



Idaho State Police Forensic Services

BIOLOGY/DNA CASEWORK ANALYTICAL METHODS

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Revision History

Revision #	Description of Changes
1	Original Issue in new template
2	Removed DNA Packets (BI-102), added EZ1 Advanced XL, replaced PP16HS with Fusion, removed source attribution, clarifications and clerical errors
3	Updated Brentamine working stock preparation, added option of digital imaging software other than Photoshop, removed Chelex extraction, added kinship statistics in PopStats, clarifications and clerical errors
4	Added Y-screen (BI-132), updated for ILIMS DNA module, clarifications and clerical errors
5	Added microscopic examination on penile swabs, added EZ1 Advanced XL 40 μ l elution volume, added DNA SpeedVac, clarified negative amplification control conditions equal to positive amplification control, clarified manual interpretation procedure, clerical errors
6	Added Y-STRs (BI-214), updated Y-screen, updated manual interpretation procedure, updated off-ladder allele nomenclature for STRmix, added LR reporting requirement for inconclusive STRmix comparisons
7	Removed Creatinine (BI-128), updated manual interpretation procedure, removed extraction control requirement, removed micro exam on penile swabs, updated to reflect batch processing and forwarding cuttings for DNA
8	Added reinterpretation of legacy data
9	Clarified legacy data reinterpretation to include technical review, added comment regarding STRmix minimum allele frequency
10	Ability to repurify extracts if needed, updated hair and sperm photo/verification requirements, removed closed microamp negative tube requirement, updated BI-212 background/references section, administrative updates
11	Updated driftcon section to reflect new electronic QC process.
12	Added ability to use male negative orifice swabs as victim references after y-screen, updated traditional screening for kits request requirements, removed cutting from tip statement from y-screen section, added option to add DTT to non-differential samples if needed, removed QIAcube procedure, added ability to increase run time if needed, added clarification regarding rejected data at CE, added guidance for unresolved peaks, updated EC/SP in sample names section to

	reflect F1/F2 update, clarified the requirements for manual exclusions, updated YSTR wording
13	Updated manual throughout for Fusion 6C, 3500 genetic analyzer, and STRmix v 2.9 implementation in casework, added direct amplification procedure for references, updated hair photo requirements, added whole swab y-screen procedure
14	Updates for 7500 SDS v2.3 and Plexor Analysis Software v1.6
15	Added additional Fusion 6C interpretation guidance, removed tube brand specifications, updated wording for Y23

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Examination of Bloodstained Evidence (MBI-100)

1.0 Background/References

1.1 Examination of items of evidence for the presence and identification of human blood is routinely performed in Forensic Biology using visual examination, presumptive screening and confirmatory testing for identification of blood and determination of the species of origin.

1.2 References

1.2.1 Forensic Science Handbook, Chapter 7: Identification and Grouping of Bloodstains, pp.267-337, Prentice-Hall, 1982.

1.2.2 Sourcebook in Forensic Serology, Immunology and Biochemistry U.S. Department of Justice, NIJ, 1983 p. 73-133.

1.2.3 Cox, M. A Study of the Sensitivity and Specificity of Four Presumptive Tests for Blood. Journal of Forensic Sciences, September 1991; 36(5): 1503-1511.

