction, and the duration of that reaction.

It is important to note that luminol will react to produce a false positive with any agent that can oxidize the chemical directly. Some cleaning agents (i.e. Drano and Pine-sol) as well as metallic halides can produce false positives with the luminol reagent. A positive reaction obtained with the luminol reagent, even a strong, long lasting chemiluminescence, is not enough to confirm the presence of blood.

It should also be noted that luminol will not interfere with further presumptive testing, human origin through testing by HemaTrace, or subsequent DNA testing.

COLLECTION:

General evidence collection guidelines should be used for the collection of luminol positive areas. If a luminol-positive stain is going to be collected using a swab, use the sponge-tipped swab provided specifically for the collection of luminol-positive stains. The preservation steps are the same although drying time is longer.

DOCUMENTATION:

Case notes regarding luminol application should always be taken as with any forensic procedure. Documentation through photography is highly recommended (where possible). Documentation of luminol reactions can be accomplished with digital photography as well as film-based photography. Photographic documentation of the luminol reaction is best accomplished in near or total darkness. It is recommended that a control (available light or flash) photograph be taken of the area to be sprayed. A reliable reference scale should be placed in the area to be photographed. It is important that the camera not be moved between the control photograph and the luminol photograph so that proper orientation and reference can be inferred.

SAFETY CONCERNS:

May be harmful by inhalation, ingestion, or skin absorption. Causes skin and eye irritation. Material is irritating to mucous membranes and upper respiratory tract. Goggles and rubber gloves should be worn. Use of a mask is required if being used in an enclosed room. Do not breathe the chemical dust. In case of contact, immediately flush eyes with water for 15 minutes and wash hands with soap and copious amounts of water.

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Do not dispose of excess luminol at the scene. It is to be brought back to the lab for disposal down the drain.

If used at a crime scene, the Department is required to leave behind a list of companies that are available for crime scene clean up.

REFERENCES:

1. Della Manna A and Montpetit S. A novel approach to obtaining reliable PCR results from Luminol treated bloodstains, J. Forensic Sci., 45:886-890, 2000.
2. RRJ Grispino. The effect of Luminol on the serological analysis of dried human bloodstains, Crime Laboratory Digest, Vol. 39(5), pp. 13-23, 1990.
3. DL Laux. Effects of Luminol on the subsequent analysis of bloodstains, J Forensic Sci., 36(5), pp. 1512-1520, 1991.
4. AM Gross, KA Harris, GL Kadlun. The effect of luminol on presumptive tests and DNA analysis using the polymerase chain reaction, J Forensic Sci., 44(4), pp. 837-840, 1999.
5. Sourcebook in Forensic Serology, Immunology and Biochemistry, National institute of Justice (1980).

Archived Version

Unit Technical Manual

Forensic Biology

Method: Abacus HemaTrace Detection of Blood

Date: 12/08/1999

Revision Date: 04/25/2016

Approved by: SAM

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 100uL 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. Extraction time will depend on the age and concentration of the stain.
3. Remove enough of the supernatant created in step 1 so that when it is added to a second tube containing 150uL of extraction buffer the color of the solution is a light straw yellow.

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

4. Load 150uL of the extraction solution onto the test card (area S).
5. 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. The test results can be documented by using a document scanner or digital camera. Once a hard copy of the test cards is obtained 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.

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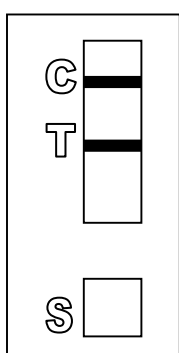
If a positive result for human blood is obtained for a given sample it may be appropriate to also test a substrate control for that sample.

INTERPRETATION:

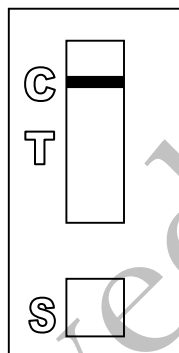
A positive test result for the presence of human blood is indicated by the presence of pink bands at both the control line "C" and the test line "T" on the test card. The presence of a pink band at the control line but the absence of a pink band at the test line indicates a negative result. A pink band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

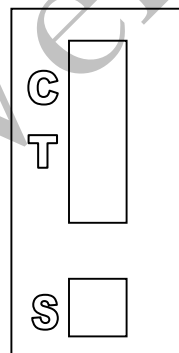
Legend: "S" = Sample loading area "T" = Test line "C" = Control line



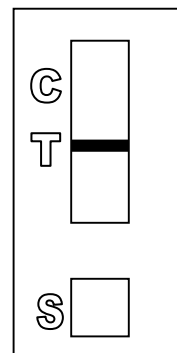
POSITIVE



NEGATIVE



INVALID



REMARKS:

The principle behind the Abacus HemaTrace Test Card is that the human hemoglobin protein present in a sample will react with a mobile monoclonal antihuman hemoglobin antibody creating a mobile antigen-antibody complex. This mobile antigen-antibody complex migrates through the absorbent test device towards the test area. In the test area "T", an antihuman hemoglobin antibody is immobilized. This immobilized antibody captures the above complex creating an antibody-antigen-antibody complex. The conjugated pink dye particles concentrate in a narrow zone on the membrane. When the human hemoglobin concentration in the samples exceeds 0.05ug/mL the pink dye particles will form a pink colored band in the test area "T" indicating a positive result. An internal positive control in area "C" consists of an immobilized anti-immunoglobulin antibody that binds all hemoglobin antibody-dye conjugates that are unable to bind to the antibody in the test area "T". The captured dye particles will form a pink band at test area "C" indicating that the test has worked properly.

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Validation work on the Abacus HemaTrace Test Card performed at the San Diego Police Department indicates that the HemaTrace card is specific for human blood, only showing some cross-reactivity (positive results) with certain higher primates. In their very concentrated form, other human body fluids can yield a positive with the HemaTrace Test Card. This may result from small amounts of hemoglobin being present in these fluids (urine for example).

Due to the positive reaction of the Abacus card with blood of higher primates and published reports of a positive reaction with blood from ferrets, it is required that reports detailing positive reactions with the Abacus card note these possibilities.

Validation of the HemaTrace Test Card by the Abacus Company revealed that treatment with Luminol, Coomassie Blue, Ninhydrin, bleach, or detergent did not interfere with obtaining results from human blood. Human blood present on 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.
7. Laux, D.L. Effects of Luminol on the Subsequent Analysis of Bloodstains, J Forensic Sci, V35 (5), p 1512-20, 1991.
8. Gerberth, V.J. Practical Homicide Investigation, Checklist and Field Guide, p 72-73, 1997.
9. Cox, M.A. A Study of the Sensitivity and Specificity of Four Presumptive Tests for Blood, Journal of Forensic Science, V36 (5), p 1503-1511, 1991.
10. Doherty, P.E., Mooney, D.J., Deciphering Bloody Imprints Through Chemical Enhancement, Journal of Forensic Science, V35 (2), p 457-465, 1990.

Unit Technical Manual

Forensic Biology

Method: Fluid Identification Interpretation Guidelines

Date: 12/08/1999

Revision Date: 09/06/2011

Approved by: SAM

The following is meant as a guide for what conclusions can be drawn based on a given combination of serological test results.

IDENTIFICATION OF SEMEN

Catalytic Test	Sperm	p30	Conclusion
Negative	Not Done	Not Done	Negative for the presumptive test for semen
Negative	Negative	Not Done	No semen was found.
Negative	Negative	Negative	No semen was found.
Positive	Negative	Negative	No semen was found.
Negative	Positive	Negative	Sperm cells were found.
Positive	Positive	Not Done	Semen was found.
Positive	Negative	Positive	Seminal fluid / semen was found.
Negative	Positive	Positive	Semen was found.
Positive	Positive	Positive	Semen was found.
Positive	Positive	Negative	Semen was found.
Positive/Negative	Inconclusive	Inconclusive	Inconclusive
Positive/Negative	Inconclusive	Inconclusive/Negative	Inconclusive

When weak AP positive results are obtained, the disclosure statement, "Acid phosphatase is an enzyme found in high concentrations in semen, but can also be detected in other body fluids and is produced by other organisms" needs to appear in the report.

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IDENTIFICATION OF BLOOD

Catalytic test	HemaTrace	Conclusions
Negative	Not Done	No blood was found.
Positive	Not Done	Presumptive test indicated the possible presence of blood.*
Positive	Negative	Presumptive test indicated the possible presence of blood; however the presence of human blood could not be confirmed.
Positive	Positive	Blood was found.**
Negative	Positive	Blood was found.**

*Analysts may come to the reasonable conclusion that a stain is apparently blood (to be stated in report as such) assuming the stain appears visually consistent with the analyst's expectation for blood, a positive catalytic test result, or the stain's existence is obvious given the context of the crime. An example would be a bloodstained t-shirt belonging to a stabbing victim. A positive HemaTrace result is mandated for an analyst to conclude blood (or human blood) is present if or when the stain is diffuse, the catalytic test result for the stain is suspect because it is difficult to visualize, the stain is on a substrate, such as rust, known to possibly cause a weak positive reaction with the catalytic test, or triggers a weak catalytic test. Analysts are encouraged where there is any doubt, or where the probative value of stain is critical, to employ the HemaTrace test to conclusively demonstrate the presence of blood.

**May also be blood of ferret or higher primate in origin. This statement must appear in report or testimony as qualifier if conclusion is presented as to human blood being present.

Unit Technical Manual

Forensic Biology

Method: **Phadebas Amylase Test**

Date: 5/1/2012

Approved by: SAM

REAGENTS:

1. PHADEBAS WORKING SOLUTION

0.02g crushed Phadebas tablet

550 μ L Nanopure 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 μ L of 1:100,000 dilution of stock standard is required for the test)
[The stock standard was reconstituted to \sim 2mg/mL with Nanopure 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 \sim 1/4 swab is used) or on filter paper (of which an \sim 5x5mm cutting is used) for each test.
3. Blank (Nanopure water)
4. Substrate control (if appropriate)

PROCEDURE:

1. Clear 1.5mL microcentrifuge tubes should be used for this test. For unknowns, add 500 μ L of nanopure water to an \sim 5x5 mm cutting or an \sim 1/4 swab. The frozen amylase standard aliquot should be diluted 1/1000 prior to use (suggested dilution is 5 μ L in 5mL). For the controls, 500 μ L of the amylase standard dilution should be used for the test, 500 μ L nanopure water should be used as a negative, and 500 μ L of nanopure water should be added to the neat saliva sample as the positive control.
2. Add 100 μ L of the Phadebas working solution to each sample and vortex.
3. Incubate the samples at 37 $^{\circ}$ C for 30 minutes.
4. Vortex, then centrifuge the samples at maximum speed for 2 minutes.

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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.

Unit Technical Manual

Forensic Biology

Method: **Detection of Urobilinogen in Fecal Stains**

Date: 1/27/98

Revision Date: 01/01/2007

Approved by: SAM

REAGENTS:

1. 10% ALCOHOLIC MERCURIC CHLORIDE (HgCl₂)
Dissolve 0.3 g of mercuric chloride into 3 mL of reagent alcohol.
Freshly prepare this reagent each time this procedure is conducted. Do not store.
2. 10% ALCOHOLIC ZINC CHLORIDE (ZnCl₂)
Dissolve 0.3 g of zinc chloride into 3 mL of reagent alcohol.
Freshly prepare this reagent each time this procedure is conducted. Do not store.

MATERIALS:

1. Ultraviolet (UV) light source
2. 6 x 50mm borosilicate glass test tubes. Note: flint glass tubes will not work for this method.

PROCEDURE:

1. Prepare an aqueous extract of the unknowns and controls. Appropriate controls include positive controls (neat, 1:5, and 1:10 dilutions), a negative control (reagents only) and any substrate controls (if applicable). Cut out approximately 0.5cm square of the stain, and place it into a 6 x 50mm borosilicate glass test tube. Add 0.25mL of distilled water to the substrate.
2. Vortex the extracts, then let the extracts stand for at least 15 minutes.
3. Remove the substrate from each tube and transfer the supernatant into a 6 x 50mm borosilicate glass test tube.
4. Add 3 drops of the 10% alcoholic mercuric chloride to each tube.
5. Add 3 drops of the 10% alcoholic zinc chloride to each tube and shake the tubes to mix.
6. Examine the extracts under UV light.

INTERPRETATION:

Under UV light the extracts will have a stable apple green fluorescence if urobilinogen is present. The controls should yield the expected results for the test to be interpretable.

A positive urobilinogen result is consistent with the presence of feces.

Unit Technical Manual

Forensic Biology

REFERENCES:

Gaensslen, R.E., *Sourcebook in Forensic Serology, Immunology, and Biochemistry*, a publication of the National Institute of Justice, U.S. Department of Justice, August 1983, pp. 197-8.

Method:

Archived Version

Unit Technical Manual

Forensic Biology

Method: **General DNA Extraction**

Date: 03/30/2000

Revision Date: 02/06/2009

Approved by: SAM

REAGENTS:

1. DIGEST BUFFER (10mM Tris-HCL, 10mM EDTA, 50mM NaCl, 2% SDS)

Mix together 1 mL of 1M Tris-HCL, pH= 7.5, 2 mL of .5M EDTA, 1 mL of 5M NaCl, 10 mL of 20% SDS (w/V) and 86 mL of nanopure water. Autoclave and store at room temperature. The solution is good for one year.

2. 10 mg/mL PROTEINASE K

Dissolve 100mg of proteinase K in 10mL of autoclaved nanopure water. Store at -20°C in 200uL aliquots. The aliquots are good for one year.

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

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

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 400-500uL of digest buffer and 15-20uL of 10mg/mL proteinase K.

[The Large Volume protocol on the EZ1 can accommodate up to 900uL of volume in the sample tubes. Analysts may choose to add more digest buffer to samples to accommodate larger sized substrates. The volume of proteinase K added to the sample should be proportional to the volume of digest buffer.]

[The QiaSymphony accommodates 500uL and 1000uL starting volumes. For the 500uL protocols, 480uL of digest buffer and 20uL of proteinase K is recommended. If more than 500uL of digest buffer and proteinase K is needed, and the QiaSymphony is to be used, the 1000uL protocols must be used.]

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[If a bloodstain appears especially dirty, the analyst may add 15uL of 1M DTT to aid in the digestion of the bloodstain. If a tissue sample is embedded in paraffin wax please refer to reference 2 for the process of removing the wax.]

2. Mix gently and incubate at 56°C for at least two hours. Following incubation the samples can be gently centrifuged to remove moisture from the lids.
3. At this point any samples can be purified using either phenol/chloroform/isoamyl alcohol, the BioRobot® EZ1, or the QiaSymphony® SP Workstation. Please refer to the desired protocol for the procedure.

REFERENCES:

1. Cetus Amplitype User Guide 1990
2. Fregeau CJ *et al.* AmpFISTR Profiler Plus and AmpFISTR 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.

Unit Technical Manual

Forensic Biology

Method: **Cartridge case/casing extraction**

Date: 06/16/2014

Revision Date: 02/20/2017

Approved by: SAM

REAGENTS:

1. DIGEST BUFFER (10mM Tris-HCL, 10mM EDTA, 50mM NaCl, 2% SDS)

Mix together 1 mL of 1M Tris-HCL, pH= 7.5, 2 mL of .5M EDTA, 1 mL of 5M NaCl, 10 mL of 20% SDS (w/V) and 86 mL of nanopure water. Autoclave and store at room temperature. The solution is good for one year.

2. 10 mg/mL PROTEINASE K (ProK)

Dissolve 100mg of proteinase K in 10mL of autoclaved nanopure water. Store at -20°C in 200uL aliquots. The aliquots are good for one year.

EQUIPMENT:

1. American Gold Series Model IS-61 Incubator
2. Eppendorf Thermomixer C
3. Microcentrifuge (capable of 10,000-15,000 x g)

PROCEDURE:

It is highly recommended that cartridge casings are worked in small batches (8-12 cartridge cases) separate from other types of samples.

1. Place the cartridge case/casing in a sterile 5mL tube with the opening of the cartridge facing up.
2. Add 800uL of digest buffer and 32uL of ProK.

[The Large Volume protocol on the EZ1 can accommodate up to 900uL of volume in the sample tubes and the CW 1000 ADV HE protocol on the QiaSymphony can accommodate up to 1mL of volume in the sample tube. Analysts may choose to add more or less digest buffer to accommodate larger or smaller caliber cartridges/casings. *The top of the digest buffer/proteinase K should not go over the top of the cartridge.*]

3. Incubate at 56°C for 30 minutes. The large Blue Bacteriological Incubator can be used for incubation of the 5mL tubes. The thermomixers have 5mL tube adapters that can be used. The

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speed of the thermomixers must be reduced to 500rpm or lower during incubation to avoid getting liquid inside the casings. The heated thermomixer lids will not fit onto the thermomixers with the 5mL adapters.

4. Use a pair of forceps to remove the cartridge case from the 5mL tube (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) and swab the entire outside of the cartridge with a sterile cotton-tipped swab.
5. Place the swab and remaining digest buffer /ProK solution into a sterile 1.5mL microcentrifuge tube or Qiagen Lyse&Spin basket tube.
6. Wash the cartridge case/casing in distilled 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 90 minutes. (*Use of the thermomixers is highly recommended*)
8. At this point any samples can be purified using either phenol/chloroform/isoamyl alcohol, the BioRobot® EZ1, or QiaSymphony®. 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

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Method: **DNA Extraction in Preparation for Y-Screen**

Date: 03/15/2012

Revision Date: 03/15/2012

Approved by: SAM

REAGENTS:

1. DIGEST BUFFER (10mM Tris-HCL, 10mM EDTA, 50mM NaCl, 2% SDS)

Mix together 1 mL of 1M Tris-HCL, pH= 7.5, 2 mL of .5M EDTA, 1 mL of 5M NaCl, 10 mL of 20% SDS (w/V) and 86 mL of nanopure water. Autoclave and store at room temperature. The solution is good for one year.

2. 10 mg/mL PROTEINASE K

Dissolve 100mg of proteinase K in 10mL of autoclaved nanopure water. Store at -20oC in 200uL aliquots. The aliquots are good for one year.

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

Dissolve .77g dithiothreitol in 5mL autoclaved nanopure water. Add 50uL 1M autoclaved sodium acetate pH= 5.2. Store at -20oC in 100uL aliquots. The aliquots are good for one year.

EQUIPMENT:

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

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 500 -750uL of digest buffer, 20-30uL of 10mg/mL proteinase K, and 20-30uL of 1M DTT.

[The QiaSymphony accommodates 500uL and 1000uL starting volumes. For the 500uL protocols, 460uL of digest buffer, 20uL of proteinase K, and 20uL DTT is recommended.]

2. Mix gently and incubate at 56°C for at least two hours. 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, or the QiaSymphony® SP Workstation and eluted in 40uL. Please refer to the desired protocol for the procedure.

REFERENCES:

1. Cetus Amplitype User Guide 1990

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Method: **DNA Extraction with Microscopic Examination**

Date: 03/30/2000

Revision Date: 01/01/2007

Approved by: SAM

REAGENTS:

1. DIGEST BUFFER (10mM Tris-HCL, 10mM EDTA, 50mM NaCl, 2% SDS)

Mix together 1mL of 1M Tris-HCL, pH= 7.5, 2mL of .5M EDTA, 1mL of 5M NaCl, 10mL of 20% SDS (w/V) and 86mL of nanopure water. Autoclave and store at room temperature. The solution is good for one year.

2. 10 mg/mL PROTEINASE K

Dissolve 100 mg of proteinase K in 10 mL of autoclaved nanopure water. Aliquot solution in 200uL aliquots and store in -20°C freezer. The aliquots are good for one year.

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 nanopure 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 50-100uL of the supernatant. Resuspend the cell pellet.
6. Refer to the procedure "Identification of Cellular Material Using Xmas Tree Staining."

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7. Add 0.5mL of digest buffer and 15-20uL of 10 mg/mL proteinase K solution. Vortex briefly. [The original substrate can be added back to increase the DNA yield from the sample].
8. Incubate the sample at 56°C for at least 2 hours.
9. At this point any samples can be purified using either phenol/chloroform/isoamyl alcohol, the BioRobot® EZ1, or the QiaSymphony® SP Workstation. Please refer to the desired protocol for the procedure.

REFERENCES:

Cetus Amplitype User Guide 1990

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Method: **Differential DNA Extraction**

Date: 06/29/2000

Revision Date: 02/05/2018

Approved by: SAM

REAGENTS:

1. DIGEST BUFFER (10mM Tris-HCL, 10mM EDTA, 50mM NaCl, 2% SDS)

Mix together 1mL of 1M Tris-HCL, pH= 7.5, 2mL of .5M EDTA, 1mL of 5M NaCl, 10mL of 20% SDS (w/V) and 86mL of nanopure water. Autoclave and store at room temperature. The solution is good for one year.

2. 10 mg/mL PROTEINASE K

Dissolve 100mg of Proteinase K in 10mL of autoclaved nanopure water. Store at -20°C in 200uL aliquots. The aliquots are good for one year.

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

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

EQUIPMENT:

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

PROCEDURE:

Note: The initial dH₂O wash and microscopic analysis of samples is optional. If this process is skipped analysts may start the differential extraction at step 8. Observations obtained from the dH₂O wash microscopic analysis can provide information that may be useful in some instances (e.g., child abuse cases and male suspect SART kit samples). The initial dH₂O wash and microscopic analysis is encouraged in those instances.

1. Place the sample (swab or other substrate) to be extracted in a sterile 1.5mL tube and add 1mL of sterile distilled water.
2. Incubate at room temperature for at least 30 minutes.

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3. Vortex the tube a couple of times during the incubation to aid in removing the cells from the substrate.
4. Remove the substrate with a pipette tip or plain wood applicator and transfer it to a second sterile microfuge tube and retain.
5. Spin the sample in a microcentrifuge for 3-5 minutes at 10,000 x g (maximum speed) at room temperature.
6. Without disturbing the cell pellet, remove and discard all but 50-100uL of the supernatant. Resuspend the cell pellet in the remaining supernatant. Place 5uL of the sample 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.
7. At this point proceed to the procedure "Identification of Cellular Material Using Xmas Tree Staining". The actual microscopic examination of this slide is considered optional. Note: In general, 100 sperm cells are needed to obtain a DNA result. The sperm cells observed microscopically, however, provide only an estimate of the total number of sperm present in a sample extract. An analyst should use appropriate judgment when deciding on how to proceed (e.g., number of washes needed) with DNA analysis on a particular sample.
8. Add 400-500uL of digest buffer and 15-20uL of 10mg/mL proteinase K to the resuspended cell pellet (more digest buffer and proteinase K may be added if the substrate is especially large). [Option: The original substrate can now be added back to increase the amount of cellular material removed from the substrate.]
9. Incubate at 56°C for 1-2 hours to lyse nonsperm cells. [Option: Incubation may be longer to ensure lysing of the nonsperm cells].
10. Spin the sample in a microcentrifuge for 3-5 minutes at maximum speed. Remove the supernatant and place in a sterile 1.5mL microcentrifuge tube. This supernatant contains DNA from any non-sperm cells present in the sample. The sample can be put aside until DNA purification. [The reagent blank supernatant will act as a control for the non-sperm fraction].
11. [Option: A second digest with digest buffer and proteinase K may be performed in order to ensure that the sperm fraction is free of non-sperm cells].

Note: An analyst may elect to skip directly to step 14 in order to immediately perform the microscopic analysis. Steps 12 and 13 should be performed prior to step 16 if sperm cells are observed in the samples. In this instance step 14 does not need to be repeated.

12. 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 50-100uL and

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discard.

13. Repeat wash step 12 a total of four or five times. [Additional washes are critical when the number of sperm is low and the number of non-sperm cells is high].