2.0 Scope

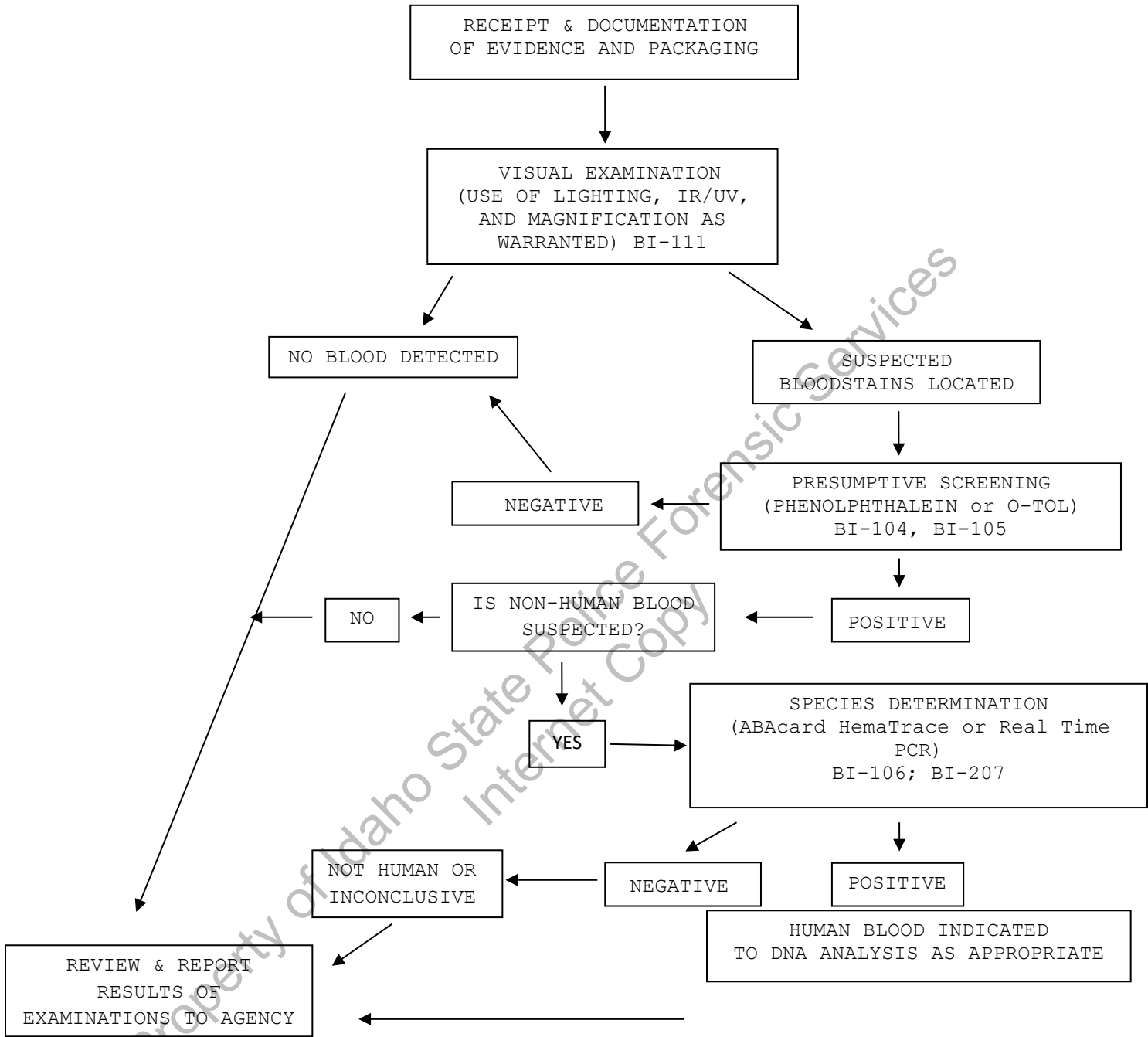
2.1 To provide uniform processing of evidentiary material for the presence of blood.

3.0 Equipment/Reagents

3.1 Various lighting conditions, including IR, and magnification may be used in general evidence examination to enhance the observation of blood. Reagents for blood detection and identification are listed in the appropriate processing protocols.

4.0 Procedure

4.1 See Flow Chart on following page.



5.0 Comments

5.1 In determination of species, the amount and condition of the stain should be considered in reporting a negative determination.

5.2 Discretion should be used in testing small and or poor condition samples for species determination if DNA testing is necessary.

Examination of Evidence for Semen (MBI-102)

1.0 Background/References

1.1 Examination of items of evidence for the presence and identification of human semen is routinely performed in Forensic Biology using visual examination, presumptive screening and confirmatory testing for identification.