Note: The number of total washes (both digest buffer and distilled water) performed on the sperm fractions should be appropriate for the nucleated epithelial and sperm cell counts observed during microscopic examination, or based on the information from prior screening of the sample (i.e., total human and male DNA quantification data). For example: a sample with high sperm cell counts should have a minimum of three digest buffer washes and one water wash performed. A sample with low sperm cell counts (or low male DNA present in the sperm fraction) and high epithelial cell counts (or high total DNA content in the non-sperm fraction) should have more digest buffer washes performed (e.g., five) in an attempt to obtain a sperm fraction free of non-sperm DNA.

14. Resuspend the pellet in 0.5-1.0mL sterile distilled water and vortex briefly. Spin the cells in a microcentrifuge for five minutes at maximum speed. Remove and discard all but 50-100uL of the supernatant. Resuspend the cells in the remaining supernatant.
15. Prepare a microscope slide in accordance with the procedure "Identification of Cellular Material Using Xmas Tree Staining". Microscopic examination of this slide will only be performed if the confirmation of the presence of sperm cells is specifically requested.

Note: If the sperm fraction is probative, it should contain a detectable amount of male DNA to proceed with DNA analysis. If the sperm fraction is being typed, the associated non-sperm fraction should be DNA typed.

If the non-sperm fraction is probative and male DNA is detected in the sperm fraction, both the sperm and non-sperm fractions must be DNA typed.

If the non-sperm fraction is probative and no male DNA is detected in the sperm fraction, only the non-sperm fraction needs to be DNA typed.

If no male DNA is detected in the sperm fraction and the non-sperm fraction has no male DNA or a ratio of human:male DNA greater than 75:1, the analysis of both fractions can be discontinued.

16. Add 400-500uL digest buffer, 20uL 1M DTT and 15-20uL of 10 mg/mL proteinase K solution to the re-suspended sperm cell pellet.
17. Incubate for at least 2 hours at 56°C.
18. At this point any samples (either non-sperm or sperm fraction) can be purified using either

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phenol/chloroform/isoamyl alcohol, the BioRobot® EZ1, or the QiaSymphony® SP Workstation. Please refer to the desired protocol for the procedure.

REFERENCES:

Cetus Amplitype User Guide 1990

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Method: **DNA Extraction from Hair**

Date: 03/30/2000

Revision Date: 03/01/2010

Approved by: SAM

REAGENTS:

1. **DIGEST BUFFER** (10mM Tris-HCL, 10mM EDTA, 50mM NaCl, 2% SDS)
Mix together 1mL of 1M Tris-HCL, pH= 7.5, 2mL of .5M EDTA, 1mL of 5M NaCl, 10mL of 20% SDS (w/V) and 86mL of nanopure water. Autoclave and store at room temperature. The solution is good for one year.
2. **10 mg/mL PROTEINASE K**
Dissolve 100mg of Proteinase K in 10mL of autoclaved nanopure water. Store at -20°C in 200uL aliquots. The aliquots are good for one year.
3. **DTT** (1M DTT, 10mM Sodium Acetate, pH= 5.2)
Dissolve .77g dithiothreitol in 5mL autoclaved nanopure water. Add 50uL 1M autoclaved sodium acetate pH= 5.2. Store at -20°C in 100uL aliquots. The aliquots are good for one year.

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:

1. Wash hair to reduce surface dirt and potential contaminants as follows:
2. For loose hairs, fill a sterile 50mL plastic screw-topped tube with sterile nanopure water and place the hair in the tube using a pair of sterile forceps. Wash each hair separately.

For mounted hairs, freeze the slide in a -20°C freezer for 20 minutes. Remove cover slip by cracking the cover slip with a diamond scribe. Alternatively, the cover slip may be removed by soaking in xylene for several hours after cracking the cover slip with a diamond scribe. Wash away the mounting medium by squirting the slide with xylene. Place the hair in a 1.5mL microcentrifuge tube and add 100% ethanol. Finally, wash the hair in the microcentrifuge tube by removing and discarding the ethanol and then adding water.

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3. Examine the hair under a dissecting microscope. Note the possible presence of body fluids on the hair. Cut off 0.5cm to 1cm of the proximal (root) end 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. [Note: All evidentiary hairs should be photographed prior to performing DNA testing on them.]
4. Place the hair sample and its control into separate sterile 1.5mL microcentrifuge tubes. Add 0.5mL digest buffer, 20uL 1M DTT and 15-20uL 10mg/mL proteinase K.
5. Incubate at 56°C for at least 6 hours.
6. Add an additional 15-20uL 1M DTT and 15uL of 10 mg/mL proteinase K to the samples.
7. Incubate at least 6 hours or until the hair is dissolved.
8. At this point any samples can be purified using either phenol/chloroform/isoamyl alcohol or the BioRobot® EZ1, or the QiaSymphony® SP Workstation. Please refer to the desired protocol for the procedure.

REFERENCES:

Cetus Amplitype User Guide 1990

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

Date: 06/21/2005

Revision Date: 04/01/2008

Approved by: SAM

REAGENTS:

1. LIQUID NITROGEN (16 liters is needed as a minimum) obtained from the 50 gallon tank located in the Trace Evidence section.

2. 10-20% BLEACH (Sodium Hypochlorite)

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

3. DIGEST BUFFER (10mM Tris-HCL, 10mM EDTA, 50mM NaCl, 2% SDS)

Mix together 1mL of 1M Tris-HCL, pH= 7.5, 2mL of 0.5M EDTA, 1mL of 5M NaCl, 10mL of 20% SDS (w/V) and 86mL of nanopure water. Autoclave and store at room temperature. The solution is good for one year.

4. 10mg/mL PROTEINASE K

Dissolve 100mg of proteinase K in 10mL of autoclaved nanopure water. Store at -20°C in 200uL aliquots. The aliquots are good for one year.

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

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

6. AMMONIUM OXALATE (saturated, pH= 3.0)

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

7. 0.5M EDTA (pH= 8.0)

Dissolve 186.1g disodium ethylenediamine tetraacetate-2H₂O to 800mL of distilled water. Stir vigorously and adjust the pH to 8.0 by adding NaOH pellets (approximately 20g). Adjust final volume to one liter with distilled water and autoclave.

EQUIPMENT:

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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 10% 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 nanopure 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 a 10-20% bleach solution for approximately 15 minutes. Rinse the bone section twice with nanopure 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

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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 a 50ml conical plastic tube.
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

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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 50mL plastic tube.

Steps #20-22 are optional and involve decalcifying the bone powder over a three day period using 0.5M EDTA. In some cases, the decalcifying step will increase the amount of DNA recovered from the ground bone. If a BioRobot® EZ1, or QiaSymphony® SP Workstation purification is to be performed then the bone sample must be decalcified. A BioRobot® EZ1, or QiaSymphony® SP Workstation purification is only recommended with relatively fresh bone samples.

20. Decalcifying is accomplished by adding 40mL of 0.5M EDTA, pH = 8.0 to the 50mL plastic tube containing the ground bone. Place the tube on a rocking shaker overnight and the following morning spin the tube in the swinging bucket rotor at approximately 2500rpm for 5 minutes. Pour the supernatant off into another 50mL plastic tube. Save this tube for step #21. Add 40mL 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 approximately 4-5 times over the three day period.
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 supernatants 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 40mL of sterile nanopure water, resuspending the pellet and centrifuging the sample for 5 minutes at approximately 2500rpm. Discard the supernatant.
23. To the bone powder pellet (after decalcification), add 600-700uL (for BioRobot® EZ1 DNA purification) of extraction buffer, 200uL of 10 mg/mL proteinase K, and 100uL of 1M DTT and mix. Incubate the tube at 56°C for at least 4 hours.
24. To the initial incubation add another 200uL of 10 mg/mL proteinase K and 100uL of 1M DTT. Incubate the tube at 56°C at least 6 hours.

Please refer to the BioRobot® EZ1 DNA purification procedure for steps on purifying the DNA from the bone sample(s).

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25. If needed, the purified DNA sample can now be concentrated. Please refer to the protocol for the use of the Centricon-100 centrifugal filter devices (DNA Purification/Concentration Using Centricon YM-100 Centrifugal Filter Devices) to concentrate the purified samples. Each purified bone sample may require several filter devices.

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.

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Method: **BioRobot® EZ1 DNA purification of Evidence Samples**

Date: 01/01/2007

Revision Date: 12/28/2012

Approved by: SAM

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 or BioRobot® EZ1 Advanced XL

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.

Optional: The addition of RNA may improve the DNA yields of samples that contain low amounts of DNA. Analysts may add 1ug of RNA to the sample lysates prior to EZ1 purification of the samples.

- a) Add 310uL of TE buffer to the supplied tube of carrier RNA (310ug) to obtain a solution of 1ug/uL.
- b) Dissolve the carrier RNA thoroughly. Add 1uL of the carrier RNA to your sample lysates. Continue following the protocol at step 2.

Note: The dissolved carrier RNA should be stored at -20°C when not being used.

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

2. 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 BioRobot® EZ1 and turn the instrument on.
3. Press "START" to display the protocols menu, then select the desired protocol. The Large Volume protocol should be selected if evidence samples are being extracted.

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4. Select the desired eluent (TE or water).
5. Select the desired elution volume based on the expected yield; 40, 50, 100, and 200uL are options. Note that the elution volume will be the same for all samples on the same BioRobot® EZ1.
6. Press any key to proceed through the text displayed in the LCD. The text in the LCD prompts the loading of the BioRobot® EZ1.

The following steps deal with the loading of samples and reagents into the BioRobot® EZ1.

7. Invert the reagent cartridges (1-6 per extraction run per instrument on the EZ1 or 1-14 per extraction run on the EZ1 Advanced XL) 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 cartridge rack. Additional samples can be accommodated in additional instruments or in subsequent instrument runs.
8. Load the cartridge rack (with cartridges) into the BioRobot® EZ1. Ensure that the reagent cartridges are seated properly.
9. Load 1-6 (or 1-14) tip holders containing the filter-tips into row 2 of the tip rack.
10. Load 1-6 (or 1-14) opened sample or 2.0mL collection tubes (from Step 3.) into row 4 of the tip rack.
11. Load 1-6 (or 1-14) uncapped appropriately labeled elution tubes into row 1 of the tip rack. Check to ensure that the elution tubes are in the order corresponding to the sample tube order so that no sample mix-ups occur. Close the workstation door.
12. Press “START” to start the extraction protocol.
13. When the protocol ends, the LCD displays “FINISHED”. If you intend to run another extraction protocol, press “ESC” to return to the “Protocols” menu. Otherwise, press “STOP” twice to return the motors to the home positions.
14. Open the workstation door. Remove and cap the elution tubes containing your purified DNA.

The BioRobot® EZ1 can be cleaned by rinsing the tube rack, tip rack, and cartridge rack, briefly, in a solution of 5% Bleach. The UV cross linker may be used to decontaminate the BioRobot® EZ1 components. The EZ1 Advanced XL has a built in UV cross linker that can be used to decontaminate the instrument for between 20 and 60 minutes. The BioRobot® EZ1 components may also be cleaned using a diluted neutral soap followed by water.

Unless another extraction protocol is being performed the BioRobot® EZ1 may be switched “OFF”.

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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. 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.

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Unit Technical Manual

Forensic Biology

Method: **QiaSymphony SP Workstation DNA Purification of Samples**

Date: 06/01/2014

Revision Date: 10-27-2014

Approved by: SAM

REAGENTS:

QiaSymphony DNA Investigator reagent cartridges (for either 192 x 200µl preps, 144 x 500µl preps, or 96 x 1000µl preps). The cartridges contain all reagents required for the DNA extraction and purification of samples on the QiaSymphony workstation. The reagents in the cartridges include guanidine thiocyanate/guanidine hydrochloride, the paramagnetic silica coated beads, ethanol, TE buffer, and sterile water.

MATERIALS/EQUIPMENT:

1. QiaSymphony SP Workstation. The QiaSymphony SP performs all steps of the sample purification procedure after lysis according to the pretreatment protocols.
2. QiaSymphony DNA Investigator kit (QIAGEN Catalogue #931436)
3. Sample Prep Cartridges, 8-well cartridges (cat. no. 997002)
4. 8-Rod Covers (cat. no. 997004)
5. Filter-Tips, 200µL and 1500µL (cat. nos. 990332 and 997024)
6. TopElute Fluid (60mL) (cat. no. 1055628)

PROCEDURAL NOTES:

The QiaSymphony SP workstation has been validated for use in DNA casework for use with four different instrument protocols:

Protocol	Input Lysate Volume	Elution Volume
CW500ADV (Casework 500uL Advanced)	Maximum 500uL ¹	100, 150, or 200uL
CW500ADV-HE (Casework 500uL Advanced – High Efficiency ³)	Maximum 500uL ¹	30, 40, 50, 60, 70, or 80uL
CW1000ADV (Casework 1000uL Advanced)	501uL to 1000uL ²	100, 150, or 200uL
CW1000ADV-HE (Casework 1000uL Advanced – High Efficiency ³)	501uL to 1000uL ²	30, 40, 50, 60, 70, or 80uL

¹Suggested 480uL digest buffer and 20 ProK or 460uL digest buffer, 20uL ProK, and 20uL DTT

²Suggested 960uL digest buffer and 40 ProK or 920uL digest buffer, 40uL ProK, and 40uL DTT

³HE (High Efficiency) protocols use the TopElute Fluid (TOPE) to overlay eluates during the elution process

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Each QiaSymphony DNA Investigator kit comes with three sets of: 1 reagent cartridge, 1 enzyme rack containing carrier RNA, 1 unidirectional piercing lid, and 1 package of Reuse Seal Strips.



FIGURE 1 – KIT COMPONENTS

Preparation of New Kits

Preparation of the Carrier RNA

- Dissolve the lyophilized carrier RNA (red cap) in 1.6mL TE Buffer.
- Transfer 400 μ L to each of the tubes in positions 1 and 2 of the enzyme rack on the reagent cartridge.
- Add additional 1.2mL TE Buffer to each tube and mix by pipetting up and down several times.
- Label the side of the carrier RNA with the date the carrier RNA is made and analyst's initials.

Note: It is important that the final volume of carrier RNA in the tubes of the enzyme rack is exactly 1.6mL the first time you make it. Dissolved carrier RNA is stable for 4 weeks when stored at 2–8°C. For longer periods, store carrier RNA at –20°C.

Preparation of Reagent Cartridges

- Remove the magnetic-particle trough from the reagent cartridge frame, vortex it vigorously for at least 3 minutes, and replace it in the reagent cartridge frame before the first use.
- Check that Buffer QSL3 does not contain a precipitate. If necessary, remove the trough containing Buffer QSL3 from the reagent cartridge and incubate for 30 minutes at 37°C with occasional shaking (do not vigorously shake) to dissolve precipitate.
- Place the reagent cartridge into the gray reagent cartridge holder.
- Place the enzyme rack containing the RNA onto the side of the base unit. The caps of the prepared carrier RNA tubes need to be removed (they can be stored on the slots below the enzyme rack).

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- Place the unidirectional piercing lid over top of the reagent cartridge and pushed down until it snaps in place.
- Remove the silver seal on the magnetic bead trough.



FIGURE 2 – ASSEMBLED COMPONENTS OF THE REAGENT CARTRIDGE

Preparation of Used Kits (within two week time frame)

Carrier RNA

- Remove already prepared Carrier RNA from the reagent refrigerator.

Preparation of Reagent Cartridges

- Remove the magnetic-particle trough from the reagent cartridge frame, vortex it vigorously for at least 1 minute, and replace it in the reagent cartridge frame.
- Remove Reuse Seal Strips from buffer troughs.
- Place the reagent cartridge into the gray reagent cartridge holder (if it is not already in one).

PROCEDURE:

1. Starting the Instrument

- a. Ensure that the QiaSymphony SP is switched on. The blue power button is located at the bottom, left corner of the QiaSymphony SP.
- b. Press the “Log In” button. Log into the instrument using the **SDPD** user (press the “SDPD” button). Enter password “**sdpd1401**” into the pink text box. Press “Ok”.

Note: If the workstation has been scanned with a previously loaded QiaSymphony DNA Investigator reagent cartridge, sufficient consumables are available, and the waste drawer has sufficient space, proceed to step 5.

2. Preparing and Scanning the “Waste” Drawer

- a. Open the “Waste” drawer.

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- b. Remove any full containers (containing used rod covers or sample prep cartridges), dispose the containers and its contents in the trash, and replace them with empty unit boxes (make sure the box spacer is removed).
- c. Make sure the tip chute is free from clogged tips.
- d. If liquid waste is full, dispose of liquid properly (see FB lab tech).
 - i. The scan will indicate if full.

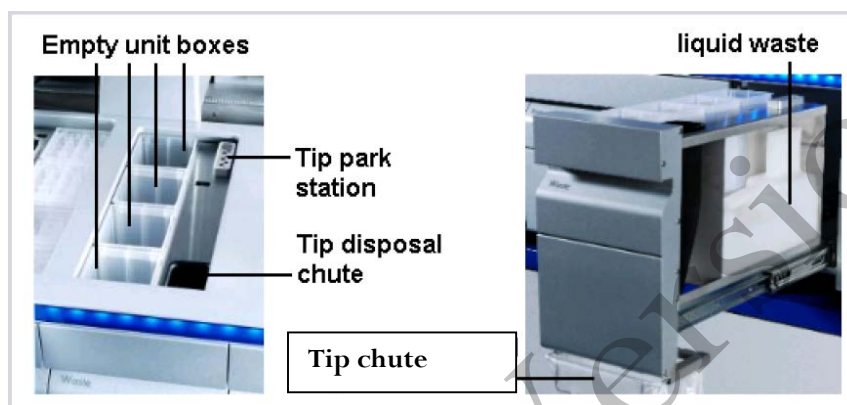


FIGURE 3 – WASTE DRAWER

- e. Shut the drawer and the message “Do you want to start the inventory scan on “waste” drawer?” will appear. Press the “Scan” button.

Note: The instrument will not display a message when done, the arm will simply stop moving, the blue lights will stop flashing, and the drawers will unlock.

Note: You can proceed to step 3 while the waste drawer scan is ongoing.

3. Preparing and Scanning the “Reagents and Consumables” Drawer

This step can be skipped if previously performed.

- a. Open the “Reagents and “ drawer.
- b. Slide the fully assembled reagent cartridge(s) into the reagent cartridge slot.
 - i. Assembled = reagent cartridge in gray base, silver seal removed from the magnetic bead trough, reseal strips removed, the unidirectional piercing lid snapped on top, and the enzyme rack on side with caps removed from tubes 1 and 2.

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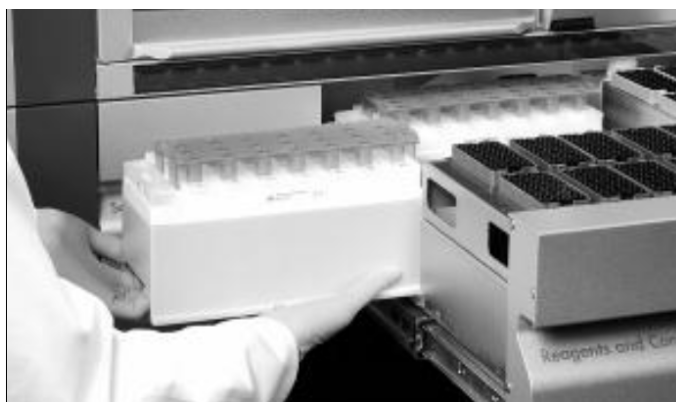


FIGURE 4 – SLIDING IN THE REAGENT CARTRIDGE

- Re-fill any empty tip cartridges (blue = 200uL, black = 1500uL), rod cover unit boxes, and reagent prep cartridge unit boxes. There are four slots in the “Reagents and Consumables” drawer for unit boxes.

Note: Keep empty unit boxes for use in waste drawer at a later time. A drawer in extraction area has been decided for their storage.

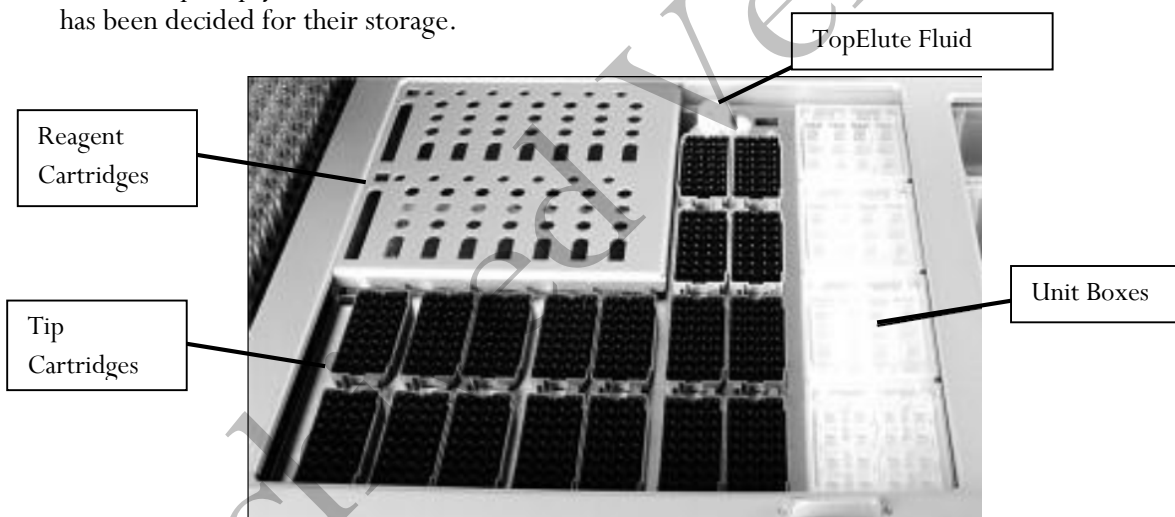


FIGURE 5 – REAGENT AND CONSUMABLES DRAWER LAYOUT

Note: If there is a tip in the back left corner (first tip) and the front right corner (last tip), the instrument will assume that the entire tip cartridge rack is full. If the first or last tip is missing, that entire tip cartridge will be scanned for the exact placement of tips. Make sure that the rod covers and reagent prep cartridges are sitting evenly in the containers. If they are off-set it can cause an error.

- c. Place an open bottle of TopElute Fluid into the “Reagents and Consumables” drawer
 - i. Press the “R+C” button
 - ii. Press the “Bottle ID” button
 - iii. Touch the pink text box to place the curser in the right place.
 - iv. Scan the barcode of the bottle of TopElute Fluid with the handheld bar code scanner.

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- v. Press “OK”.
- d. Shut the drawer and the message “Do you want to start the inventory scan on “Reagents and Consumables” drawer?” will appear. Make sure that “Yes” is selected for all categories. Press the “Scan” button.

Note: The instrument will not display a message when done, the arm will simply stop moving, the blue lights will stop flashing, and the drawers will unlock.

4. Optional: **Using the Sample Calculator**

- a. You can check to see how many samples can be run with the current load of consumables by using the Sample Calculator.
- b. Press the “R+C” button
- c. Press the “Sample Calc.” button
- d. Choose the protocol that you plan on using under the “Custom Protocols” list and the calculator will give you the number of samples that can be run with the loaded reagents and the limiting reagent/consumable.
- e. When finished viewing, press “Ok”.

5. **Loading the “Eluate” Drawer**

- a. Open the “Eluate” drawer.
- b. Place the elution rack onto the deck (if not already on the deck) in the slot that corresponds with the sample rack position that you are going to use. See Figure 6 at the end of the protocol.
 - i. Elution racks stored in labeled drawer in extraction area.

Note: The spot furthest to the back of the instrument is for the “cooling rack” only. The other three spots are for the non-cooling racks. The cooling function does not work with the current protocols, however, and so elutants should not be left on the instrument overnight.