1.2 References

1.2.1 Sourcebook in Forensic Serology, Immunology and Biochemistry U.S. Department of Justice, NIJ, 1983 p.149-181.

2.0 Scope

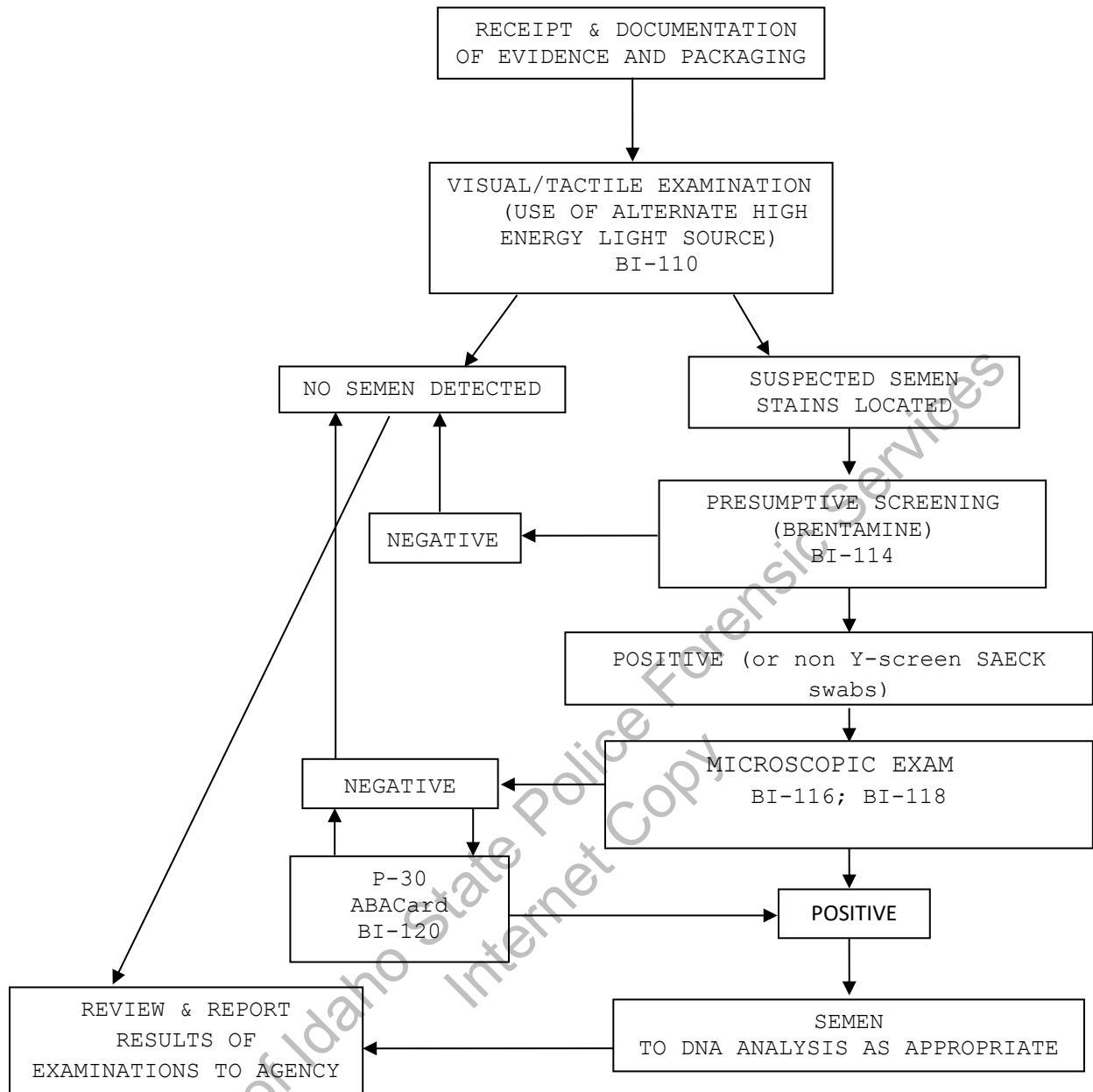
2.1 To provide uniform processing of evidentiary material for the presence of semen.

3.0 Equipment/Reagents

3.1 Normal room lighting conditions and the use of an alternate light source to view fluorescence emitted from semen stains. Reagents for semen detection and identification are listed in the appropriate processing protocols.

4.0 Procedure

4.1 See Flow Chart on following page.



5.0 Comments

- 5.1 When examining pants/panties, a presumptive AP screening will always be performed on crotches (even in absence of visual cue).
- 5.2 A P-30 test need not be performed on item(s) which yielded a positive microscopic exam.
- 5.3 The presence of spermatozoa is confirmatory for semen; however, the absence of spermatozoa without additional testing is insufficient to conclude that no semen was detected and the report will reflect this limitation by only making reference to the absence of spermatozoa.

Examination of Evidence for Body Fluids (MBI-104)

1.0 Background/References

1.1 Examination of items of evidence for the presence of body fluids and substances other than blood or semen is sometimes requested and several methods are available to detect the presence of saliva, urine and feces.

1.2 References

1.2.1 Sourcebook in Forensic Serology, Immunology and Biochemistry U.S. Department of Justice, NIJ, 1983 pp. 197-198; 183-189; 191-195.

2.0 Scope

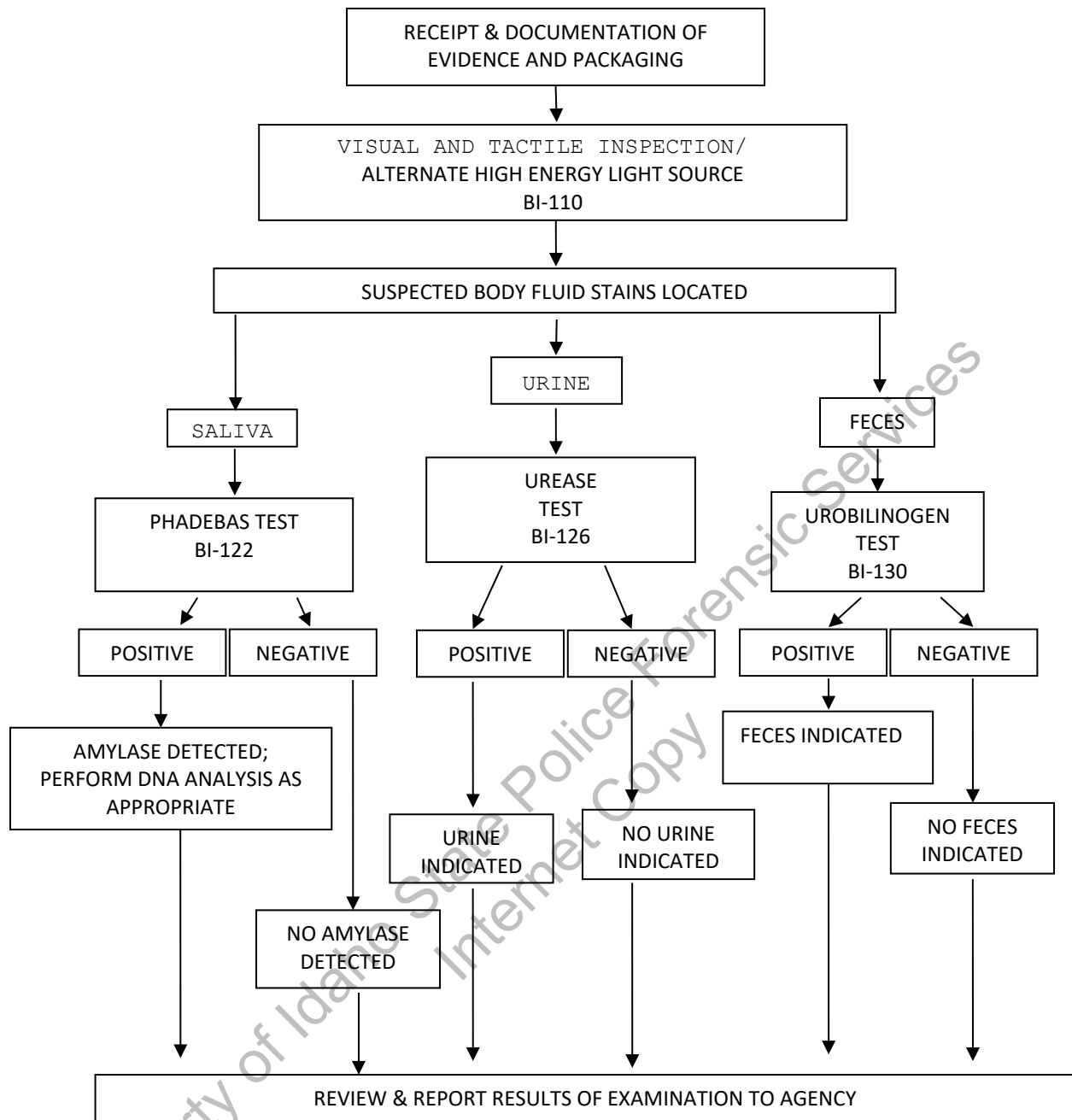
2.1 To provide uniform processing of evidentiary material for the presence of saliva, urine or feces.

3.0 Equipment/Reagents

3.1 Normal room lighting conditions and the use of an alternate light source to view fluorescence and assist in the localization of possible body fluid stains. Reagents for analysis of the detected substances are listed in the appropriate processing protocols.