- c. Place your elution tubes in the elution rack.
 - i. For placement of sample tubes in the sample rack and corresponding elution tubes in the elution rack see Figure 6 at the end of the protocol. (It is essential that samples in the sample rack in the same order as their corresponding elution tubes in the elution rack to prevent sample mix-ups. This should be double checked prior to instrument protocol activation. Follow Figure 6 for correct placement.)
- d. Shut the drawer.
- e. Press the Slot button on the left of the touch screen that corresponds with the slot on the deck which holds the rack in which you placed your elution tubes.
- f. Press the “Tube 1.5mL” button.
- g. Press the “SAR#72.607 T1.5 Screw” button. (Will work with the EZ1 elution tubes or the Sarstedt screw-capped tubes.)
- h. Press the Press “Ok”.
- i. The instrument will perform a short scan automatically.

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Note: The instrument only scans for the presence/absence of the elution rack, not the actual elution tubes. The type of tube is manually entered by the user and the number of samples scanned by the instrument in the “Sample” drawer will determine where on the elution rack the instrument pipettes into.

The instrument will assume the previous’ run elution tubes are still on the instrument unless the previous run information has been cleared (either manually by the user or automatically when the instrument is switched off). You can check this by pressing the Slot button on the touch screen corresponding with the desired elution rack and then pressing the “View” tab. If there are colored and numbered wells, then the instrument still thinks the previous elution tubes are on the instrument. See step 9 to clear this.

6. Loading the “Sample” Drawer

Note: All samples loaded into a sample rack must be run with the same protocol and elution volume.

- a. Place your uncapped samples into the 24-tube sample carrier.
 - i. Sample carriers stored in labeled drawer in extraction area.
- b. Load the sample carrier into the “Sample” drawer in the position that corresponds with the elution rack slot you are using. See Figure 6 at the end of the protocol.
 - i. The carrier must be brought to the line on the drawer before the instrument prepares the barcode reader to read the tube carrier.
 - ii. Wait for the green light on the available position to flash prior to sliding the samples into the sample drawer. If the lights turn red, the sample loading must be repeated.
 - iii. The light will turn orange if the sample loading was done correctly.

Note: Only positions 1 through 4 are available for samples. The position labeled A is not used for any of the validated protocols.

- c. Shut the Sample drawer

7. Queuing a Run

- a. Once the Sample drawer has been loaded, the next available line on the “S” screen’s status should read “LOADED”.
- b. Press the blue “SP Batch” button to the left of this line.
- c. The number of samples on the next screen should correspond with the number of samples in your sample rack. The default tubes “SAR#72.693 T2.0 Screw” will work with both the EZ1 sample tubes or the Qiagen Lyse & Spin tubes.
- d. Press the “Select All” button.
- e. Press the “Next” button.
- f. On the following screen press the “Select All” button again.

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- g. Press the “Custom Procotols” button. Choose the correct protocol from the drop down menu (a hand symbol should appear next to each sample on the sample list after this step).
- h. Press “Next”.
- i. The elution slot and volume screen will appear.

Note: Alternatively, the “Fast Setup” button can be used. Using the Fast Setup by-passes the need to input the sample tube information, or protocol (it uses the previously selected protocol) and skips to the elution selection options. This will be most useful when you are loading multiple tube carriers that use the same protocol, or you know the previous user was running the protocol required.

- j. Select the desired eluate volume from list (30-80ul for HE protocols and 100, 150, or 200 for non-HE protocols)
- k. Press the Slot (1-4) on the left side of the screen corresponding with the eluate rack that you loaded your elution tubes into.
- l. A circled number will appear the box corresponding with your run number (not the lane you loaded your sample rack into).
- m. Press “Queue”.
- n. After information about the batch has been entered, the status changes from “LOADED” to “QUEUED”.
- o. Option: Double check that the correct protocol(s) have been selected and your elution tubes are placed in the correct location:
 - i. Press the “E” button.
 - ii. Select the Slot on the left that has your run number (circled number next to Batch)
 - iii. Press the “View” tab and the touch screen will show the corresponding location on the elution rack where your samples will be eluted into.
 - iv. Press “OK”.
 - v. Press the “S” button.
 - vi. Press the “Sample View” button.
 - vii. Press the “Sample Preparation” tab and the touch screen will show you an overview of the runs completed and queued, including sample position, the protocol selected for each sample, the status (unprocessed/completed), volume In (max input volume), and volume out (elution volume).
- p. When ready to run press the “Run” button.

Note: If a run is already in progress when you Queue your run, you do not need to press “Run” (nor will it be an option). The instrument will automatically purify the next run in the Queue.

8. Removing Samples

- a. At the end of the protocol run, the status of the batch changes from “RUNNING” to “COMPLETED”.
- b. Open the “Eluate” drawer, cap and remove your samples. (The touch screen will automatically go to the “E” screen.)

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- c. Remove the eluate rack.
- d. Shut the “Eluate” drawer.
- e. In the “E” screen press the Slot box corresponding with your run.
- f. Press the “Remove” button and a screen will appear asking “Do you want to remove the rack from slot X”.
- g. Press “Yes”.
- h. Press “Ok”. The instrument will perform a short scan of the “Eluate” drawer.

Note: The elution rack must be manually unloaded for the instrument to clear the elute wells for future users (unless the instrument is going to be turned off, since turning off the instrument will clear all previous run information) and clear the positions for the next user.

Note: If you are putting on a run while another run is going, you cannot open the “Reagents and Consumables” or “Waste” drawers. This instrument will let you open the “Eluate” drawer while the instrument is running only if the instrument is not eluting current sample. If you are loading a new elution rack in the “Eluate” drawer while the instrument is in the middle of a run, it may pause or take several minutes to perform the eluate drawer scan, depending on where in the run the instrument is. When the instrument is ready it will let you open the “Eluate” drawer, un-pause, or finish the scan and let you continue setting up your run.

- i. Open the “Sample” drawer and remove your sample rack.
- j. Close the “Sample” drawer unless you are loading additional racks.
- k. Dispose of your empty sample tubes. (These tubes should be completely empty.)

9. Storing Reagents

- a. **Partially used reagent cartridges can be stored for a maximum of two weeks.
- b. If a reagent cartridge is partially used, seal the buffer troughs with the provided Reuse Seal Strips.
- c. Cap the carrier RNA tubes with screw caps and place in the reagent refrigerator.

Note: To avoid evaporation, the reagent cartridge should be open for a maximum of 15 hours (including run times) at a maximum environmental temperature of 30°C.

10. Turn the Instrument Off

- a. Should be done after the last user
- b. Press the blue button to shut off

PERFORMING UV DECONTAMINATION

UV decontamination should be performed on a weekly basis, but can be performed more often on an as needed basis.

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1. Ensure that the QiaSymphony SP is switched on. The power switch is located at the bottom, left corner of the QiaSymphony SP. Log in to the instrument using the SDPD user. The password is “sdpd1401”.
2. Go to **Tools>Maintenance SP>Start UV Light**
3. Remove all samples, consumables, and racks from the instrument. Select the amount of time for the UV light to run.
4. Follow on-screen instructions. Replace consumables, and racks as needed.

TESTING O-RING TIGHTNESS AND CHANGING THE O-RINGS

The tightness of the o-rings needs to be checked on a weekly basis. The o-rings need to be changed on a monthly basis for optimum pipetting performance.

1. Ensure that the QiaSymphony SP is switched on. The power switch is located at the bottom, left corner of the QiaSymphony SP. Log in to the instrument using the SDPD user. The password is “sdpd1401”.
2. Go to **Tools>Service SP**
3. Select the desired protocol (CheckPipettingChannelORing.lua or ChangePipettingORing.lua)
4. If changing the o-rings, place the Replacement O-Ring tool with a new o-ring on the spindle into slot 11 in the reagents and consumables drawer.
5. Select the channel to replace the o-ring for. Follow the on-screen instructions to replace the o-ring.
6. Repeat for all o-rings that need replacing.

REFERENCES:

1. QiaSymphony® DNA Investigator® Handbook. Qiagen February 2014
2. QiaSymphony Users Manual: General Description vMay2013
3. QiaSymphony Users Manual: Operating the QiaSymphony SP vApril2012
4. Qiagen Protocol notes Casework 500 ADV HE V8. Qiagen November 2012
5. Qiagen Protocol notes Casework 1000 ADV HE V8. Qiagen November 2012
6. Qiagen Protocol notes Casework 500 ADV V7. Qiagen November 2011
7. Qiagen Protocol notes Casework 1000 ADV V7. Qiagen November 2011

Unit Technical Manual

Forensic Biology

Method: **DNA Extraction from Reference Samples Using the BioRobot® EZ1**

Date: 06/02/2003

Revised: 10/28/2011

Approved by: SAM

REAGENTS:

1. DIGEST BUFFER (10mM Tris-HCL, 10mM EDTA, 50mM NaCl, 2% SDS)

Mix together 1mL of Tris-HCL, pH = 7.5, 2mL of 0.5M EDTA, 1mL of 5M NaCl, 10mL of 20% SDS (w/v) and 86mL of nanopure water. Autoclave and store at room temperature. The solution is good for one year.

2. 10mg/mL PROTEINASE K

Dissolve 100mg of proteinase K in 10mL of autoclaved nanopure water. Store at -20°C in 200uL aliquots. The aliquots are good for one year.

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:

[Note: Although the QiaSymphony® SP workstation can be used to purify reference samples, only 500µL and 1000µL starting volume protocols have been validated. If the QiaSymphony® SP workstation is used for reference sample purification, it is recommended that 480µL of digest buffer and 20µL of proteinase K be used for sample lysis. The CW500ADV protocol should be used.]

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-

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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.

4. 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 BioRobot® EZ1 and turn the instrument on.
5. Press “START” to display the protocols menu then select the “Trace”, “Trace TD”, “Norm”, or “Norm TD” protocol. The Normalization protocols will limit the DNA yield of the samples by employing a limited amount of paramagnetic beads in the extraction. In the TD protocols the protocol uses a Tip-Dance technique that can accommodate the purification of a sample with up to a maximum of ¼ swab in the tube. If this option is selected the substrate, if it is less than or equal to ¼ swab, need not be removed prior to purification on the EZ1.
6. Select the desired eluent (TE or water).
7. Select the desired elution volume; 200uL is recommended for most reference sample extractions (additional elution volumes available are 40, 50, and 100µL).
8. Press any key to proceed through the text displayed in the LCD. The text in the LCD prompts the loading of the BioRobot® EZ1.

The following steps deal with the loading of samples and reagents into the BioRobot® EZ1.

9. Invert the reagent cartridges (1-6 per extraction run per instrument on the EZ1 or 1-14 per extraction run on the EZ1 Advanced XL) 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 cartridge rack. Additional samples can be accommodated in additional instruments or in subsequent instrument runs.
10. Load the cartridge rack (with cartridges) into the BioRobot® EZ1. Ensure that the reagent cartridges are seated properly in the cartridge rack.
11. Load 1-6 (or 1-14) tip holders containing the filter-tips into row 2 of the tip rack.
12. Load 1-6 (or 1-14) opened sample or collection tubes (from Step 3.) into row 4 of the tip rack.
13. Load 1-6 (or 1-14) uncapped appropriately labeled elution tubes into row 1 of the tube rack. Check

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to ensure that the elution tubes are in the order corresponding to the sample tube order so that no sample mix-ups occur. Close the workstation door.

14. Press "START" to start the extraction protocol.
15. When the protocol ends, the LCD displays "FINISHED". If you intend to run another extraction protocol, press "ESC" to return to the "Protocols" menu. Otherwise, press "STOP" twice to return the motors to the home positions.
16. Open the workstation door. Remove and cap the elution tubes containing your purified DNA.

The BioRobot® EZ1 can be cleaned by rinsing the tube rack, tip rack, and cartridge rack, briefly, in a solution of 5% Bleach. The UV cross linker may be used to decontaminate the BioRobot® EZ1 components. The EZ1 Advanced XL has a built in UV cross linker that can be used to decontaminate the instrument for between 20 and 60 minutes. The BioRobot® EZ1 components may also be cleaned using a diluted neutral soap followed by water.

Unless another extraction protocol is being performed the BioRobot® EZ1 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.

Unit Technical Manual

Forensic Biology

Method: **qPCR with the Quantifiler™ Human DNA Quantification Kit using the NIST SRM Human DNA Quantitation Standard**

Date: 05/01/2012

Approved by: SAM

REAGENTS:

1. TE Buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0)

Mix together 10mL of 1M Tris-HCl, pH = 8.0 with 0.2mL of 0.5M EDTA and 990mL nanopure water. Aliquot into bottles, autoclave and store at room temperature. This solution is good for one year.

2. Quantifiler Human DNA Quantification Kit: Applied Biosystems (P/N 4343895)

- A. Quantifiler Human Primer Mix
- B. Quantifiler PCR Reaction Mix
- C. Quantifiler Human DNA Standard

The Reaction Mix should be removed from the kit upon receipt and stored at 2 to 8°C. The remaining components of the kit should be stored at -15 to -25°C. 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. 96- Well Optical Reaction Plates Applied Biosystems (P/N N801-0560)
2. Optical Adhesive Covers Applied Biosystems (P/N 4311971)
3. MicroAmp Splash free Support Base Applied Biosystems (P/N 4312063)

EQUIPMENT:

ABI Prism 7500 Sequence Detection System Applied Biosystems

PROCEDURE:

1. Thaw the Quantifiler Primer Mix and concentrated DNA standard (stored at -20C). Vortex the Quantifiler Primer Mix and the refrigerated Quantifiler PCR Reaction Mix for 5 seconds. Spin the Primer Mix and DNA standard tubes briefly in a microcentrifuge to remove any liquid from the caps.

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2. Prepare a 1/10 and a 1/50 dilution of the human DNA standard in TE buffer. These dilutions will serve as calibrators for the quantitation assay.
3. 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 1.5ml microcentrifuge tube:

10.5ul of Quantifiler Primer Mix	x	# of Samples
12.5ul of Quantifiler PCR Reaction Mix	x	# of Samples

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

4. Vortex the master mix at medium speed for 5 seconds and spin the tube briefly in a microcentrifuge to remove any liquid from the cap.
5. With the 96-well optical reaction plate in a support base dispense 23uL of the master mix into each sample well.
6. Add 2uL or 8uL of sample, and 2uL calibrators, and the appropriate volume of controls (2 or 8uL) to the appropriate wells.
7. 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.
8. Transport the reaction plate to the ABI 7500 SDS in the amplification area.
9. Turn on the computer (if necessary) and then the SDS instrument.
10. Push the plate door in and slide it out until it provides room to insert the plate.
11. Position the plate in the thermal block so that well A1 is in the upper left corner and the notched corner is in the upper right corner.
12. Gently close the door to by sliding it backward into position.
13. Launch the 7500 SDS Software and set up a plate document (File> New) either by using a blank document or opening a plate document template. The plate document shows the location of samples/standards on the reaction plate. If a sample import file (.txt) has been created it can be imported by selecting **File>Import Sample Setup** and browsing for the appropriate file. If the sample setup was imported then skip to step 18.

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14. If required, add detectors to the plate document. If the detectors for the Quantifiler kit have been created, they will be listed in the Detector Manager: **Tools>Detector Manager**. Highlight the Quantifiler Human and IPC detectors in order to make them available to the plate. Click "Add to Plate Document".
15. Highlight the entire plate document, then from the Well Inspector (**View>Well Inspector**) add the detectors (Quantifiler Human and IPC) to all the wells of the plate document.
16. Add sample information for each well by double clicking on it (or highlight and **View>Well Inspector**). The Well Inspector box will appear. Add the sample name. If clicking on a well where a quantitation standard is located two changes need to be made to the Quantifiler Human detector. Change task from "unknown" to "standard" and add the concentration of the standard to the "Quantity" column.
17. When finished filling out the plate document save it as a SDS document (*.sds).
18. Select the Instrument tab and review the Quantifiler amplification profile which should consist of:
 - Initial incubation at 95°C for 10 minutes.
 - 40 cycles of the following profile:
 - 95°C for 15 seconds
 - 60°C for 1 minute

If need be, delete the initial 50°C incubation step and change the reaction volume to 25uL. If any changes were made save the file and click "Start" or simply click on "Start".
19. Following completion of the run the plate document can be analyzed. Verify the analysis settings (**Analysis>Analysis Settings**) which should be:
 - Detector: All
 - Threshold: .200000
 - Baseline Start (cycle): 6
 - Baseline End (cycle): 15
20. Once you have verified the settings are correct, analyze your data. **Analysis>Analyze**
21. Save your file.
22. Export the Ct values of your samples by selecting **File>Export>Ct**. The 7500 software can be closed at this point and the instrument can be turned off..
23. Import the Ct values into the SDPD Worksheet Maker's "**Quant Data**" worksheet. The data is

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automatically compared against the NIST standard curve in the workbook and the results are available in the “**Quant Results**” worksheet of the workbook. The “Quant 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 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.

Assessment of sample quality:

If a sample has a high human Ct value and a normal IPC Ct value (generally less than 28) the sample has a low amount of DNA.

If a sample has a low human Ct value and a normal or slightly high IPC Ct value (slightly greater than 28) 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 human Ct value and a high IPC Ct value (>>28) the sample is likely inhibited. Appropriate troubleshooting measures should be employed with the sample to overcome the potential inhibitors.

REFERENCE:

Quantifiler™ Human DNA Quantification Kit User's Manual

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Method: **qPCR with the Quantifiler[®] Duo DNA Quantification Kit using the NIST SRM Human DNA Quantitation Standard**

Date: 05/01/2012

Revision Date: 04/25/2015

Approved by: SAM

REAGENTS:

1. TE Buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0)

Mix together 10mL of 1M Tris-HCl, pH = 8.0 with 0.2mL of 0.5M EDTA and 990mL nanopure water. Aliquot into 100mL or 250mL bottles, autoclave and store at room temperature. This solution is good for twelve months.

2. Quantifiler Duo DNA Quantification Kit: Applied Biosystems (P/N 4387746)

- A. Quantifiler Duo Primer Mix
- B. Quantifiler Duo PCR Reaction Mix
- C. Quantifiler Duo DNA Standard

The Reaction Mix should be removed from the kit upon receipt and stored at 2 to 8°C. The remaining components of the kit should be stored at -15 to -25°C. 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. 96- Well Optical Reaction Plates Applied Biosystems (P/N N801-0560)
2. Optical Adhesive Covers Applied Biosystems (P/N 4311971)
3. MicroAmp Splash free Support Base Applied Biosystems (P/N 4312063)

EQUIPMENT:

ABI Prism 7500 Sequence Detection System Applied Biosystems

PROCEDURE:

1. Thaw the Quantifiler Duo Primer Mix and concentrated DNA standard (stored at -20C). Vortex the Quantifiler Duo Primer Mix and the refrigerated Quantifiler Duo PCR Reaction Mix for 5 seconds. Spin the Primer Mix and DNA standard tubes briefly in a microcentrifuge to remove any liquid from the caps.

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2. Prepare a 1/10 and a 1/50 dilution of the human DNA standard in TE buffer. These dilutions will serve as calibrators for the quantitation assay.
3. 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 1.5ml microcentrifuge tube:

10.5ul of Quantifiler Duo Primer Mix	x	# of Samples
12.5ul of Quantifiler Duo PCR Reaction Mix	x	# of Samples

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

4. Vortex the master mix at medium speed for 5 seconds and spin the tube briefly in a microcentrifuge to remove any liquid from the cap.
5. With the 96-well optical reaction plate in a support base dispense 23uL of the master mix into each sample well.
6. Add 2uL or 8uL of sample, and 2uL calibrators, and the appropriate volume of controls (2 or 8uL) to the appropriate wells. Note: For quantitation for Y-Screen DNA analysis, add 8uL of sample to the appropriate wells.
7. 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.
8. Transport the reaction plate to the amplification area.
9. Turn on the computer (if necessary) and then the 7500 SDS instrument.
10. Press the tray door on the SDS instrument to open it.
11. Position the reaction plate in the plate holder so that well A1 is in the upper left corner and the notched corner is in the upper right corner and close the tray door.
12. Launch the 7500 SDS Software and set up a plate document (File> New) either by using a blank document or opening a plate document template. The plate document shows the location of samples/standards on the reaction plate. If a sample import file (.txt) has been created it can be imported by selecting **File>Import Sample Setup** and browsing for the appropriate file. If the sample setup was imported then skip to step 18.
13. If required, add detectors to the plate document. If the detectors for the Quantifiler kit have been

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created, they will be listed in the Detector Manager: **Tools>Detector Manager**. Highlight the Dual Quant Human, Dual Quant Y and Dual Quant IPC detectors in order to make them available to the plate. Click "Add to Plate Document".

14. Highlight the entire plate document, then from the Well Inspector (**View>Well Inspector**) add the detectors (Human, Y and IPC) to all the wells of the plate document.
15. Add sample information for each well by double clicking on it (or highlight and **View>Well Inspector**). The Well Inspector box will appear. Add the sample name. If clicking on a well where a quantitation standard is located, two changes need to be made to the Dual Quant Human and Dual Quant Y detectors. Change task from "unknown" to "standard" and add the concentration of the standard to the "Quantity" column.
16. Select the Instrument tab and review the Quantifiler Duo amplification profile which should consist of:
 - Incubation at 50°C for 2 minutes
 - Incubation at 95°C for 10 minutes
 - 40 cycles of the following profile:
 - 95°C for 15 seconds
 - 60°C for 1 minuteChange the sample volume to 25uL and ensure that the 9600 emulation box is checked.
17. When finished filling out the plate document save it as a SDS document (*.sds) and click "Start" to start the run.
18. Following completion of the run the plate document can be analyzed. Verify the analysis settings (**Analysis>Analysis Settings**) which should be:
 - Detector: All
 - Threshold: .200000
 - Baseline Start (cycle): 3
 - Baseline End (cycle): 15
19. Once you have verified the settings are correct, analyze your data. **Analysis>Analyze**.
20. Save your file.
21. Export the Ct values of your samples by selecting **File>Export>Ct**. The 7500 software can be closed at this point and the instrument can be turned off.
22. Import the Ct values into the SDPD Worksheet Maker's "**Quant Data**" worksheet. The data is automatically compared against the NIST standard curve in the workbook and the results are available

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in the “**Quant Results**” worksheet of the workbook. The “Quant 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 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.

Assessment of sample quality:

If a sample has a high human Ct value and a normal IPC Ct value (generally less than 30.75) the sample has a low amount of DNA.

If a sample has a low human Ct value and a normal or slightly high IPC Ct value (slightly higher than 30.75) 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 human Ct value and a high IPC Ct value ($\gg 30.75$) the sample is likely inhibited. Appropriate troubleshooting measures should be employed with the sample to overcome the potential inhibitors.

The relative amount of male and female DNA in a mixture can be determined by dividing the total human DNA quantitation by the male DNA quantitation and subtracting one. As an example, if the total DNA quantitation is four times the male DNA quantitation, there is approximately three times as much female DNA as male DNA.

Assessment of samples quantified for Y-Screen analysis:

Based on the results of the Y-screen validation, the following general guidelines should be taken into account for submission of screened samples for DNA testing. Although higher amounts are more likely to provide complete and robust results, in general, samples having as low as 0.06ng of total male DNA may provide some informative results using autosomal typing kits and samples having as low as 0.03ng of total male DNA may provide some informative results using Yfiler. If ¼ swab was tested for Y-screen analysis, the following are recommended (assuming ng/4ul, not ng/ul):

Quant	Submission recommendations
0.001ng/uL	1¾ swabs should provide informative Yfiler results
0.003ng/uL	¾ swab for Yfiler (minimum) and/or 1¾ swab for autosomal typing kits
0.008ng/uL	¾ swab for autosomal (minimum) and/or remaining Y-screen extract may provide Yfiler information

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Note that other factors, such as the likelihood of multiple semen donors and the ratio of male to female DNA in the sample, may require the submission of a greater amount of sample for DNA testing.

REFERENCE:

Quantifiler[®] Duo DNA Quantification Kit User's Manual

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

Date: 04/17/2013 **Revision Date: 10/05/2015** **Approved by: SAM**

REAGENTS:

1. TE Buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0):

Mix together 10mL of 1M Tris-HCl, pH = 8.0 with 0.2mL of 0.5M EDTA and 990mL nanopure water. Aliquot into bottles, autoclave and store at room temperature. This solution is good for twelve months.

2. Quantifiler Human (or Duo) DNA Quantification Kit: Applied Biosystems (P/N 4343895 or 4387746 (Duo))
 - A. Quantifiler Human (or Duo) Primer Mix
 - B. Quantifiler Human (or Duo) PCR Reaction Mix
 - C. Quantifiler Human DNA Standard.

The Reaction Mix should be removed from the kit upon receipt and stored at 2 to 8°C. The remaining components of the kit should be stored at -15 to -25°C. 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 Plates: Applied Biosystems (P/N N8010560)
2. Optical Adhesive Covers: Applied Biosystems (P/N 4311971)

EQUIPMENT:

1. Hamilton Microlab Nimbus 4 Liquid Handling Workstation: Hamilton Robotics
2. ABI Prism 7500 Sequence Detection System: Applied Biosystems

PROCEDURE:

1. This protocol is to setup the 96-well optical plate for qPCR DNA quantitation using Quantifiler™ (Human or Duo) quantitation kits and the NIST Quantitation Standard Curve.

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

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- 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. Thaw the appropriate Quantifiler Primer Mix and concentrated DNA standard (which is stored at -20°C). Vortex the Primer Mix and refrigerated qPCR Reaction Mix for 5 seconds. Spin the Primer Mix and DNA standard tubes briefly in a microcentrifuge to remove any liquid from the caps.
 3. Prepare a 1/10 and a 1/50 dilution of the human 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 calibrators and one qPCR blank. Create a master mix by combining the following volumes of reagents in a 2.0mL screw-cap tube.
 - a. 10.5µL of Primer Mix x # of Samples
 - b. 12.5µL of qPCR 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 1205µL of master mix and the second tube should contain the remaining volume.

5. 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. If appropriate, edit the volume for the samples from 2 or 8µL (do not change the volume of the standards).
 - 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.

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6. Empty the tip waste (if necessary).
7. Refill the tip racks on the Nimbus.
8. Place the 96-well optical plate in the appropriate location on the deck.
9. 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.

10. 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 4 above).

11. Remove the caps from the samples and master mix.
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 qPCR and GF PCR Setup** method within the software. Alternatively, a two-step process can be employed by using the similarly named files with (master mix only) or (samples only) in the file names.
 - b. Click the **“Run”** button (▶).
 - c. In the user prompt, navigate to the worklist input file (from step 5 above), select **“qPCR Setup”**, 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”**.
14. 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.
15. Click **“OK”** to acknowledge completion of the run.

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16. 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 ABI 7500 SDS.
17. The qPCR analysis can be continued by following the “Quantitative PCR DNA with the Quantifiler™ Human DNA Quantification Kit” or “Quantitative PCR with the Quantifiler Duo DNA Quantification Kit” protocol at 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™ Human DNA Quantification Kit User’s Manual. Applied Biosystems.
Quantifiler® Duo DNA Quantification Kit User’s Manual. Applied Biosystems.

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

Date: 05/01/2012

Revision Date: 02/05/2018

Approved by: SAM

The chart below represents the results obtained for the sensitivity study of the GlobalFiler, Yfiler, and Minifiler kits using data obtained with the NIST standard. The target ranges represent target amounts of DNA that should result in peak height between 3000-10,000rfu.

This information is presented for analysts as a guide to determine the amplification targets for single source samples. Sample type 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	0.3 - 0.6ng
YFiler Plus	0.4 - 0.8ng

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Method: **Concentrating DNA Extracts with Microcon YM-100 Filters**

Date: 04/01/2011

Approved by: SAM

REAGENTS:

TE BUFFER (10mM Tris-HCL, 0.1mM EDTA pH= 8.0)

Mix together 10mL of 1M Tris-HCL, pH= 8.0 with 0.2mL of 0.5M EDTA and 990mL nanopure water. Aliquot into 100mL bottles, autoclave and store at room temperature. The solution is good for twelve months.

MATERIALS:

1. Microcon-YM 100 Centrifugal Filter Devices (Amicon)
2. Microcentrifuge (capable of at 1,000 x g)

PROCEDURE:

Note: The capacity of the Microcon-YM100 reservoir is approximately 500uL. 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-YM100 unit by inserting the sample reservoir into the filtrate vial.
2. Add approximately 50uL of TE buffer to the upper Microcon-YM100 reservoir.
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 spin-column in a microcentrifuge for 12-15 minutes at 2300rpm (~500 x g). The DNA sample will be concentrated in about 5-15uL of TE buffer in the bottom of the upper Microcon-100 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 vial. Place the Microcon spin-column right-side up into a new into filtrate vial.

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8. Add 3-6uL of TE buffer to the Microcon-100 column (depending on desired final volume). Gently vortex the filter assembly.
9. Collect the DNA sample by inverting the reservoir into a new microcon 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 2000

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Method: **DNA Concentration Using the DNA Speed-Vac DNA-100**

Date: 05/01/2012

Revision Date: 08-15-2016

Approved by: SAM

REAGENTS:

TE BUFFER (10mM Tris-HCL, 0.1mM EDTA pH= 8.0)

Mix together 10mL of 1M Tris-HCL, pH= 8.0 with 0.2mL of 0.5M EDTA and 990mL nanopure water. Aliquot into 100mL bottles, autoclave and store at room temperature. The solution is good for twelve months.

EQUIPMENT: Savant DNA Speed Vac DNA-100

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, or by more than a factor of 12 if purified organically and containing purchased TE buffer. 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-100.
2. Turn on the DNA Speed-Vac DNA-100. Select one of the three heat level options (low, medium, and high). Generally either medium or high are selected. The heat level will influence how quickly evaporation occurs.
3. Close the lid firmly and the DNA Speed-Vac will commence concentrating the samples through a combination of heat and the application of a vacuum to the inner chamber of the Speed-Vac.
4. After the appropriate amount of volume reduction has occurred, remove and cap your sample tubes. Any samples whose volume was reduced more than desired can be brought up to the appropriate volume using either TE buffer or nanopure water.
5. Turn off the DNA Speed-Vac.

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

Date: 10/27/2014

Revision Date: 08-15-2016

Approved by: SAM

REAGENTS:

TE BUFFER (10mM Tris-HCL, 0.1mM EDTA pH= 8.0)

Mix together 10mL of 1M Tris-HCL, pH= 8.0 with 0.2mL of 0.5M EDTA and 990mL nanopure water. Aliquot into 100mL bottles, autoclave and store at room temperature. The solution is good for twelve months.

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, or my more than a factor of 12 if purified organically and containing purchased TE buffer. 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 heat level options (low, medium, and high). Generally either medium or high are selected. The heat level will influence how quickly evaporation occurs.
3. Choose drying rate (Low = ambient temperature, medium = 43°C, high = 65°C; medium or high is recommended) using switch "D".
4. The Speed-Vac DNA 120 model is capable of both "Manual" mode and "Auto" mode (where you can set a timer):

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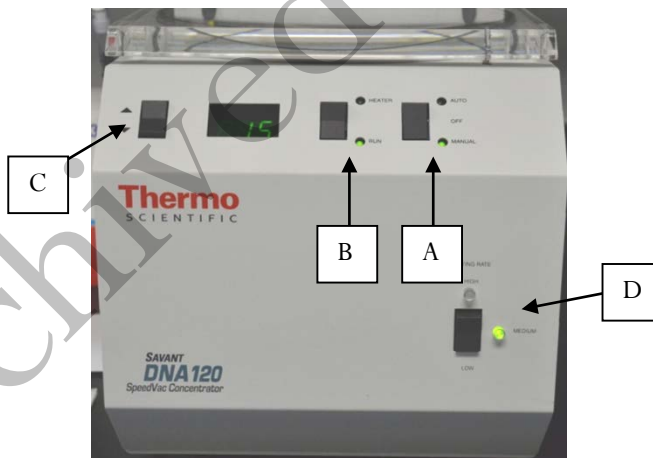
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MANUAL MODE

- Choose the MANUAL setting using switch “A”. 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

- Choose the RUN timer setting using switch B”.
- 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).
- Choose the HEATER timer setting using switch “B”.
- 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.*
- 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.



50uL TE to 10uL	Old Speed Vac	New Speed Vac
Medium Heat	35 minutes	20 minutes
High Heat	15 minutes	10 minutes
40uL TE to 10uL	Old Speed Vac	New Speed Vac
Medium Heat	20 minutes	10 minutes

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High Heat	15 minutes	5-10 minutes
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Approximate Times

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Method: Amplification with the GlobalFiler® kit

Date: 10/05/2015

Revision Date: 02/20/2017

Approved By: SAM

REAGENTS:

1. TE Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)

Mix together 10 mL of 1 M Tris-HCl, pH = 8.0 with 0.2 mL of 0.5 M EDTA and 990 mL nanopure water. Aliquot into 100 mL bottles, autoclave and store at room temperature. This solution is good for one year.

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 kit components are good up to the expiration date. The primer sets and the ladder are light sensitive, and should be stored protected from light.

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 or P/N 4379983)
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, then vortex for 3 seconds and centrifuge briefly before opening the tubes or bottles.

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.

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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 for 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 for 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 ng of target DNA in a volume equal to or less than 15 µL. *Note:* 0.6 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.
 - Add 4-6 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).

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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 anytime after reaching 4°C. Long term storage of amplified DNA should be at -15 to -25°C.

REFERENCES:

GlobalFiler® PCR Amplification Kit User Guide

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

Date: 02/05/2018

Approved by: SAM

REAGENTS:

1. 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

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. 10uL of Yfiler™ Plus Master Mix

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- b. 5.0uL 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 for 3 seconds then centrifuge briefly.

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

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

7. If less than 10uL 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 10uL. 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 300µL). Target 0.6ng of the DNA Control 007 (10µL of the suggested dilution). If less than 10uL of Control DNA is added, the difference is made up by adding TE buffer.
- Add 10uL 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:

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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

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

Date: 04/17/2013

Revision: 02/05/2018

Approved by: SAM

REAGENTS:

1. TE Buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0):

Mix together 10mL of 1M Tris-HCl, pH = 8.0 with 0.2mL of 0.5M EDTA and 990mL nanopure water. Aliquot into bottles, autoclave and store at room temperature. This solution is good for one year.

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
 - 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 kit components are good up to the expiration date. The primer sets and the ladder are light sensitive, and should be stored protected from light.

3. Yfiler[™] Plus PCR Amplification Kit: Applied Biosystems (P/N 4484678 and 4482730)
 - A. Yfiler[™] Plus Master Mix
 - B. AmpF/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 contain a light sensitive dye, 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)

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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 (Note: The Nimbus requires at least 8 samples to run the amplification protocol). 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

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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/ μ L] 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 μ L) vs. Yfiler[™] Plus (total to 10 μ L).

- 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 300 μ L of TE in the appropriate place on the deck.
11. Remove the caps from the samples, master mix, and TE.

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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 qPCR and GF PCR Setup** method within the software. The method is also available in versions that will perform master mix transfer only and TE and samples transfer only. These have been named with the same file name as before, but with the (master mix only) and (samples only) additions.
 - 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=A1₁, 2=A2₁, ..., 96=H12₁, 97=A1₂, 98=A2₂, ..., 192=H12₂) 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 anytime after reaching 4°C. Long term storage of amplified DNA should be at -15 to -25°C.

REFERENCES:

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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.

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Method: **3500 Genetic Analyzer Instrument Preparation, Maintenance, and Calibration**

Date: 7/29/15

Revision Date: 02/05/2018

Approved by: SAM

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 kit, and labeled with 6 dyes: 6-FAM, VIC, NED, TAZ, SID, and LIZ).
6. DS-33 Matrix Standard kit (Dye Set G5): Applied Biosystems (P/N 434583), 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 Yfiler™ Plus kits, and labeled with 5 dyes: 6-FAM, VIC, NED, PET, and LIZ).
7. HIDI Formamide: Applied Biosystems (P/N 4311320)
Dispense the formamide into 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.

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

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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

OVERVIEW OF MAINTENANCE SCHEDULE:

Weekly/Biweekly (recommended in the following order):

- Run the Wash Pump and Channels Wizard (weekly)
- Clean anode buffer cup pin-valve assembly (weekly)
- Place new Anode Buffer Container on instrument (**Hard Stop**) (bi-weekly)
- Replace Cathode Buffer Container (**Hard Stop**) (bi-weekly)
- Restart computer and instrument (weekly)

Monthly:

- Flush the Pump Trap
- Replace septa on Cathode Buffer Container
- Clean the autosampler and drip tray
- Defragment Hard drives

As needed:

- Replace Polymer
- Spatial Calibration
- Spectral Calibration
- Replace Capillary Array (also requires spatial and spectral)
- Pump cleaning

PROCEDURE:

Powering on the 3500 and launching the Data Collection software

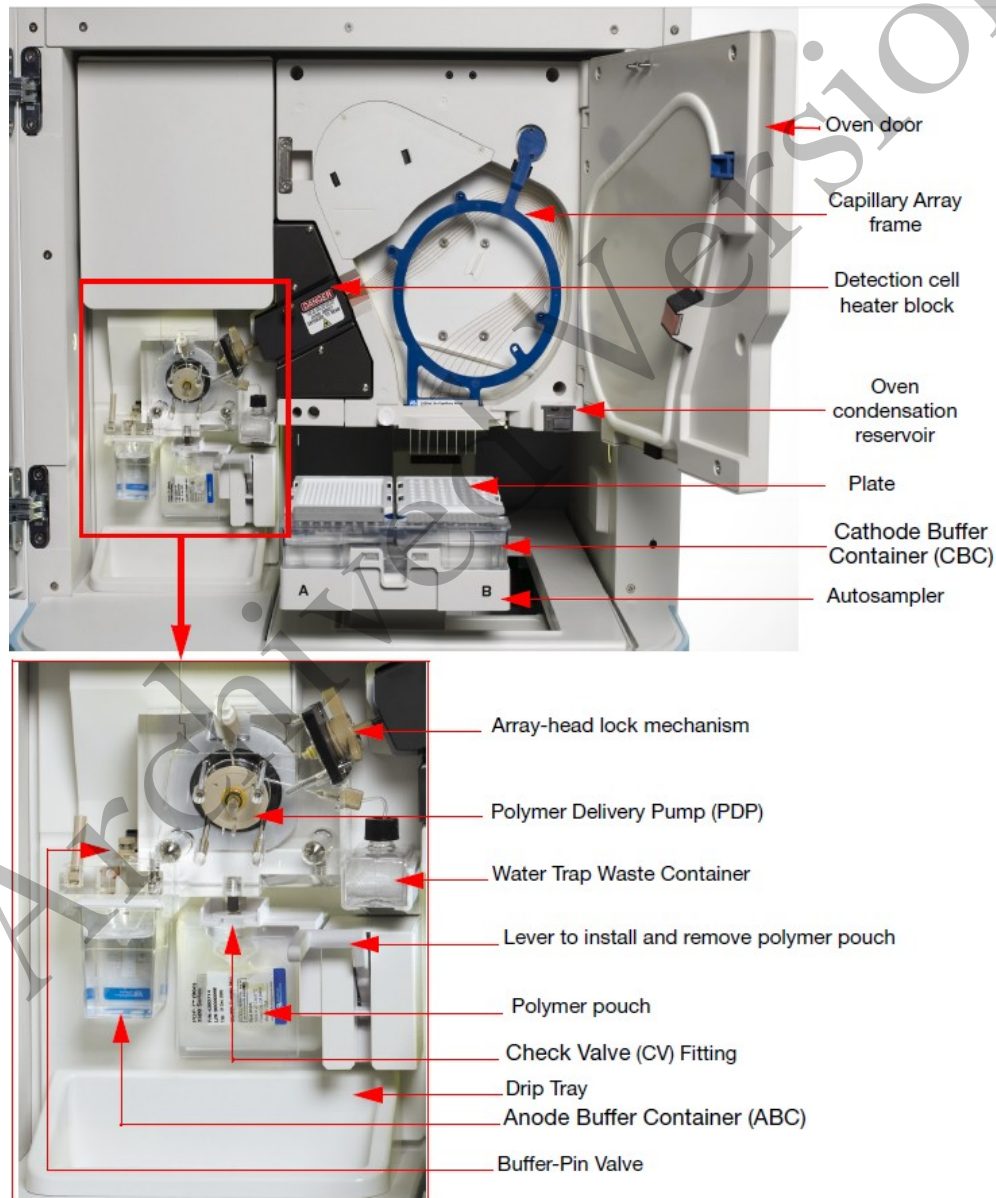
1. Power on the computer. At the Log On prompt, log in to the computer.
2. Before turning on the 3500 instrument, ensure that the oven door is closed and locked, that the instrument doors are closed, and the computer is powered on.
3. Power on the instrument by pressing the on/off button on the front of the instrument. Ensure that the green status light is on and not flashing before proceeding.

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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.

INSTRUMENT INTERIOR COMPONENTS:



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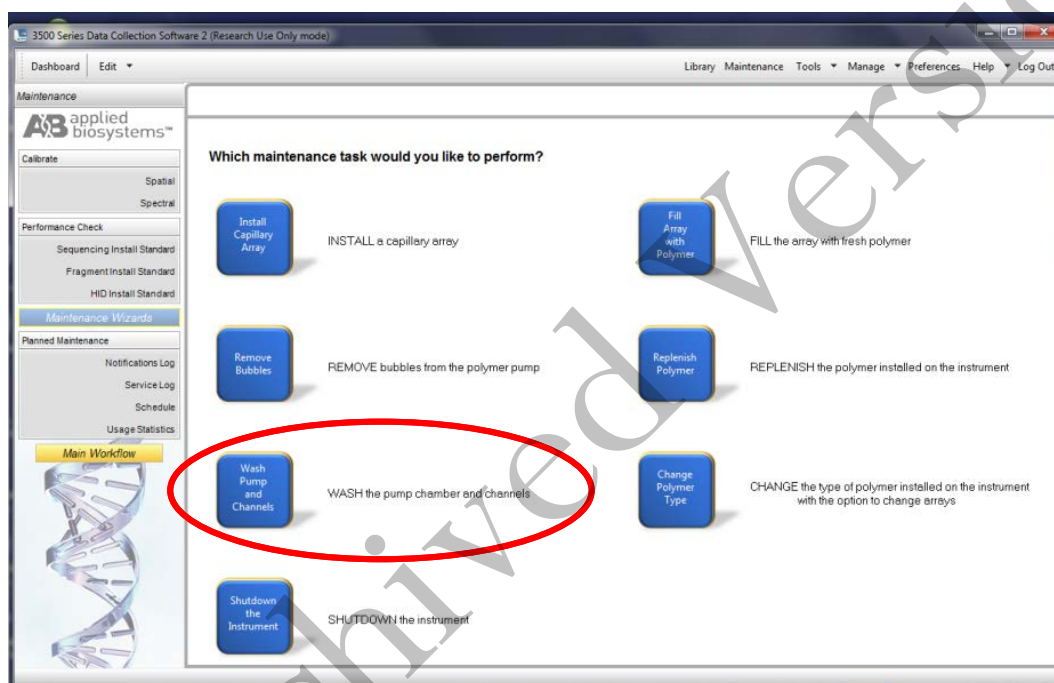
Weekly Maintenance

At least 30 minutes prior to beginning weekly maintenance, bring Conditioning Reagent, POP-4 Polymer*, Anode buffer container, and Cathode Buffer Container to equilibrate to room temperature.

*If polymer remains before the expiration date, and there are still injections left in the pouch, polymer can be reused for a total of approximately four weeks on the instrument before it needs to be replaced.

Run the Wash Pump and Channels Wizard

This wizard takes about 40 minutes from start to finish. Click **'Maintenance'** on the top panel, and **'Maintenance Wizards'** on the left to find the **'Wash Pump and Channels'** wizard.



Follow the wizard prompts to empty the anode buffer (NOTE: The EMPTY anode buffer container is placed back on the instrument for the water wash), remove polymer, insert conditioning reagent, and insert polymer to flush the pump chamber and channels.

Clean anode buffer container valve pin assembly

Following pump chamber and channels wash, and before installing the new anode buffer container, use a clean lint-free cloth and deionized water to wipe clean the anode buffer container valve pin assembly on the polymer delivery pump.

Place new Anode Buffer Container on instrument

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1. Remove Anode Buffer Container from outer plastic wrap. Ideally, this is done after the wash pump and channels wizard.
2. Tilt it slightly to make sure that most of the buffer is in the larger side of the container. There should be less than 1 mL of buffer remaining in the small side of the container. Verify that buffer is to the fill line.
3. Gently peel off the plastic seal on the top without spilling any into the small side of the container. If it does spill into the overflow side, pipette it back into the reservoir so that the liquid reaches the fill line.
4. Place the Anode Buffer Container (label side first) under the valve pin assembly, and slowly slide the lip of the container into the locks.

Replace Cathode Buffer Container (replacing septa only once per month)

1. Remove Cathode Buffer Container from outer plastic wrap and peel off plastic seal from the top, ensuring that it is peeled off of every circular opening.
2. Wipe off any buffer on top of the container with a lint-free cloth. Ensure that the top of it is dry.
3. Press **tray** on the front of the instrument to bring the autosampler forward. Remove the old cathode buffer by pinching in the middle.
4. Place the appropriate cathode buffer septa on both sides of the cathode buffer container. These only need to be replaced once per month. Septa can be washed with water and returned to the new cathode buffer.
5. Install the new buffer by clicking it into the tabs on the autosampler.
6. Close the instrument door to re-initialize.

Restart 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.

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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).

Monthly Maintenance

Flush the Pump Trap

The water trap must be flushed once per month (or more often as needed) to prolong the life of the pump by cleaning any diluted polymer and clearing air bubbles.

1. Fill the supplied 20 mL, all-plastic Luer lock syringe with nanopure water. Do not use a syringe smaller than 20 mL. Doing so may generate excessive pressure within the trap. Expel any bubbles from the syringe.
2. Attach the syringe to the forward-facing Luer fitting at the top of the pump block. Hold the fitting with one hand while threading the syringe onto the fitting with the other hand.
3. Open the Luer fitting by grasping the body of the fitting and turning it to loosen. Attached syringe and turn counterclockwise approximately one half turn.

IMPORTANT! Do not use excessive force when you push the syringe plunger as this may damage the trap seals. Take approximately 30 seconds to flush 5 mL of nanopure water through the trap.

4. Remove the syringe from the Luer fitting. Hold the fitting with one hand while turning the syringe counterclockwise with the other hand.
5. Close the Luer fitting by lightly turning clockwise until the fitting seals against the block.
6. Dispose the excess water inside the overflow container.

Replace septa on Cathode Buffer Container

Once per month, change both of the septa on the Cathode Buffer Container during the weekly maintenance.

Clean the autosampler and drip tray

Wipe them down with a lint-free cloth and either deionized water or absolute ethanol

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Defragment hard drives

Click the **Windows Start icon** to find **Disk Defragmenter** and open it. Click on each disk and click '**Analyze**' to determine the degree of fragmentation. Click '**Defragment**' for every disk that is fragmented. This may take several minutes to hours depending on the degree of fragmentation.

As Needed Maintenance

Replace 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.