4.0 Procedure

4.1 See Flow Chart on following page.



5.0 Comments

5.1 Generally, feces samples and urine stains are not processed for DNA. However, exceptions may be made in instances where the sample represents the only probative evidence.

5.2 Sample size, and the significance of indicating saliva as the DNA source, should be considered before consuming sample for amylase testing.

Individualization of DNA Sources by STR Analysis (MBI-200)

1.0 Background/References

1.1 Once a DNA source has been detected and identified as to 'source type' where applicable and feasible, it is often important to attribute the DNA sample to a particular individual inasmuch as possible. Current DNA technology, in the analysis of STR loci, offers individualization potential. However, the individualization of a particular sample occurs through a comparative process. This process requires a DNA profile from a 'known' sample to which the evidence sample profile can be evaluated. DNA analysis will only be performed when all necessary 'known' or 'reference' samples, for the given case, have been submitted to the laboratory unless extenuating circumstances prohibit submission of such samples.

Although the analysis of STR loci offers individualization potential, analysis may not be necessary or performed on every case and/or sample submitted to the laboratory. DNA analysis will only be performed on cases and/or individual samples, which have the potential for resolving a probative and forensically significant question/issue regarding the given case, unless analysis is mandated by statute or court order. If the analysis of a sample resolves a given question, additional samples submitted for the resolution of the same question within the case will likely not be analyzed. Items for which DNA testing has already been performed will not be retested, except in the instance where new technology has become available and has the potential to resolve a question that could not be answered with the original technology. Additionally, DNA testing may establish identity, but does not establish timeframe or consent.

2.0 Scope

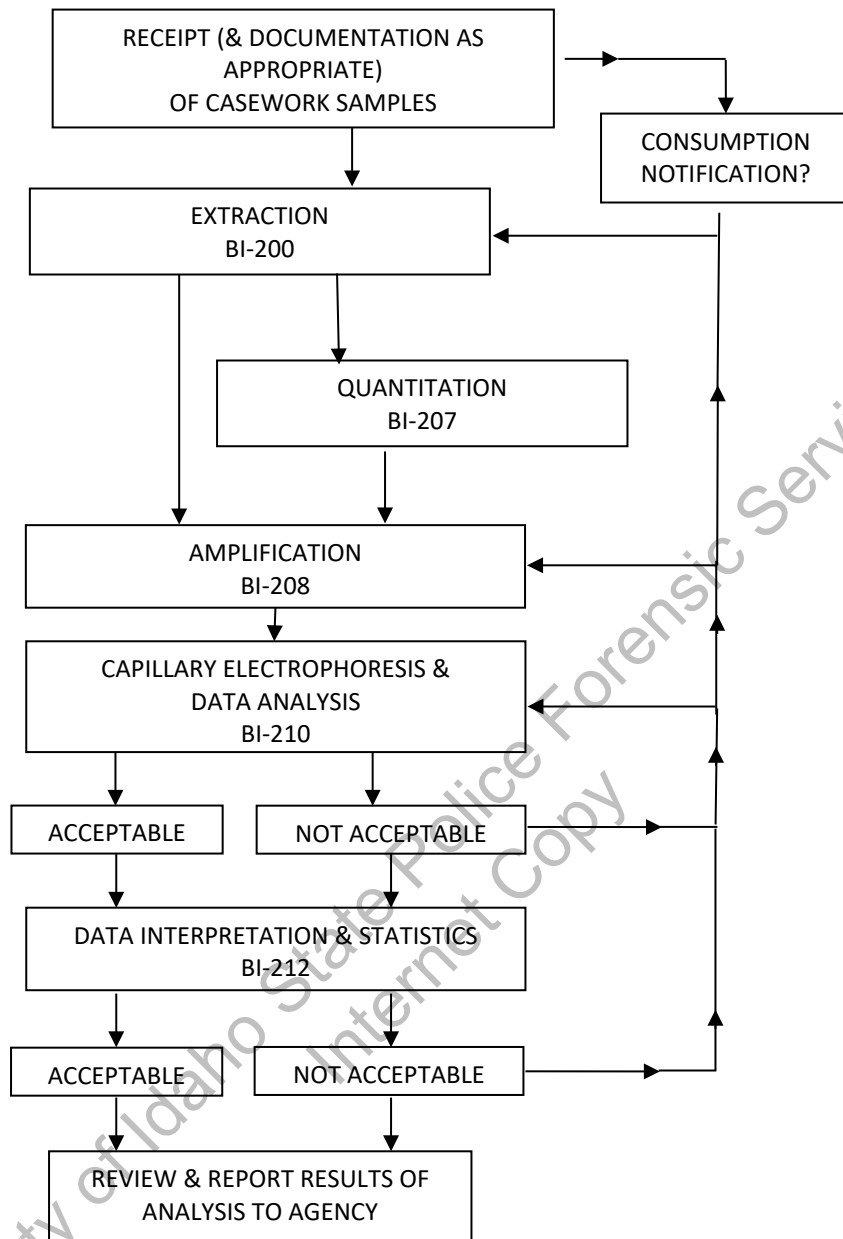
2.1 To provide uniform processing of DNA samples to achieve high quality data and consistent interpretation.