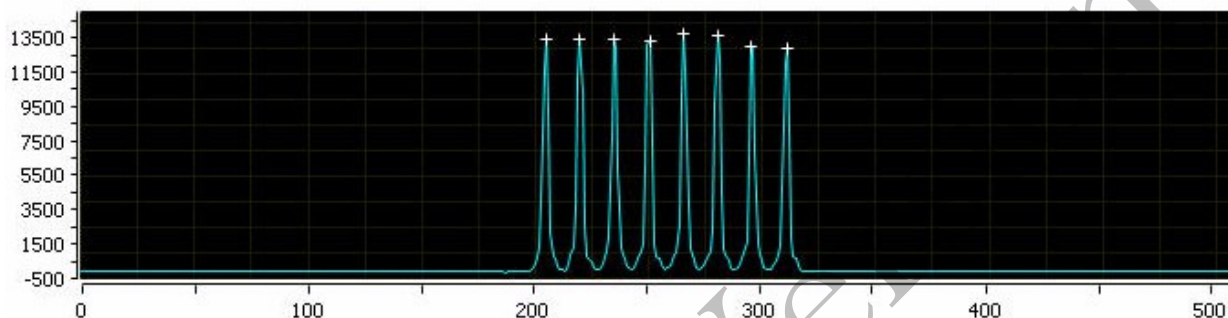
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- One marker (+) at the apex of every peak. No off-apex markers.
- An even peak profile (all peaks about the same height).
- The Max Spacing – Min Spacing should be no more than 2 pixels.

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**'.

Note: The software does not save historical calibration results; only the most recent spatial calibration is maintained in the software.

7. In order to save a record of these reports, click '**Print**'. In the Printer dialog box, select **CutePDF Writer** as the printer to print the report to a PDF file.
8. Save the file to **D:AB SW & DATA > Maintenance & Calibration Reports** to keep a historical record of this calibration for the lab.

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.

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- 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 μ L
- b. HIDI Formamide: 290 μ L

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

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

5. Load 10 μ L of matrix standard/HIDI mixture into wells A1-H1 of a 96-well reaction plate.
Note: multiple matrix samples (i.e., 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.

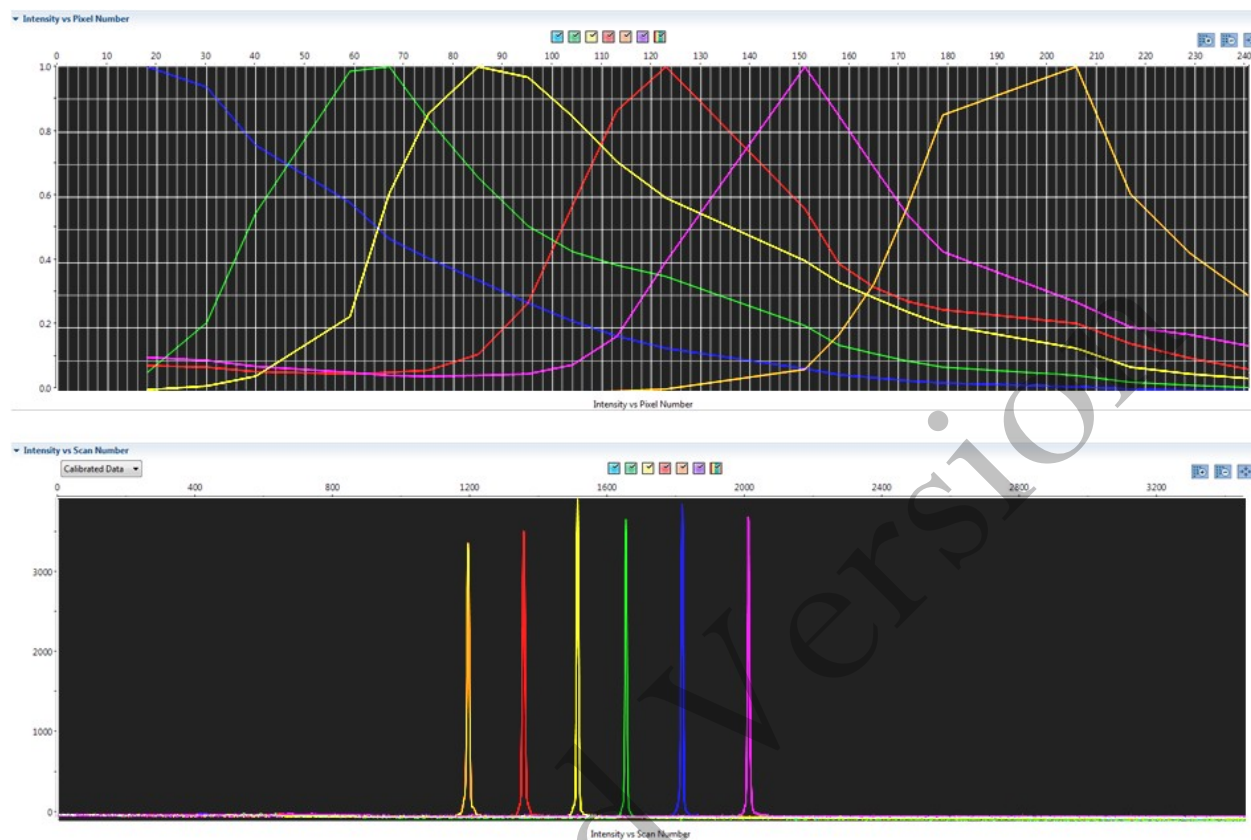
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8. After the denaturation protocol is finished, chill the reaction plate immediately in the 96-well cooling block (-20°C) for at least 3 minutes.
9. Place the plate into a 3500 Series 96-well plate base (it will only fit in one orientation) and cover 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.

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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.

Note: The software does not save historical calibration results; only the most recent spatial and spectral calibration is maintained in the software.

16. In order to save a record of these reports, click '**View Spectral Calibration Reports**' and then '**Print**'. In the Printer dialog box, select **CutePDF Writer** as the printer to print the report to a PDF file.
17. Save the file to **D:AB SW & DATA > Maintenance & Calibration Reports** to keep a historical record of this calibration for the lab.

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.

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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

Unit Technical Manual

Forensic Biology

Method: **Preparation of samples for analysis on the Applied Biosystems 3500 Genetic Analyzer**

Date: 7/29/15

Revision Date: 02/05/2018

Approved by: SAM

REAGENTS:

1. HIDI Formamide: Applied Biosystems (P/N 4311320)
Dispense the formamide into 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 when handling.

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:

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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%)
 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:** 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

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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

Archived Version

Unit Technical Manual

Forensic Biology

Method: **Preparation of amplified samples for analysis on the Applied Biosystems 3500 using the automated CAS-1200**

Date: 7/29/15

Revision Date: 02/05/2018

Approved by: SAM

REAGENTS:

1. HIDI Formamide: Applied Biosystems (P/N 4311320)
Dispense the formamide into 500uL 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 when handling.
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:

6. MicroAmp Optical 96-Well Reaction Plates: Applied Biosystems (P/N N8010560)
7. MicroAmp Splash Free Support Base: Applied Biosystems (P/N 4312063)
8. Septa for 3500 Genetic Analyzers, 96 well: Applied Biosystems (P/N 4412614)
9. Retainer and Base for 3500 Genetic Analyzer, 96 well: Applied Biosystems (P/N 4410228)
10. 96-well cooling block: Stored in the reagent freezer

EQUIPMENT:

1. CAS-1200: Corbett Robotics
2. Veriti® Thermal Cycler: Applied Biosystems
3. 3500 Genetic Analyzer: Applied Biosystems

PROCEDURE:

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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 tapered tube:

19.2 µL formamide x (# of samples + 20%)

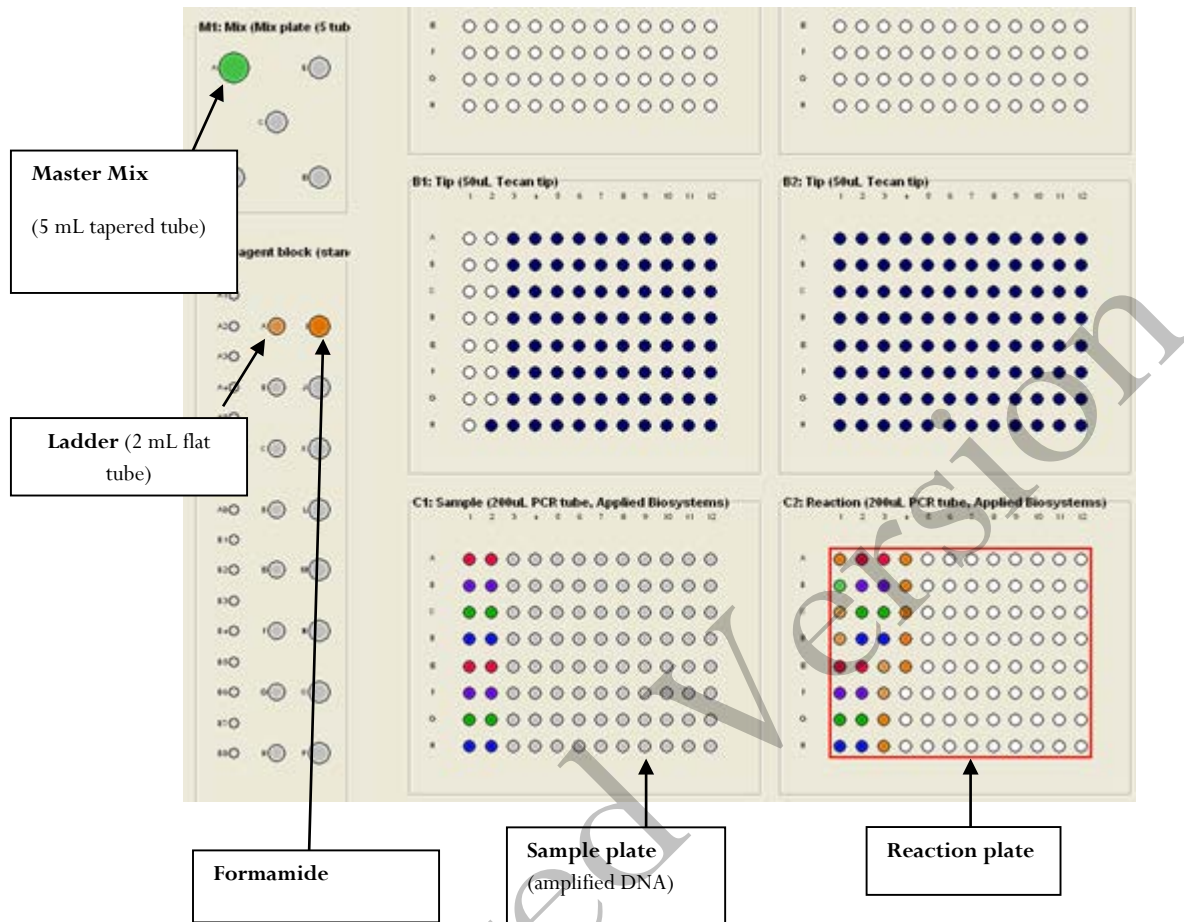
0.8 µL GeneScan-600 [LIZ] x (# of samples + 20%)

2. Vortex the master mix. Place the tube of master mix into Well A on the M1 (Mix Plate) deck on the CAS-1200. **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 of the CAS-1200. Place a new 96-well optical reaction plate on the C2 Reaction plate area on the deck of the CAS-1200.
5. Remove the cover from the 96-well optical reaction plate that contains amplified samples.

Deck Layout Diagram

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- Place a 2 mL flat tube containing ample kit-dependent Allelic Ladder in Well A on the R1 (Reaction Plate) deck on the CAS-1200. Make sure the Ladder has been mixed (via vortexing) and spun down (in a microcentrifuge) prior to loading on the deck.
- Place a 1.5 mL tapered tube containing at least **250 µL** formamide into Well I on the R1 (Reaction Plate) deck on the CAS-1200.
- 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) 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).

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Right click and select '**Add selected wells to sample bank**'

In the Update Sample Bank window, select '**Existing bank**' (the only choice is 'Amplified samples GlobalFiler'). Click '**Add selection**'. 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, and 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**'

In the Update Sample Bank window, click '**Delete selection**'. 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, and 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 μ L 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. To signal the CAS-1200 to begin the protocol, 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 CAS-1200 will now add the master mix, formamide blanks, ladders and DNA samples to the 96-well optical reaction plate.

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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 in 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 using the CAS-1200:

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
CAS-1200 Liquid Handling System Manual

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Method: **Decontaminating the CAS-1200 using the automated UV protocol**

Date: 04/01/2008

Approved by: SAM

EQUIPMENT:

1. CAS-1200 Corbett Robotics

PROCEDURE:

1. Remove the box of ejected tips and use the plug that is attached to the side of the tip ejector chute to plug the tip ejector chute.
2. With the 3500 Plate Setup 2 μ L program open, click on the UV Light icon (the yellow light bulb).
3. In the UV lamp control window, choose the length of time you wish the UV lamp to be active by sliding the flashing cursor right or left (a minimum of 5 minutes is recommended).
4. Select Start.
5. When the UV light operation has completed, click OK, unplug the tip ejector chute, and re-position the ejected tips box.

REFERENCES:

CAS-1200 Liquid Handling System Manual

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Method: **Capillary Electrophoresis of Amplified Samples on Applied Biosystems 3500 Genetic Analyzer**

Date: 7/29/15

Revision Date: 02-05-2018

Approved by: SAM

MATERIALS:

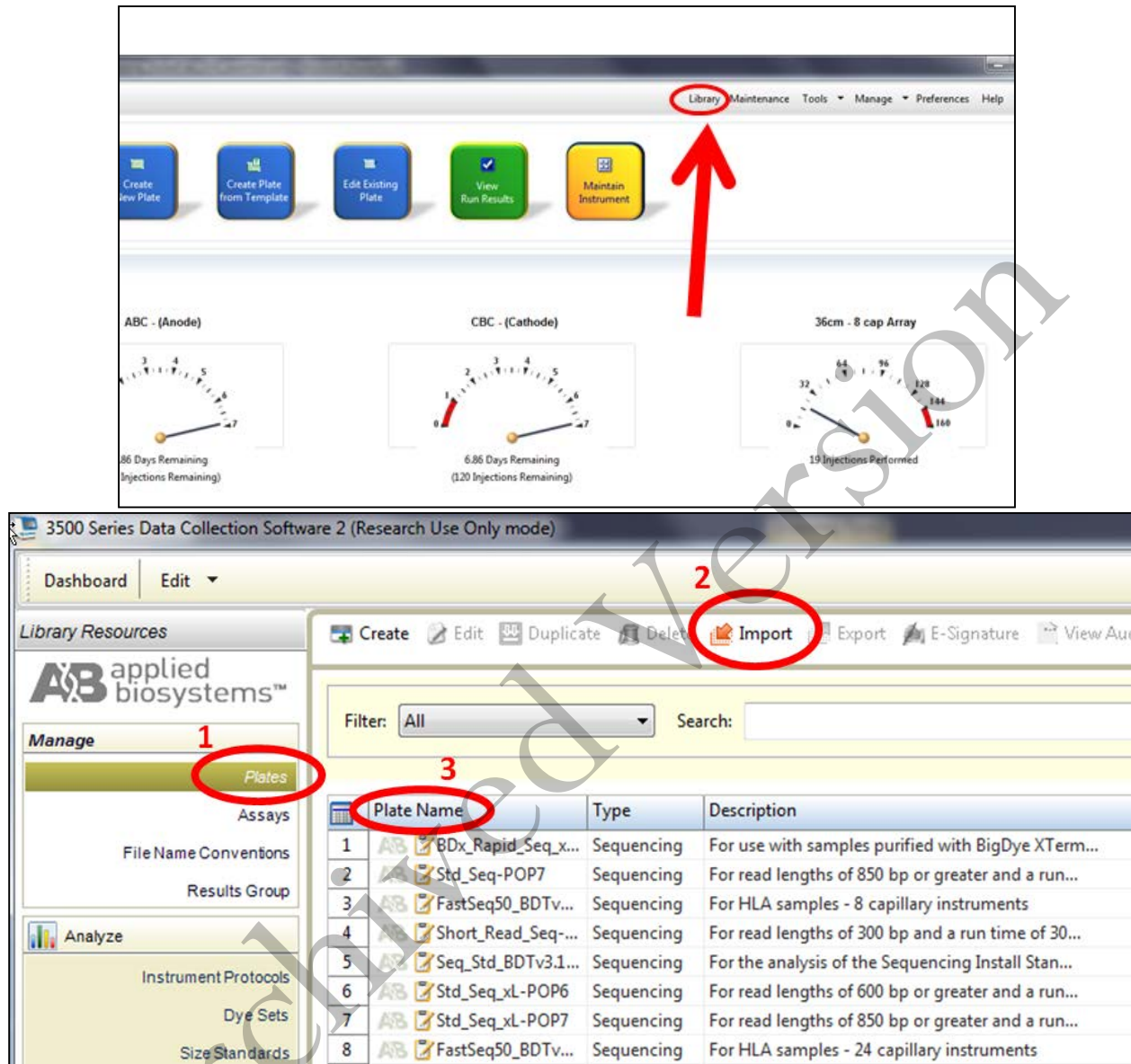
1. 3500 Data Collection Software (Series 2)
2. Optional: Plate record file (.txt) from SIMS worksheet

PROCEDURE:

1. Launch the **3500 Series Data Collection Software 2**.
2. Check the dashboard to verify that maintenance has been done, and 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).

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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.

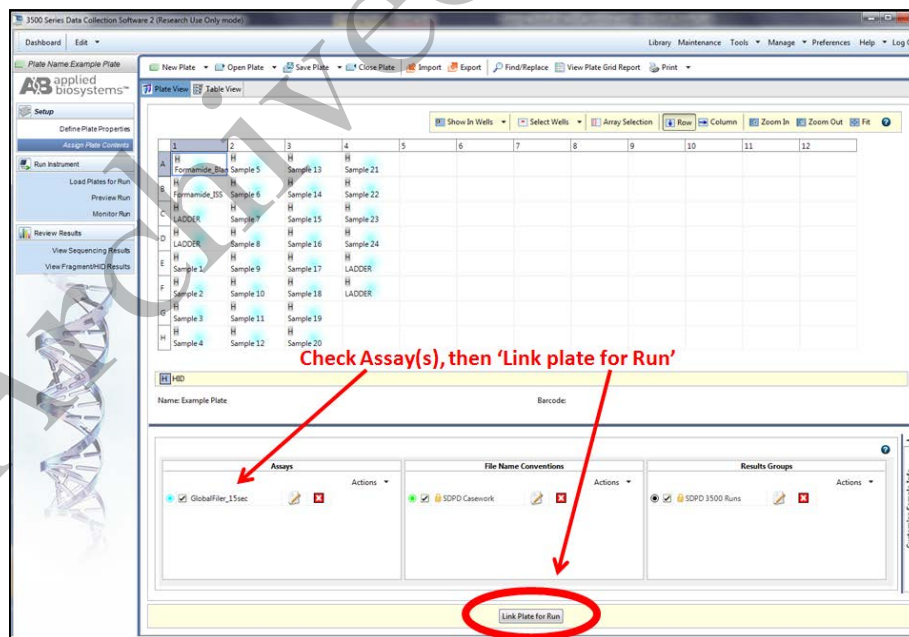
Alternatively, a plate can be created by clicking **Create New Plate** on the dashboard screen, manually

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entering plate name and assigning plate contents.

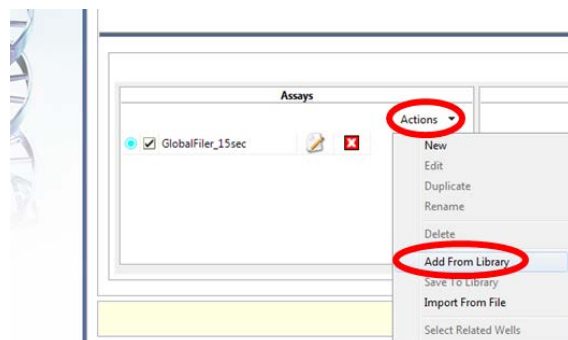
- a. Sample Name: Analyst designated name for each sample and control
- b. Sample Type: Each sample should be designated appropriately
 - i. Allelic Ladder
 - ii. Positive Control
 - iii. Negative Control
 - iv. Sample
- c. Assay Name:
 - i. **GlobalFiler_15sec** - (Default protocol)
 - ii. **GlobalFiler_24sec** - (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



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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

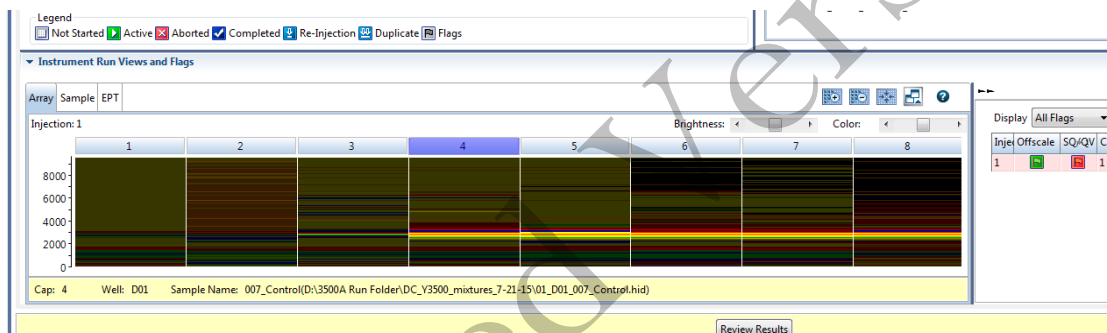
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help to determine if any samples need to be re-injected without having to transfer them to a computer with GeneMapperID-X. Peak heights and shape can be assessed with this viewer. Use the cursor on the axes to zoom. Note: OL alleles will not be flagged 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 is 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.



- Run will automatically complete.
- 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.

- Click the blue button in the dashboard **Edit Existing Plate**.
- Find your previous plate and open it.
- 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.
- On the plate view page in the Assays box in the lower left, add all the assays you want to use by

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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

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Method: **GeneMapper ID-X™ Analysis of Data**

Date: 06/01/2006

Revision Date: 10/05/2015

Approved by: SAM

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 AmpFISTR panels and bins are pre-installed in the Panel Manager 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 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 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:\3130-310 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**

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Method, Panel, Size Standard (see below). Unknowns should be designated Sample, Allelic Ladders should be designated Allelic Ladder, the positive PCR control should be designated as Positive Control, and any reagent blanks, amplification blanks, or formamide blanks should be designated as Negative Controls.

Kit	Analysis Method	Panel	Size Standard
GlobalFiler	GF_reference	GF_Reference_2016	GS600_LIZ
GlobalFiler	STRmix_GF_evidence	GF_evidence_2017	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 GeneMapperID 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:)>GeneMapper ID Projects>Analysts folder**) 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)].

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9. The original project should be deleted from the GeneMapperID 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 GeneMapperID projects. Open **GeneMapper Manager** and from the **Projects** tab click **Import**. Browse to **Forensic Biology on FB server (H:) > GeneMapper ID Projects** and select the desired project. Click **OK**. The project will have been imported to the GeneMapperID 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/STR Identifiler Plus™ PCR Amplification Kit User's Manual
Yfiler™ Plus PCR Amplification Kit User's Manual
GeneMapper™ ID Software version 3.1 User Manual.
GeneMapper™ ID Software Tutorial.

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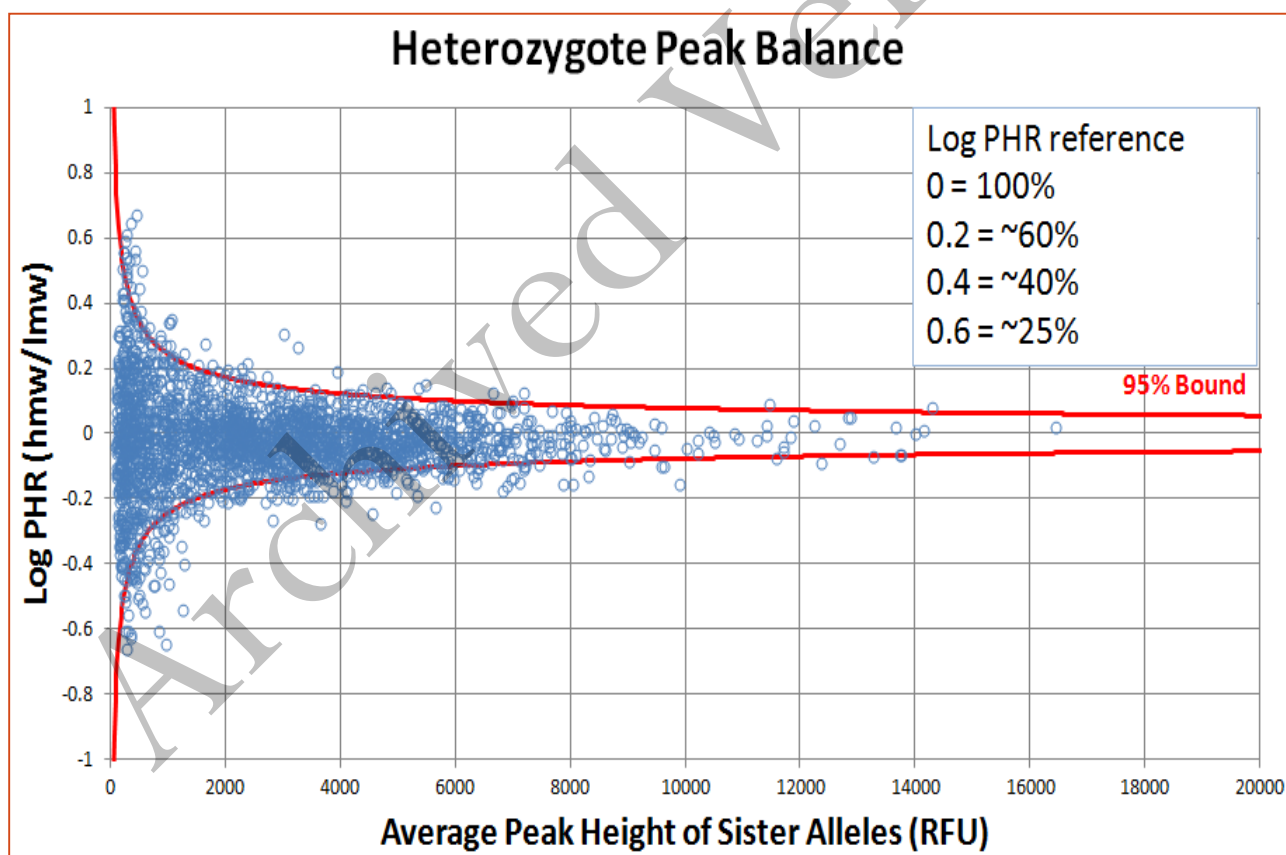
Method: **GlobalFiler Interpretation Thresholds**

Date: 10/05/2015

Revision Date: 11/28/2016

Approved by: SAM

- 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.



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- 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 without filtering n-1 repeat stutter, however; n+1 repeat and n-1/2 repeat stutter will be filtered at the mean plus two standard deviations. 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:

GF_Reference_2016

Locus	Stutter Filter (%)			
	GMID-X <i>N-1</i>	GMID-X <i>N+1</i>	GMID-X <i>N-.5</i>	GMID-X <i>N-2</i>
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

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The stutter cutoff values used by the software for evidence sample analysis are based on our internal validation and are presented below:

GF_Evidence_2017

Locus	Stutter Filter (%)
	GMID-X <u>N-.5</u>
D3S1358	0
vWA	0
D16S539	0
CSF1PO	0
TPOX	0
D8S1179	0
D21S11	0
D18S51	0
DYS391	0
D2S441	0
D19S433	0
TH01	0
FGA	0
D22S1045	0
D5S818	0
D13S317	0
D7S820	0
SE33	5.15
D10S1248	0
D1S1656	5.50
D12S391	0
D2S1338	0

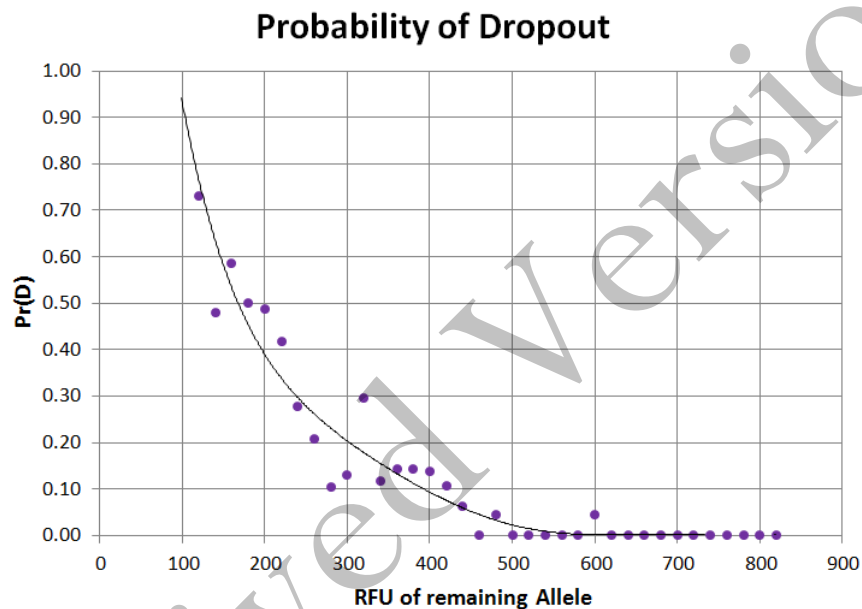
Another type of stutter artifacts include n-2 repeat stutter. n-2 repeat stutter general is detected when amplification of the parent peak is robust. The SDPD validation studies have established that the expectations for the ratio of the n-2 repeat stutter peak to the n-1 repeat stutter peak is equivalent 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

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stutter percentage. Analysts should use caution when making determinations on n-2 stutter peak removal from mixtures.

- 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

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Method: **Yfiler™ Plus Interpretation Thresholds**

Date: 02/05/2018

Approved by: SAM

- 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 more rare. 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				

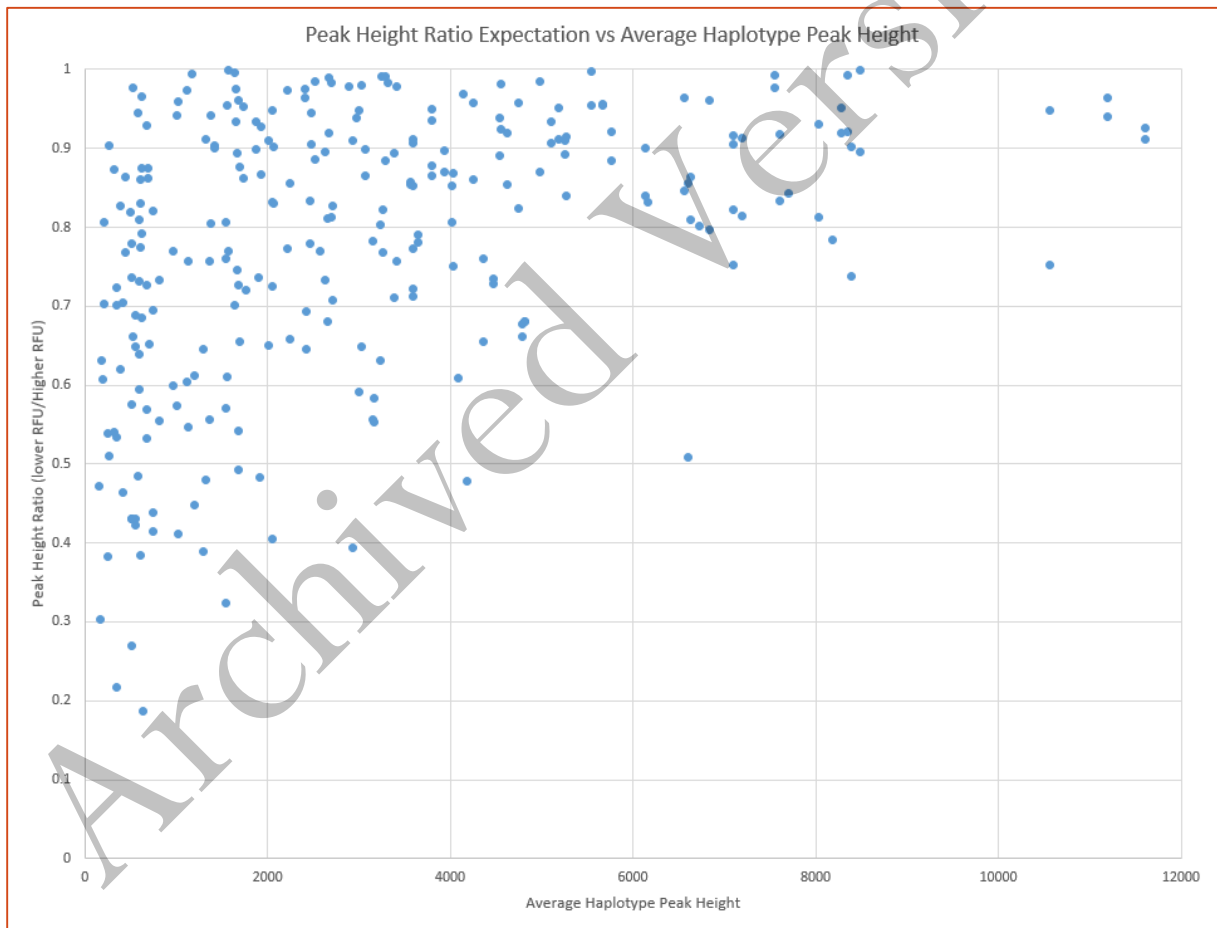
Another type of stutter artifacts include $n-2$ repeat stutter. $n-2$ repeat stutter general is detected when amplification of the parent peak is robust. The SDPD validation studies have established that the expectations for the ratio of the $n-2$ repeat stutter peaks in the stutter study for Yfiler™ 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-

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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 DYS387S1a/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.



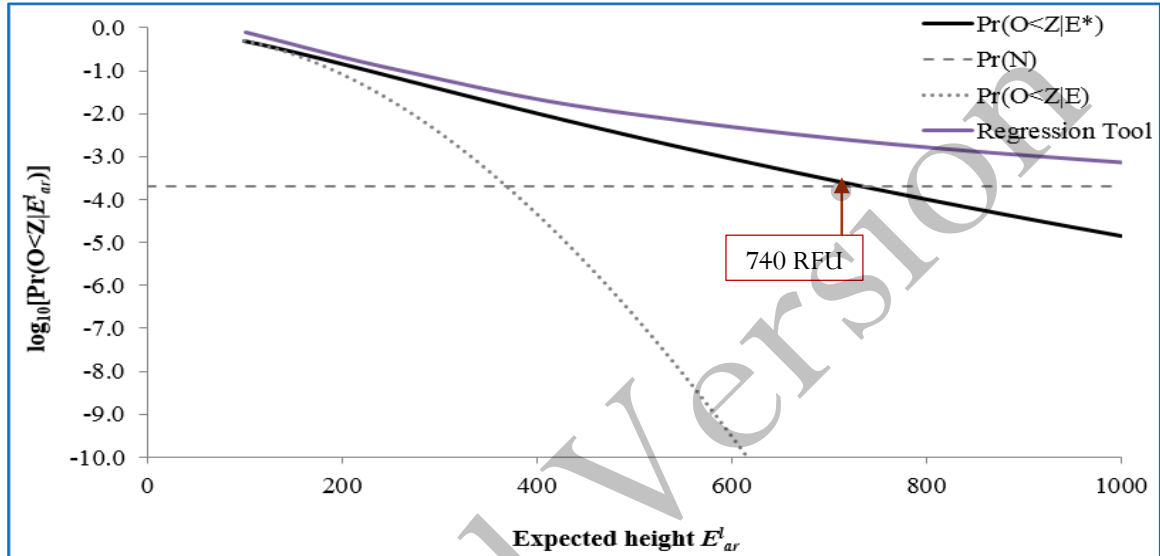
- Stochastic Threshold (probability of drop-out): There are two ways in which drop-out can be considered; either dropout has occurred for one allele of a duplicated locus, or dropout has occurred for a non-duplicated locus and so no information is seen. There is also the possibility that the absence of

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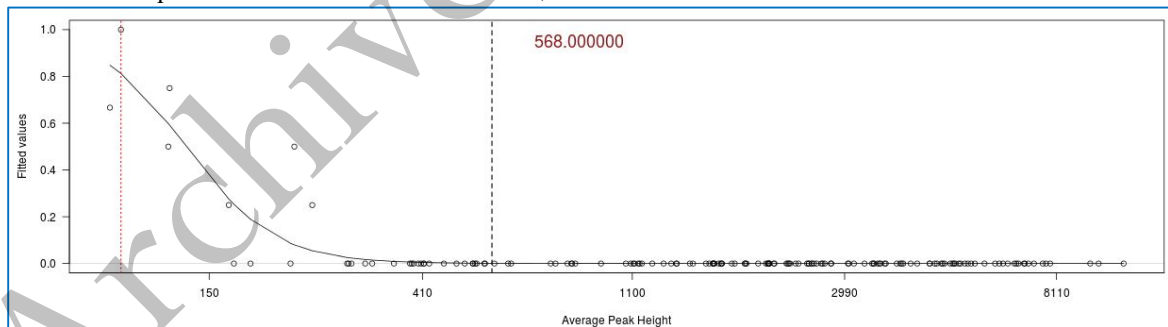
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information at a locus is due to a silent allele. The comparison between the probability of drop-out and the probability of a silent allele for duplicated and non-duplicated loci yields two interpretational thresholds:

- 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].

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REFERENCES:

SDPD Yfiler™ Plus Validation studies
Yfiler™ Plus User's Manual

Archived Version

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METHOD: **STRmix™ Analysis**

Date: 10/05/2015

Revision Date: 08/15/2016

Approved by: SAM

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 GeneMapperID-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:

An attempt was made to run this mixture through the interpretational software. The results exceed the computing capacity of the software and are unsuitable for comparisons.

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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, a point estimate of the 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 GeneMapperID-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”, “Marker”, 16 “Allele” columns, and 16 corresponding “Size” columns. Even though there should be a max of 2 alleles at each locus, the number of allele columns is set to 16 in order to accommodate the evidence files, and minimize setting changes in STRmix™.
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”, 16 “Allele” columns, 16 corresponding “Size” columns, and 16 corresponding “Height” columns across the top row.
4. **Export table**, and save to desired location.
5. Close these plots.

Manual creation of files for STRmix™ input

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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 sites in common. For this analysis both the questioned sample and the reference sample have to input from .txt files. This function also works with LR from Previous Analysis, provided .txt files are input.

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”, “Marker”, 16 “Allele” columns, and 16 corresponding “Size” columns.

If a STRmix™ deconvolution of a GlobalFiler evidence sample requires an assumed person, whose reference is in Identifiler format, it is recommended that the 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).

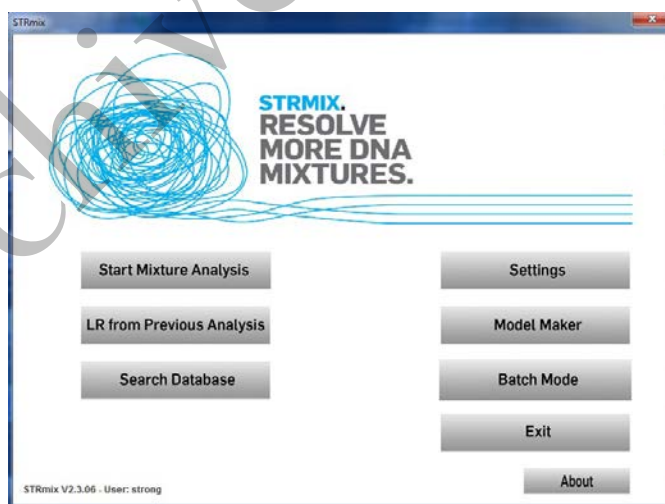


FIGURE 1

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Each of the functions within STRmix™ can be accessed from the Startup screen. To exit the software click the Exit button, *the top right-hand X button has been disabled.*

Single Sample Analysis

1. Select Start Mixture Analysis to open the Sample Summary window (see Figure 2). This initiates a three-step process for the interpretation of single source and mixed DNA profiles.

The screenshot shows the 'STRmix' software interface. The window title is 'STRmix'. It has a standard Windows-style title bar with a close button (X) in the top right corner. The main content area is divided into two sections. The top section is for case information, with the following fields: 'Case Number' (text box containing 'Training Mixture'), 'Sample ID' (text box containing 'E1'), and 'Case Notes' (text area containing 'optional comments'). The bottom section is titled 'Step 1: MCMC settings' and contains: 'Number of contributors' (text box containing '2'), 'DNA kit used' (dropdown menu showing 'SDPD GlobalFiler'), '# MCMC accepts' (text box containing '500000'), and '# burnin accepts' (text box containing '100000'). At the bottom of this section are three buttons: 'Other Settings', 'Cancel', and 'Confirm'. The status bar at the bottom left of the window reads 'STRmix V2.3.06 - User: strong'. A large, semi-transparent watermark 'Archived Version' is overlaid diagonally across the center of the image.

FIGURE 2

Fill in the Case Number, Sample ID, any case notes (optional), the number of contributors for the analysis. Select the appropriate DNA kit (SDPD GlobalFiler or SDPD GlobalFiler 24sec) for the analysis. The number of MCMC accepts and burn-in accepts should only be changed if longer analyses are required to better resolve complex mixtures. All five person mixtures must be run in low memory mode (Figure 3). Low memory mode can be selected by clicking the “Other Settings” button in the Sample Summary Window (Figure 2).

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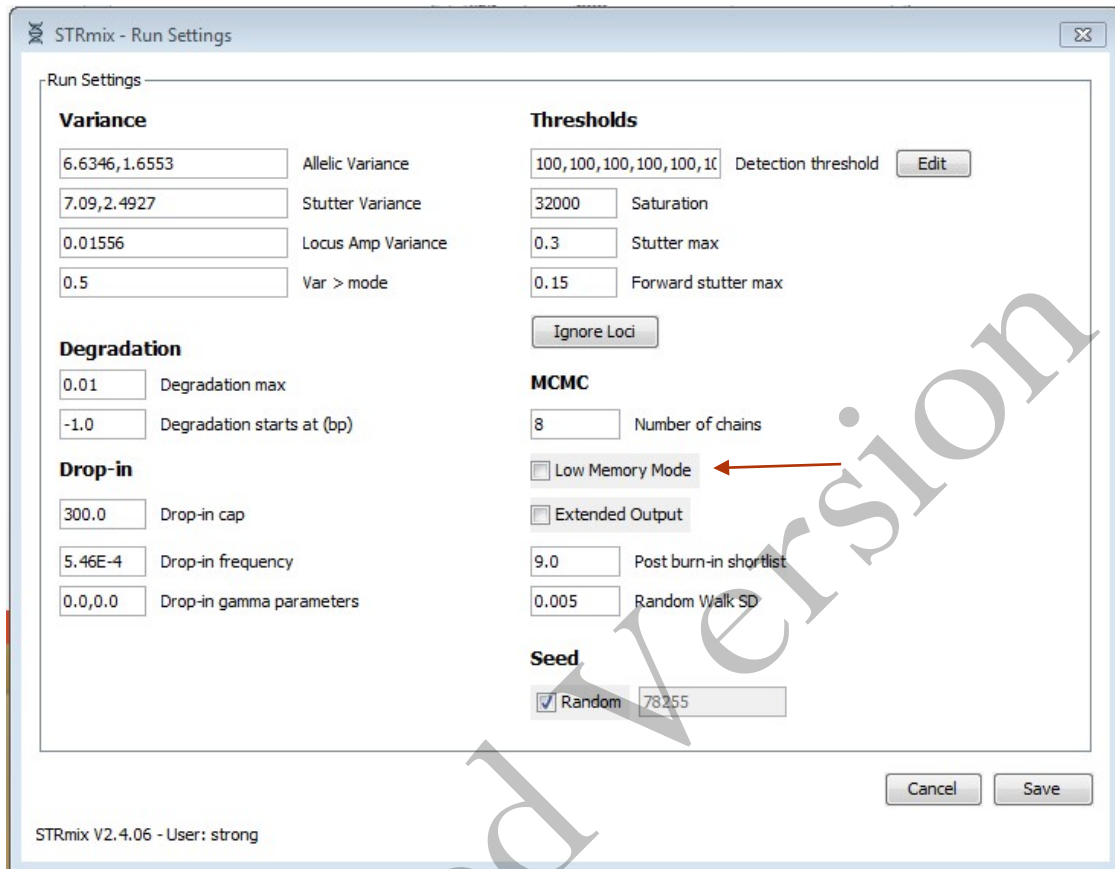


FIGURE 3

Any Other Settings (e.g., ignore loci or degradation max) should only be changed if needed.

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.

Select confirm.

2. In the Set Evidence EPGs screen select Add EPG to open Add EPG window (see Figure 4)

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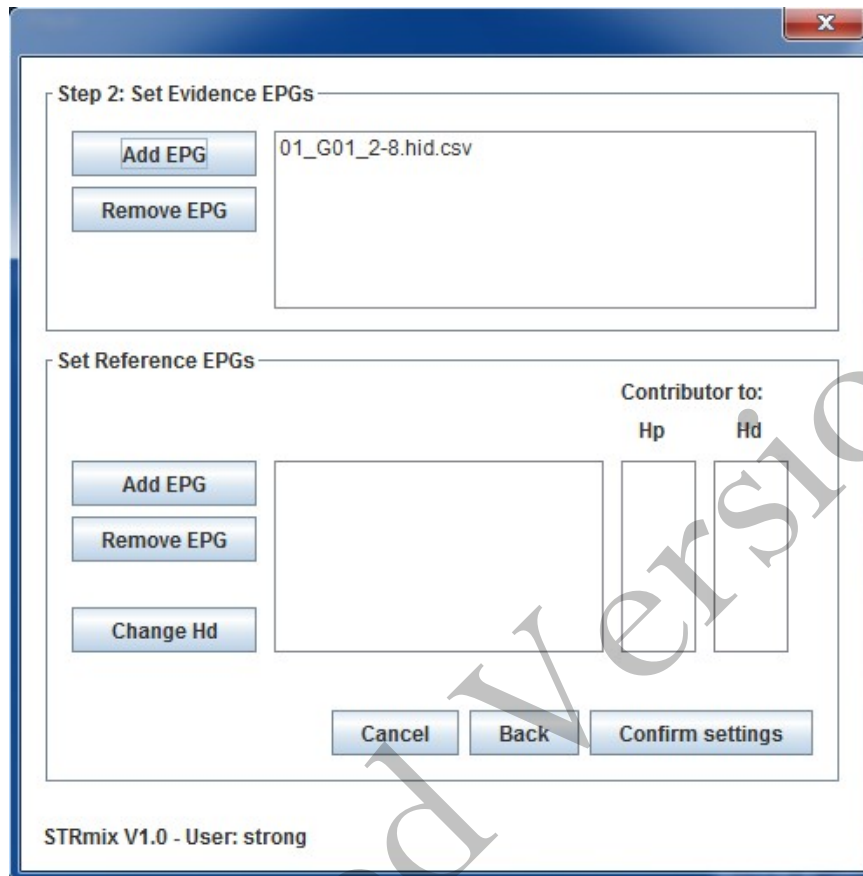


FIGURE 4

3. Select Find Text file, Find STRmix™ file or Create File as appropriate to enter an evidence input file. Navigate to the appropriate file and select the sample to be analyzed.

Select Add EPG to add the input file and proceed or Cancel to return to the Add EPG screen. Multiple EPGs of multiple amplifications of the same DNA extract can be added together. This feature should not be used for samples that have been re-injected.

4. In the Set Reference EPGs screen repeat the steps above for any reference input file/s as appropriate. Reference input files will automatically be assigned as a known contributor under Hp. If any references can reasonably be assumed, they may be added at this step. Should you require a reference sample to be an assumed contributor under Hd then select the reference of interest with the cursor so it is highlighted then press the Change Hd button. In general, persons of interest will be compared after initial deconvolution by the software.

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Select Confirm Settings to proceed to the next step (refer to Figure 4).

5. Select the appropriate population/s from the drop down menu. If reference samples have been added as unknowns, population groups for any likelihood ratio calculations can be added at this step by choosing Add Pop for each highlighted population of interest from the drop-down menu. Population settings are only required when reference samples have been selected in Step 2. If no reference samples are selected the settings in Step 3 are disabled (as in Figure 5).

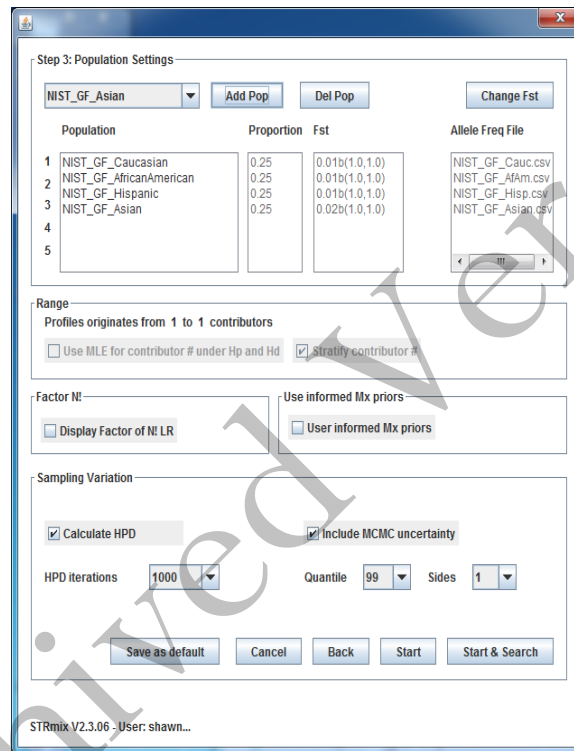


FIGURE 5

6. Select Start to start the calculation.
7. Once the run is complete, **Run Report** and **Create PDF**. A PDF report cannot be created after the fact without re-running this mixture. Close the Advanced report box, and **Finish**.
8. Results for this run will be located on the local computer in the STRmix™ Results folder. Transfer the folder to the FB network.

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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-person mixtures can surpass an hour each. 4- and 5-person mixtures can take hours/days to complete.

Batch Sample Analysis

1. Multiple samples may also be added to STRmix™ to be processed sequentially by selecting Batch Mode from the screen in Figure 1.

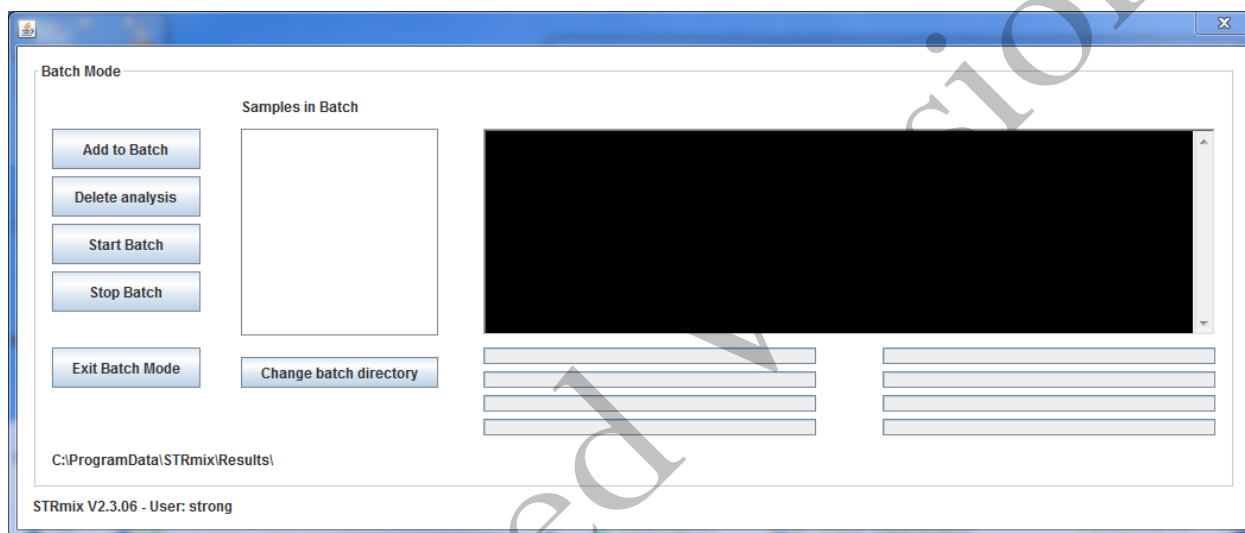


FIGURE 6

2. The process proceeds in the same way as Single Sample Analysis by going through steps 1 through 6. After step 6, when Start is selected, the software does not actually start a deconvolution, but will return you to the screen in Figure 6.
3. Once at the screen in Figure 6, additional STRmix™ analyses can be queued by repeating step 2 above. Once all the samples for the batch have been added, selecting Start Batch will commence the deconvolutions. The deconvolutions will proceed in alpha-numeric order.

Database searching

STRmix™ has a database searching feature which offers the option of searching an unresolvable mixture against a database 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:

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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™ mixture analysis. The likelihood ratios in this feature are run without the Balding and Nichols adjustments to improve run time. Individuals giving an LR above some list management value are investigated. A suggestion is to use 1000 to reduce the risk of adventitious hits.

The SDPD elimination DNA database has been created with the required Excel formatting. All mixture deconvolutions should be searched against the elimination DNA database. Database searches can occur within CODIS or can be performed within STRmix. The STRmix database search function must be performed on any mixture with components that will not or cannot be searched in CODIS. The STRmix database search will be more specific than CODIS searches due to the nature of how each program performs searches.

There are two options for searching this database within STRmix™.

Option 1

1. From the STRmix™ main menu (Figure 1), select Search Database.
2. Navigate to the previously analyzed sample folder of choice, highlight the settings.ini file and select Open.
3. Select Choose Database to open a file chooser. Navigate to the SDPD staff database and select Open. The number of profiles within the database appears in the window. Select the Minimum LR (e.g., 1000) and the population for the search. Check Standard to undertake a standard database search. Select Save as default to save the settings for the next search session.
4. Select Start to start the search or Cancel to return to the main menu. On completion of the search, select Finish to close the window. Results are automatically saved in a results directory (the file path is summarized at the bottom of the Results window) once Finish is selected. Database searches with no results need not be printed, but the database search files should be saved. If any matches to staff are obtained, the DB search file can be opened in Microsoft Word, or Excel and printed. Alternatively, a screen capture can be done and printed for any samples with an LR above the cutoff. Refer to the Forensic Biology Policy Manual section 4.4 for additional information on staff matches.

Option 2

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1. During either a single sample analysis or batch analyses, from the Populations Settings window (Figure 4), select Start and Search.
2. Follow steps 3 and 4 from Option 1.

Calculation of a Likelihood Ratio in STRmix™

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 (see step 4 in the Single Sample Analysis section above).

LR from Previous Analysis

1. When performed after the initial deconvolution, select LR from Previous Analysis from the STRmix™ main menu (Figure 1).
2. Navigate to the previously analyzed sample folder of choice, highlight the settings.ini file and select Open.
3. The window in Figure 2 will open, but analysis settings will be unavailable for change. Fill in the Case Number, Sample ID, and any case notes (optional). The sample ID box should be altered to contain the initials for the reference sample to be compared (e.g., XXXXXXXX LR Ref XX). Any loci to be ignored can be set in Others Settings. Select confirm.
4. The window in Figure 4 will open. Only the Set Reference EPGs will be available. If any contributors need to be assumed at this point, the deconvolution should be repeated as this is not an available feature when conducting an LR from Previous Analysis. Navigate to the reference input file/s as appropriate. Reference input files will automatically be assigned as a known contributor under Hp. Select Confirm Settings.
9. The window in Figure 5 will open. Select the appropriate population/s from the drop down menu and choosing Add Pop. Select Start to start the calculation.
10. Once the run is complete, **Run Report** and **Create PDF**. A PDF report cannot be created after the fact without re-running this mixture. Close the Advanced report box, and **Finish**.

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- Results for this run will be located on the local computer in the STRmix™ Results folder. Transfer the folder to the FB network.

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

All calculations will be generated for the Caucasian, African American, Hispanic, and Asian populations using the DNA allele frequency data taken from Forensic Science International: Genetics 7 (2013) e82-83. If a different database is used for calculations, it shall be included in the administrative documents 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 7. 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.

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	0
3	5	0	0	0	0	0	0	0	0	0	0
4	6	0	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	

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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 7

Once the new database has been reviewed it needs to be added to the STRmix™ software. From the STRmix™ main menu (Figure 1) select Settings. Select Edit/View population. 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 Fst values. Select Save Pop

Analysts are to include a copy of all non-standard database papers in their case file as an Administrative Document. Reviewers don't need to re-check already validated databases but need to ensure that the correct population has been used.

STRmix™ settings

The following are the STRmix default settings:

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STRmix - Default Settings

Default Settings

MCMC settings

8 # MCMC chains

500000 MCMC accepts

100000 Burnin accepts

9.0 Post burn-in shortlist

0.005 Random Walk SD

Low Memory Mode

Inputs and Outputs

Extended Output

16 Alleles per locus

Summary:

Analysis

LR

Parameters

Weightings

Settings

Inputs

Interpretations

Default Kit: SDPD GlobalFiler

Likelihood Ratio

1000 HPD iterations

99.0 Sig value

1 Sides

Factor of N! LR

Include MCMC uncertainty

Default Text File Directory: H:\Casework Batches

Default STRmix File Directory: C:\ProgramData\STRmix

Cancel Save

STRmix V2.4.06 - User: strong

Note: the Default Text File Directory and Default STRmix File Directory do not need to conform to the above locations; however, all other default parameters should have these settings.

The following are the GlobalFiler settings:

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Add/ Edit DNA profiling kit

DNA profiling kit: SDPD GlobalFiler [Edit Kit] [Delete Kit]

Kit name: SDPD GlobalFiler

Stutter File: SDPD_GF_Stutter.txt [Find File] [Edit File]

Stutter Exceptions File: SDPD_GF_StutterExceptions.csv [Find File] [Edit File]

Number of Loci: 24 Gender Locus: AMEL

Locus Order: D3S1358,WAD16S539,CSF1PO,TPOX,Yindel,AMEL,D8S1179,D21S11,D18S51,DYS391,D2S441,D19S433,TH01,FGA,D22S1045,D5S818,D13S317,D7S820,SE33,D10S1248,D1S1656,D12S391,D2S1338

Include Loci: Y,Y,Y,Y,N,Y,Y,Y,N,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y [Ignore Loci]

Detection Threshold: 100,100 [Set Td]

0.3 Stutter max 300 Drop-in cap 5.6346,1.6553 Allelic Variance
32000 Saturation 5.46E-4 Drop-in frequency 7.0900,2.4927 Stutter Variance
-1.0 Degradation starts at 0.0 Drop-in parameters 0.1 Var > mode
0.01 Degradation max 0.01556 Locus Amp Variance

[Cancel] [Save Kit]

STRmix V2.3.07 - User: shawnm

STANDARD OR REDUCED INJECTION SETTINGS

Add/ Edit DNA profiling kit

DNA profiling kit: SDPD 24s GlobalFiler [Edit Kit] [Delete Kit]

Kit name: SDPD 24s GlobalFiler

Stutter File: SDPD_GF_Stutter.txt [Find File] [Edit File]

Stutter Exceptions File: SDPD_GF_StutterExceptions.csv [Find File] [Edit File]

Number of Loci: 24 Gender Locus: AMEL

Locus Order: D3S1358,WAD16S539,CSF1PO,TPOX,Yindel,AMEL,D8S1179,D21S11,D18S51,DYS391,D2S441,D19S433,TH01,FGA,D22S1045,D5S818,D13S317,D7S820,SE33,D10S1248,D1S1656,D12S391,D2S1338

Include Loci: Y,Y,Y,Y,N,Y,Y,Y,N,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y [Ignore Loci]

Detection Threshold: 100,100 [Set Td]

0.3 Stutter max 510 Drop-in cap 11.63659,1.455022 Allelic Variance
32000 Saturation 9.28E-4 Drop-in frequency 8.354948,2.273458 Stutter Variance
-1.0 Degradation starts at 0.0 Drop-in parameters 0.1 Var > mode
0.01 Degradation max 0.017811 Locus Amp Variance

[Cancel] [Save Kit]

STRmix V2.3.07 - User: shawnm

24-SECOND INJECTION SETTINGS

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The following are the settings for the NIST population databases for the GlobalFiler kit:

The figure displays four screenshots of the 'Add/ Edit Population' dialog box in STRmix V2.3.06 software, showing settings for different population groups. Each window includes fields for Population, Pop Name, Allele freq File, Pop proportion, Applies to kit, Default Fst, Multiplier x beta(Alpha, Beta), Population size, Children per family, Generate proportions, Siblings, Niece/Nephew, Parents, Grandparent, Children, Grandchild, Uncle/Aunt, Cousin, and Unrelated. Buttons for Edit Pop, Delete Pop, Find File, Edit File, Cancel, and Save Pop are also present.

Top Left: African American Population

- Population: NIST_GF_Afric...
- Pop Name: GF_AfricanAmerican
- Allele freq File: NIST_GF_AfAm.csv
- Pop proportion: 1.0
- Applies to kit: SDPD GlobalFiler
- Default Fst: 0.01b(1.0,1.0)
- Multiplier x beta(Alpha, Beta):
- Population size: 0
- Children per family: 0
- Generate proportions: [Button]
- Siblings: 0.0
- Niece/Nephew: 0.0
- Parents: 0.0
- Grandparent: 0.0
- Children: 0.0
- Grandchild: 0.0
- Uncle/Aunt: 0.0
- Cousin: 0.0
- Unrelated: 1.0

Top Right: Asian Population

- Population: NIST_GF_Asian
- Pop Name: NIST_GF_Asian
- Allele freq File: NIST_GF_Asian.csv
- Pop proportion: 1.0
- Applies to kit: SDPD GlobalFiler
- Default Fst: 0.02b(1.0,1.0)
- Multiplier x beta(Alpha, Beta):
- Population size: 0
- Children per family: 0
- Generate proportions: [Button]
- Siblings: 0.0
- Niece/Nephew: 0.0
- Parents: 0.0
- Grandparent: 0.0
- Children: 0.0
- Grandchild: 0.0
- Uncle/Aunt: 0.0
- Cousin: 0.0
- Unrelated: 1.0

Bottom Left: Caucasian Population

- Population: NIST_GF_Cauc...
- Pop Name: NIST_GF_Caucasian
- Allele freq File: NIST_GF_Cauc.csv
- Pop proportion: 1.0
- Applies to kit: SDPD GlobalFiler
- Default Fst: 0.01b(1.0,1.0)
- Multiplier x beta(Alpha, Beta):
- Population size: 0
- Children per family: 0
- Generate proportions: [Button]
- Siblings: 0.0
- Niece/Nephew: 0.0
- Parents: 0.0
- Grandparent: 0.0
- Children: 0.0
- Grandchild: 0.0
- Uncle/Aunt: 0.0
- Cousin: 0.0
- Unrelated: 1.0

Bottom Right: Hispanic Population

- Population: NIST_GF_Hisp...
- Pop Name: NIST_GF_Hispanic
- Allele freq File: NIST_GF_Hisp.csv
- Pop proportion: 1.0
- Applies to kit: SDPD GlobalFiler
- Default Fst: 0.01b(1.0,1.0)
- Multiplier x beta(Alpha, Beta):
- Population size: 0
- Children per family: 0
- Generate proportions: [Button]
- Siblings: 0.0
- Niece/Nephew: 0.0
- Parents: 0.0
- Grandparent: 0.0
- Children: 0.0
- Grandchild: 0.0
- Uncle/Aunt: 0.0
- Cousin: 0.0
- Unrelated: 1.0

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The following are the settings for the NIST population databases for the GlobalFiler kit:

The figure displays four screenshots of the 'Add/Edit Population' dialog box in STRmix V2.3.06, showing settings for different NIST population databases for the GlobalFiler kit. Each screenshot shows the same dialog box with different population-specific settings.

Top Left Screenshot (African American):

- Population: NIST_GF24_Afr...
- Pop Name: F24_AfricanAmerican
- Allele freq File: NIST_GF_AfAm.csv
- Pop proportion: 1.0
- Applies to kit: SDPD 24s GlobalFiler
- Default Fst: 0.01b(1.0,1.0)
- Multiplier x beta(Alpha, Beta):
- Population size: 0
- Children per family: 0
- Siblings: 0.0, Niece/Nephew: 0.0
- Parents: 0.0, Grandparent: 0.0
- Children: 0.0, Grandchild: 0.0
- Uncle/Aunt: 0.0, Cousin: 0.0
- Unrelated: 1.0

Top Right Screenshot (Asian):

- Population: NIST_GF24_As...
- Pop Name: NIST_GF24_Asian
- Allele freq File: NIST_GF_Asian.csv
- Pop proportion: 1.0
- Applies to kit: SDPD 24s GlobalFiler
- Default Fst: 0.02b(1.0,1.0)
- Multiplier x beta(Alpha, Beta):
- Population size: 0
- Children per family: 0
- Siblings: 0.0, Niece/Nephew: 0.0
- Parents: 0.0, Grandparent: 0.0
- Children: 0.0, Grandchild: 0.0
- Uncle/Aunt: 0.0, Cousin: 0.0
- Unrelated: 1.0

Bottom Left Screenshot (Caucasian):

- Population: NIST_GF24_Ca...
- Pop Name: T_GF24_Caucasian
- Allele freq File: NIST_GF_Cauc.csv
- Pop proportion: 1.0
- Applies to kit: SDPD 24s GlobalFiler
- Default Fst: 0.01b(1.0,1.0)
- Multiplier x beta(Alpha, Beta):
- Population size: 0
- Children per family: 0
- Siblings: 0.0, Niece/Nephew: 0.0
- Parents: 0.0, Grandparent: 0.0
- Children: 0.0, Grandchild: 0.0
- Uncle/Aunt: 0.0, Cousin: 0.0
- Unrelated: 1.0

Bottom Right Screenshot (Hispanic):

- Population: NIST_GF24_Hi...
- Pop Name: NIST_GF24_Hispanic
- Allele freq File: NIST_GF_Hisp.csv
- Pop proportion: 1.0
- Applies to kit: SDPD 24s GlobalFiler
- Default Fst: 0.01b(1.0,1.0)
- Multiplier x beta(Alpha, Beta):
- Population size: 0
- Children per family: 0
- Siblings: 0.0, Niece/Nephew: 0.0
- Parents: 0.0, Grandparent: 0.0
- Children: 0.0, Grandchild: 0.0
- Uncle/Aunt: 0.0, Cousin: 0.0
- Unrelated: 1.0

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METHOD: **Autosomal STR Interpretation Guidelines**

Date: 06/01/2004

Revision Date: 11/28/2016

Approved by: SAM

Introduction

The Scientific Working Group on DNA Analysis Methods (SWGDM) 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)

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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 60-460bp are required. For MiniFiler analysis, GS600 LIZ peaks from 80-400bp 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.

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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 (www.cstl.nist.gov/strbase/var_tab.htm). 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 administrative documents 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. *Tri-allelic patterns* 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* is often the result of over-amplification and can cause several other interpretational difficulties, such as: “minus-A” products, increased stutter, and pull-up (see

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below). If off-scale data is 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-4bp in tetranucleotide loci) or larger (e.g., n+4bp in tetranucleotide loci), or sometimes one half repeat smaller (e.g., n-2bp 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 repeat stutter products, and evidence samples destined for STRmix™ analysis should have n-1 repeat stutter peaks labeled. Additional stutter products (i.e., n+1 repeat and 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

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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 may need to be re-extracted. Not all contamination events will necessitate reanalysis 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

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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.

- 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 must be jointly evaluated by the DNA analyst and the DNA Technical Manager (or his/her designee). On a case-by-case basis, the DNA Technical Manager will evaluate the results obtained and 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. The evaluation of the control results and the DNA Technical Manager's approval (or his/her designee's) of the process must be documented in the analyst's case notes and, in an Unexpected Results Summary (see FB policy manual section 1.6).

If the results from samples associated with the failed positive control are deemed usable, the failure of the positive control must be included 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.

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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 for each kit should be consulted).
- Generally, evidence of a mixed DNA sample exists at multiple loci.

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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).

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, alleles in 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 number of contributors determination. 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 has 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

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sample), 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 “Inconclusive number of contributors – cannot be analyzed” 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 3.2). 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.

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.

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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)

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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 indicates that three or more contributors may be present)).

2.6 Samples that are unsuitable for comparisons

Mixtures of 6 people or more are considered unsuitable for comparisons.

Samples amplified with GlobalFiler that have data at five autosomal markers or less and MiniFiler amplifications that have data at three autosomal markers or less are unsuitable for comparisons.

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 or less are unsuitable for comparisons.

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 less
- 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 indicates 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.

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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. *Work conducted on samples prior to 3500*

STRmix™ requires biological modeling of STR data in order to be used. The Forensic Biology Unit has developed biological models for GlobalFiler and MiniFiler, both run on the 3500. There may be instances where further work may be required in a case where data was originally run using another STR kit and/or electrophoresis instrument (such as Identifiler on a 3130). We have no biological models for data generated on these instruments and no ability to analyze data produced using them. There are three options:

- If DNA extract remains, the sample(s) can be re-amplified and run with our current kit on our current instrument.
- 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 old sample (pre-3500) has not been previously reported and now requires reporting (e.g. through a database link to an old case) and there is no DNA extract left for analysis, then the interpretation will need to be carried out using the last available interpretation guidelines for that data and reported.

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.

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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).

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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 LR:

- 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 supports the presence of an assumed contributor, this should be documented and can be used as a basis for using an assumption.

This evaluation can take into account peak height ratio expectations, apparent mixture ratios, and stutter ratio expectations, which are all available from 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.

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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. 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 (i.e. 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 (i.e. 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.

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3.5. 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 most iterations or the more appropriate saturation cap 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.

3.6. 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 n-1 repeat 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 make sense 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 intuitively seem correct. Examples of this are:

- The mixture proportions do not reflect what is observed
- The degradation does not reflect what is observed
- The interpreted contributor genotypes are not intuitively correct
- Large LRs (greater than 1) are obtained for each locus, except one where the LR is 0, and the POI reference appear consistent with the scene profile

Causes for these issues include:

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- The MCMC has not run for enough iterations.
- The number of contributors has not been correctly chosen.
- The PCR has been affected (i.e., 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 (i.e., re-amplify or re-extract).
- Run STRmix™ at an increased number of iterations.
- Correct the STRmix™ input file.

Additional diagnostics information within the summary output of STRmix™ v2.3 may indicate that a STRmix™ v2.3 deconvolution has not converged on the best sample space. There are six main 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. Below is an example of data that is present within the raw output. Each is discussed in turn below.

3.6.1. 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.

3.6.2. Effective Sample Size

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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.

3.6.3. Average log(likelihood)

This value shows the average $\log_{10}(\text{likelihood})$ 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 has been removed that was real, particularly stutter peaks, and must now be described in STRmix™ by dropout
- Artfactual peaks have been left labeled and must now be accounted for in STRmix™ by drop-in.

A low or negative value for the average $\log_{10}(\text{likelihood})$ may indicate to users that the analysis requires additional scrutiny. To note, good quality mixed DNA profiles are likely to give higher average $\log_{10}(\text{likelihood})$ values than good quality single source profiles. So low average $\log_{10}(\text{likelihood})$ values alone are not necessarily an indicator of an issue.

3.6.4. 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

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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.

3.6.5. 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 $\log_{10}(\text{likelihood})$, 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 $\log_{10}(\text{likelihood})$ and an average variance constant.

If some data has been omitted, left on or misinterpreted this should result in a low average $\log_{10}(\text{likelihood})$ and high variances.

3.6.6. Troubleshooting

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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. You may wish to extend the total iterations, for example if the acceptance rate is low, the ESS is low and/or the GR value is above 1.2.

3.7. 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 ignoring the locus with the biological alteration. 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 alteration.

If the fit to Hp is poor, then the profile should be deconvoluted still ignoring the locus and be reported as “POI excluded”. This is to be conducted for all reference profiles provided in the case, not just the reference sample of the individual with alteration. If it is not possible to clearly exclude an individual due to the complexity of the mixture the LR calculated by STRmix™ should be reported.

The following caveat should be included in the report for use in these circumstances:

*The reference DNA profile of John SMITH is tri-allelic at locus FGA. This means that instead of two alleles at this location, as expected, there are three alleles. This is a recognized but rare biological occurrence. As a result, locus FGA has been omitted from the statistical calculations as the STRmix™ program is unable to use tri-allelic loci for reference comparisons.

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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™.

3.8. Quality Assurance Check of STRmix Results

QA checks are designed to identify potential DNA contamination by checking deconvoluted samples against staff elimination DNA profiles. In addition, the elimination database contains randomly generated non-contributor DNA profiles that can be used to determine the quality of the DNA profiles (or components of the DNA profiles) that have been deconvoluted. The QA checks are carried out using the 'Search Database' function of STRmix™. The checks are carried out by each individual analyst and should be conducted prior to the submission of a file for technical review.

Elimination database files exist for the GlobalFiler and MiniFiler test kits and are in the STRmix™ validation folder (H:\QA-QC files\Validations\3500 and GlobalFiler and STRmix\STRmix\Settings for SDPD kits\Databases).

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 settings.ini file that contains the deconvolution of interest
- Ensure STRmix™ is navigating to the correct database file; C:\Program Data\STRmix\Databases\SDPD_kit_date.csv
- Ensure the 'minimum match' level is set to 1,000. Lower match thresholds (e.g., 100) may be required if the amount of information in the evidence profile is limited (e.g., MiniFiler results from casings).
- Ensure the default population is the relevant GlobalFiler or MiniFiler population (only one population file needs to be selected).

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- 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.
- If the profile that has linked to the QA check is to be presented in a report, an LR against the profile for that individual in the staff database must be calculated.
- All possible links to the staff profiles in the elimination database must be brought to the attention of the DNA Technical Manager and a Staff Match Report initiated.

Documentation of QA Checks:

When an LR is obtained for a POI that will be reported as either limited, moderate, or strong support for inclusion, the number of results for random non-contributor samples within the QA check that produced Database Search LRs higher than the LR_{POI} should be noted on the LR for the POI. Therefore, if for example $LR_{POI} = 500$, the number of random non-contributor samples that produced LRs greater than or equal to 500 should be noted on the LR sheet (e.g., QA check: random samples with $LR \geq LR_{POI} = X$).

3.9. Reporting Likelihood Ratios

When reporting inclusionary LR calculations the value of the 99% lower HPD interval will be reported. This LR incorporates the comparison of a reference profile to all the components of a mixture rather than just the component matching the reference. In this way the result can be reported as a comparison of a POI to the mixture as a whole. The 99% lower HPD incorporates a measure of allele and MCMC variance.

Additionally, sometimes evidence profiles may run, but a 99% lower HPD cannot be calculated because the Effective Sample Size returns NaN (not a number). If this occurs, a point estimate of the LR will be reported instead of the HPD value. This can be accomplished by deselecting Calculate HPD in the Sampling Variation box prior to running a likelihood ratio calculation. When necessary, the notes should specifically reflect the reason that the calculation presented is a point estimate of the LR.

In certain circumstances the 99% lower HPD will be dramatically different from the point LR calculation from the MCMC (i.e., greater than a 10^6 difference). This may occur when one or more loci favor exclusion, but the vast majority of loci favor inclusion. In this scenario, the point LR closest to 1 will be reported. Analysts should evaluate the data when this occurs to determine if the underlying reason for the large difference between the point LR and HPD can be identified and resolved (e.g.,

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mixture results require an additional contributor to explain the data or the presence of an unresolved allele).

For inconclusive and exclusionary LR, the point LR closest to 1 will be reported.

3.10. 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.

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 administrative documents with the case file and noted 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.

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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 statistics are identical or, where applicable, meet or exceed a laboratory-defined source attribution or minimum threshold.

Calculations provided by STRmix™ are likelihood ratio calculations of the form:

$$LR_C = \frac{\sum_j w_j \Pr(S_j | H_1)}{\sum_u w_u \Pr(S_u | H_2)}$$

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 are adjusted to posterior mean frequencies prior to the Balding and Nichols adjustment.

$$p(A) = \frac{x_a + 1/k}{N + 1}$$

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.

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In STRmix™ calculations, persons of interest are compared as possible contributors to the mixture as a whole.

4.1. Hypotheses for Likelihood Ratio Calculations in STRmix

Analysis should be set up with the first hypothesis (named H_p in STRmix™) comprising the person of interest and known or unknown individuals to fill up to the number of contributors (i.e. for a three person mixture H_p would be POI and two unknowns).

The second hypothesis (named H_d in STRmix™) should consist of all unknown individuals up to the number of contributors (i.e. for a three person mixture H_d would 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:

H_p = Assumed contributor + POI + unknown individuals

H_d = Assumed contributor + unknown individuals

As an example, for a three person mixture:

H_p = Assumed contributor + POI + 1 unknown individuals

H_d = Assumed contributor + 2 unknown individuals

4.2. Defining LR results

4.2.1. Provides support for inclusion. If a person of interest receives an $LR > 100$.

4.2.2. *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.

4.2.3. Inconclusive. LR between 0.01 and 100 for up to and including 4-person mixtures, or between 0.001 and 1000 for 5-person mixtures.

4.2.4. 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.

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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 possibly 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 LR's greater than 100, 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 LR's greater than 100, it is expected that the analyst will also run a comparison using an Hp that includes both POIs (i.e., $H_p = POI1 + POI2 + 1 \text{ unknown}$). 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 “_Report” text file. This information is included in the STRmix Advanced Report.

When comparisons to an evidentiary profile within STRmix result in inclusionary LR's 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

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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.
- 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.

4.5 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.

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.

The following represents the suggested verbal scale used to convey the strength of the likelihood ratio calculation for single source through 4-person mixtures.

Likelihood Ratio

Verbal Wording

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>10,000	Strong support
1000-10,000	Moderate support
100-1000	Limited support
0.01 -100	Inconclusive
<10 ⁻² - 0	Excluded

Studies conducted using a database of ten thousand randomly generated GlobalFiler profiles indicates that it becomes more likely to generate LR's greater than 100 for non-contributors in 5-person mixtures. Based on this information, when reporting results for 5-person mixtures the inconclusive range will extend from 0.001 to 1000. The result being that the category of limited support is eliminated for 5-person mixtures, while remaining unchanged for single source through 4-person mixtures.

Likelihood Ratio	Verbal Wording
>10,000	Strong support
1000-10,000	Moderate support
0.001 -1000	Inconclusive
<10 ⁻³ - 0	Excluded

5.1 Reporting of conclusions

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.

The contributor order will be reported and must be included in the case file.

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.

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.

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

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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.

- 5.1.1 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. 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.
- 5.1.2 If suitable for STRmix™ analysis, conclusions should state the assumed number of contributors used in the analysis. If comparisons are made, the conclusion should 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 should be included in the report.

If an inclusion is made, and if the inclusion warrants statistical weight (see section 4), the applicable LR should be stated. The verbal scale indicated by the LR will be presented in the report.

Any assumptions made regarding the comparisons and/or the probability calculations must 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.

If an evidence profile results in an unknown 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 should be included.

- 5.1.3 Statements when samples are uninterpretable:

No DNA types were detected.

Due to the low level of the detected DNA types and/or the unknown number of contributors, this/these sample(s) are uninterpretable.

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5.1.4 Statements when samples are unsuitable for comparison:

Due to insufficient information obtained, this sample is unsuitable for comparisons.

An attempt was made to run this mixture through the interpretational software. The results exceed the computing capacity of the software and are unsuitable for comparisons.

Due to a determination of greater than five contributors, this sample is unsuitable for comparisons.

5.1.5 Examples of inclusions in common scenarios:

5.1.5.1 Example of an inclusion as a contributor to a mixture when the number of contributors is assumed (likelihood ratio scenario)

It is $X \times 10^{XX}$ times more likely to obtain the DNA results if <POI> is a contributor than if he/she is not a contributor.

5.1.5.2 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 $X \times 10^{XX}$ times more likely to obtain the DNA results if <POI> is a contributor than if he/she is not a contributor.

5.1.5.3 Example of an inclusion in a paternity case

It is $X.XX \times 10^{YY}$ more likely to obtain the evidence 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%.

5.1.5.4 Inclusions to single source profiles on non-probative samples

A qualitative statement must provide sufficient information to clearly express the significance of the association:

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DNA consistent with originating from a single person was recovered from the apparent bloodstain. Jane Victim is included to the DNA results at all 21 markers.

5.1.5.5 Non-probative inclusions to mixtures

A mixture of two people was recovered from the evidence. John Doe is included as being a possible DNA contributor to the mixture at all 21 markers. There is at least one unknown DNA contributor to the mixture. DNA types foreign to John Doe will be searched against the CODIS databases.

5.1.6 Examples of wording when a comparison or the sample is inconclusive

During DNA testing of the evidence (Item 1), the positive amplification control did not provide the expected results. Therefore no conclusions will be drawn from this item.

Assuming <NAME> is a contributor to the mixture, it/It is inconclusive whether <POI> is a contributor to the mixture because the likelihood ratio result (between 0.01 and 100) does not provide sufficient support for inclusions or exclusions.

The likelihood ratio result does not provide sufficient support for inclusions or exclusions.

5.1.7 Exclusions (if applicable) will be listed out in each conclusion.

5.1.8 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 2.9 of the Laboratory Quality Manual.

5.1.8.1 Example of wording for staff matches

The DNA profile from Evidence Item X was found to match DNA from staff. No other item in this case contains this profile and therefore interpretations of these items were not affected.

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5.1.9 Streamlining conclusions

In cases without listed suspects, it may be appropriate to streamline the reporting of the conclusions. If suspects in these cases are developed and future reports are issued detailing these comparisons, streamlined conclusions would not be appropriate. The following are examples of streamlined conclusions.

5.1.9.1 Comparisons but no CODIS searches

The DNA results are unsuitable to be searched against CODIS; however, comparisons can be made if future references are obtained.

5.1.9.2 CODIS searching

The DNA recovered from the evidence is from an unknown individual and will be searched against CODIS.

A subset of the DNA types will be searched against CODIS.

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METHOD: **Y-STR Interpretation Guidelines**

Date: 06/01/2004

Revision Date: 02/05/2018

Approved by: SAM

Introduction

The Scientific Working Group on DNA Analysis Methods (SWGDM) 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)

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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:

- Greater than one allele 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).

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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 contributors 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 (i.e., 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.

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In circumstances when the number of contributors cannot be estimated, the profile should be reported as “Inconclusive number of contributors – cannot be analyzed” 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.

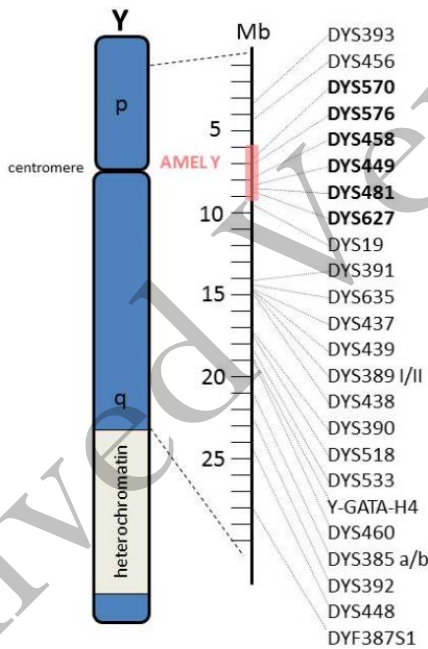


FIGURE 4 - Y-CHROMOSOME MAP OF RELATIVE LOCUS POSITIONS

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

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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 (i.e. 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 (i.e., 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 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) occurs at the same relative peak height to the average peak height of a lower level contributor to the sample (i.e., 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.

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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.

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 outlined in **Figure 1** and **2** must be met or exceeded.
- 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 is 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.

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).

When applying the interpretational thresholds in **Figures 1 and 2**, selection of the appropriate mixture ratio for the profile in question should be based upon the average ratio across the profile rather than the ratio at an individual locus. It is not advised to use the duplicated loci for determination of the mixture ratio. Peak height ratio expectation provided in the Yfiler™ Plus Interpretation Thresholds can be used to assist in the correct pairing of alleles at duplicated loci.

When the mixture ratio of a profile exceeds those given in the Tables, the value for the largest given ratio should be used (e.g., if interpreting a two person mixture with an apparent 20:1 ratio, the 10:1

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values from Table 1 should be used). Exception: do not interpret a minor component from a single peak locus when the ratio is greater than 10:1.

Not all possible mixture ratios are accounted for in **Figure 2**. When using **Figure 2**, the most appropriate ratio is that between the apparent major peaks and the peaks of the most intense minor component (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 the 2:1:1 values from **Figure 2** should be used).

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 caution and a communication with the DNA Technical Manager must be documented and included in the case file.

DNA Profile description ($x > y$)	Scenarios	Interpretation threshold (Major)	Interpretation threshold (Minor)
2 person profile with 2 peaks $O_A = x$ rfu $O_B = y$ rfu	S_1 : Major = [A], minor = [B] S_2 : Major = [B], minor = [A]	$x:y = 2:1 - 692$ rfu $x:y = 3:1 - 312$ rfu $x:y = 4:1 - n/a$ $x:y = 5:1 - n/a$	The minor is the smaller detected peak if the taller exceeds the threshold on the left
2 person profile with 1 peak $O_A = x$ rfu	S_1 : Major = [A], minor = [A] or [Q] S_2 : Major = [A] or [Q], minor = [A]	$x:y = 1:1 - 640$ rfu $x:y = 2:1 - 480$ rfu $x:y = 3:1 - 414$ rfu $x:y = 4:1 - 388$ rfu $x:y = 5:1 - 372$ rfu $x:y = 7:1 - 355$ rfu	$x:y = 1:1 - 640$ rfu $x:y = 2:1 - 720$ rfu $x:y = 3:1 - 1050$ rfu $x:y = 4:1 - 1400$ rfu $x:y = 5:1 - 1750$ rfu $x:y = 7:1 - 2380$ rfu $x:y = 10:1 - 3060$ rfu

FIGURE 1 - MINIMUM THRESHOLDS FOR INTERPRETATION OF SINGLE SOURCE COMPONENTS FROM 2-PERSON MIXTURES
 (O_B = OBSERVED PEAK A, O_B = OBSERVED PEAK B)

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DNA Profile description (x > y)	Scenarios	Interpretation threshold (Major)
3 person profile with 3 peaks $O_A = x$ rfu $O_B = y$ rfu $O_C = y$ rfu	S_1 : Major = [A], minors = [B] and [C] S_2 : Major = [B] or [C], minors = [A] and [C] or [B]	$x:y = 2:1:1 - 389$ rfu $x:y = 3:1:1 - 251$ rfu $x:y = 4:1:1 - n/a$ $x:y = 5:1:1 - n/a$
3 person profile with 2 peaks $O_A = x$ rfu $O_B = y$ rfu	S_1 : Major = [A], minors = [A] and/or [B] and/or [Q] S_2 : Major = [B] or [Q], minors = [A] and/or [Q] and/or [B]	$x:y = 2:1:1 - 3719$ rfu $x:y = 3:1:1 - 555$ rfu $x:y = 4:1:1 - 314$ rfu $x:y = 5:1:1 - 256$ rfu
3 person profile with 1 peak $O_A = x$ rfu	S_1 : Major = [A], minors = [A] and/or [Q] S_2 : Major = [Q], minors = [A] and/or [Q]	$x:y = 1:1:1 - 824$ rfu $x:y = 2:1:1 - 515$ rfu $x:y = 3:1:1 - 407$ rfu $x:y = 4:1:1 - 355$ rfu $x:y = 5:1:1 - 325$ rfu $x:y = 7:1:1 - 290$ rfu $x:y = 10:1:1 - 259$ rfu

FIGURE 2 - MINIMUM THRESHOLDS FOR INTERPRETATION OF SINGLE SOURCE COMPONENTS FROM 3 PERSON MIXTURES
 (O_A = OBSERVED PEAK A, O_B = OBSERVED PEAK B, O_C = OBSERVED PEAK C)

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 (i.e., 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 the 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 be 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

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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 the results for the database count will be reported as a match probability.

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 US YSTR database have a subset of the Yfiler™ Plus 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 used when reporting 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 more common than the

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population frequency, 5% would be less common. All the databases described below provide the 95% upper confidence interval.

4.1. Combining autosomal and Y-STR match probabilities

It is acceptable to combine autosomal and Y-STR match probabilities under the assumption of negligible dependencies between the loci. The match probabilities of autosomal and Y-STR results may only be combined when the same assumptions are used to derive the match probabilities and that the results from consistent ethnicities are used in conjunction with each other.

When autosomal match probabilities are to be combined with Y-STR haplotype match probabilities, the calculations should be performed for each of the main ethnic groups reported for autosomal STR testing. Only the lowest of the combined results needs to be included in the report.

4.2. Available Haplotype Databases

Y-STR haplotype frequencies can be calculated from a number of established databases. The laboratory has incorporated the CADOJY-Mix Tool v3.0.3 for the calculation of Y-STR haplotype frequencies. This haplotype calculator can be found on the forensic biology computer network:

<H:\Worksheets\Statistical Tool\CADOJY-Mix Tool v3.0.3>

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 4.1 of the U.S. Y-STR Database.

In addition, The US Y-STR Database website allows for the calculation of single source Y-STR haplotypes. Release 4.2 of the website contains over 35,660 haplotypes contributed from numerous sources from around the United States:

www.usystrdatabase.org

To search for a haplotype, enter the alleles of interest for each marker by using the drop-down menu. To specify a value not listed in the drop-down menu, use the corresponding text boxes. In addition to supplying the profile probability (population frequency estimate using the 95% upper confidence limit), this database also provides the match probability estimates in the form of a likelihood ratio estimate, but only for the subsets of the Yfiler™ Plus loci that are contained within the other common testing kits (Yfiler, PowerPlex Y, and PowerPlex Y23). The match probability also uses the 95% upper confidence limit for the estimates of the haplotype probability. Match probability estimates of haplotypes may be combined with match probabilities for autosomal DNA markers (see section 4.1).

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Numerous other Y-STR haplotype databases are available on-line and their use is at the discretion of the analyst. The Y-chromosome Haplotype Reference Database managed by Lutz Roewer is a powerful 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:

www.yhrd.org

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 whether a mixture of DNA was obtained, and if a mixture was obtained, the minimum number of contributors should be stated. 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 should 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.

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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 or major Y-STR haplotype

Male specific Y-STR testing of the evidence indicated the presence of DNA from a single male or male lineage. John Doe, and any paternally related male, cannot be excluded as a possible source of the male DNA from this sample. The number of times the Y-STR DNA types from the evidence were observed in the ... Database (website of appropriate database) is:

5.2.3 Example of an inclusion to an apparent single source or major Y-STR haplotype (match probability)

Male specific Y-STR testing of the evidence indicated the presence of DNA from a single male or male lineage. John Doe, and any paternally related male, cannot be excluded as a possible source of the male DNA from this sample. The match probability is estimated to be X, which means, assuming a single haplotype, the DNA match is at least Y times more likely to occur if John Doe (or a patrilineal relative) is the contributor than if the source of the evidence results is a randomly selected individual.

5.2.4 Example of an inclusion to a Y-STR mixture

During male specific Y-STR analysis of the evidence, a mixture of DNA from at least X males was detected. John Doe, and any paternally related male, cannot be excluded as a possible (major/minor) contributor to the mixture of male DNA from this sample. The approximate number of times the possible combinations of Y-STR haplotypes from this mixture were observed in the ... Database (website of appropriate database) is:

5.2.5 Example of an exclusion to an apparent single source or major Y-STR haplotype

Male specific Y-STR testing of the evidence indicated the presence of DNA from a single male or male lineage. John Doe is excluded as the source of the haplotype detected.

5.2.6 Example of an exclusion to a Y-STR mixture

During male specific Y-STR analysis of the evidence, a mixture of DNA from at least X males was detected. John Doe is excluded as a contributor to this Y-STR mixture.

5.2.7 Example of a Y-STR sample that is unsuitable for comparison

Insufficient information was obtained during male specific Y-STR analysis of the evidence. This sample is unsuitable for comparisons.

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REFERENCES:

1. Yfiler™ Plus PCR Amplification Kit User's Manual
2. SDPD AmpF/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
7. Duncan Taylor, Jo-Anne Bright, John Buckleton, Using Probabilistic Theory to Develop Interpretation Guidelines for Y-STR Profiles, Forensic Science International: Genetics <http://dx.doi.org/10.1016/j.fsigen.2015.11.010>
8. Oskar Hansson, Peter Gill, Thorn Egeland, STR-validator: an open source platform for validation and process control, Forensic Science International: Genetics (2014) 13:154-166 <https://doi.org.10-1016/j.fsigen.2014.07.09>
9. Bright J, Turkington Jnana, Buckleton J. Examination of the variability in mixed DNA profile parameters for the Identifiler multiplex. Forensic Science International: Genetics 4: 111-114.

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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).

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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/uninterpretable: an interpretation or conclusion in which the DNA typing results are insufficient, as defined by the laboratory, for comparison purposes.

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").

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.

Mixture: a DNA typing result originating from two or more individuals.

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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 due, for example, 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.

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.

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Appendix B: List of common abbreviations used in the forensic biology unit.

+	Positive	PTO	Supervising Criminalist Patrick O'Donnell
⊖	Negative	RMS	Reference Mouth Swabs
~	Approximately	RMSCK	Reference Mouth Swab Collection Kit
ALS	Alternate light source	RB	Reagent blank
AP	Acid Phosphatase	R/T or RT	Room Temperature
B/C or BC	Barcode	S or SP	Sperm fraction
BPB	Brown paper bag	SAO	Same as original
BP	Base pair(s)	SART	Sexual Assault Response Team kit
BPS	Brown paper sack	SAEK	Sexual Assault Evidence Kit
CPT	Clear plastic tube	SDPD	San Diego Police Department
CODIS	Combined DNA Index System	SDSD/SDSO	San Diego Sheriff Department
Crim	Criminalist	TMTC	Too many to count.
CSS	Crime Scene Specialist	T/S or TS	Tape sealed; sealed with evidence tape, dated and initialed
CSU	Crime Scene Unit	Undet.	Undetermined (no quantifiable DNA present)
DEIF	Deionized formamide	WBC	White blood cells
Det.	Detective		
DDA	Deputy District Attorney		
e-gram/epg	Electropherogram		
FB	Forensic Biology		
Form	Formamide		
HemaTrace	Test for the confirmation of blood		
ILS	Internal lane standard (see also ISS)		
Inc #	Incident Number		
ISS	Internal size standard (see also ILS)		
KDB	Supervising Criminalist Kristin Beyers		
LMG	Leuco Malachite Green		
MRSCK	Mouth Reference Swab Collection Kit		
N/A	Not analyzed or not applicable		
ND	Not detected		
NE	Nucleated epithelial cells		
Neg	Negative		
NS	Non-sperm fraction		
p30	seminal protein p30 (Prostate Specific Antigen or PSA)		
Pheno	Phenolphthalin		
P/R or PR	Property Room		
P/T or PT	Property Tag		