3.0 Equipment/Reagents

3.1 As listed in individual analytical procedures.

4.0 Procedure

4.1 See Flow Chart on following page.



5.0 Comments

5.1 Careful scrutiny at each step will ensure insufficiencies are identified, and compensated for where feasible, at the earliest possible point (see BI-210 for specifics).

Processing Liquid Blood (BI-100)

1.0 Background/References

1.1 Known reference standards in forensic casework may be received in the form of liquid blood, generally in a lavender top (EDTA) tube. The EDTA acts as a preservative for the DNA (even up to several years post-collection); however, if left in a liquid state for prolonged periods of time (especially post-mortem samples), these samples are more susceptible to degradation, potentially resulting in the loss of DNA. These liquid samples should be stored refrigerated to aid in their preservation until which time a bloodstain can be prepared. Bloodstains stored in a dry state, even at room temperature, may be suitable for DNA testing for many years. Bloodstains are to be prepared as soon as feasible following sample receipt (generally at the time of evidence analysis). However, if evidence processing is to be delayed beyond 2 months, it is recommended that any post-mortem blood samples associated with the case are checked out and bloodstains made for preservation.

2.0 Scope

2.1 To provide a method for the creation of stable DNA samples from blood.

3.0 Equipment/Reagents

- Blood Stain Card(s) (such as Whatman® non-FTA)
- Envelopes
- Disposable Transfer pipet or mechanical pipette with filter barrier tip

4.0 Procedure

4.1 Label stain card with a minimum of case number, item number, date and initials. Subject name may also be placed on the card for identification purposes.

4.2 Label blood sample tube with case number, item number, date, initials, and blood level. Mix the tube thoroughly by inversion.

4.3 Remove cap from blood tube and draw blood into pipet. Carefully spot, at minimum, 1 drop (~50µl) blood onto each circle.

4.4 Allow bloodstain card to air-dry completely before packaging.

4.5 Place the dried stain card into an envelope (~6½" x 9½"). Seal envelope with evidence tape on flap and label with initials and date across seal. The stain card will be sampled and given a sub-item number in ILIMS. An ILIMS barcode will be printed and placed on the envelope containing the bloodstains.

NOTE: bloodstains associated with a sexual assault evidence collection kit will be placed back in the kit without an ILIMS barcode.

5.0 Comments

5.1 Exercise caution and wear appropriate protective gear when preparing bloodstains (e.g., gloves, lab coat, protective eyewear).

5.2 Bloodstains are to be prepared either in the hood with the sash at the appropriate level, or at a workbench while wearing a disposable face shield.

5.3 Only one blood sample source should be open at a time. When processing multiple samples, close one tube before opening another and make sure stains are placed sufficiently far away from a card being processed to avoid cross-contamination.

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Phenolphthalein Test for Blood (BI-104)

1.0 Background/References

1.1 Most screening tests for blood depend on the catalytic action of the heme group. To minimize false positives, the test is frequently performed as a multi-step test. A good overview is found in the first reference.

1.2 References

1.2.1 Gaensslen, R. Sourcebook in Forensic Serology, Immunology, and Biochemistry. (1983) U.S. Dept. of Justice, Washington, D.C., p. 101-105.

1.2.2 Higaki, R.S. and Philp, W.M.S. A Study of the Sensitivity, Stability and Specificity of Phenolphthalein as an Indicator Test for Blood, (1976) Canadian Journal of Forensic Science, Vol 9, No.3, p.97-102.

2.0 Scope

2.1 To provide a method for the localization and presumptive identification of bloodstains.

3.0 Equipment/Reagents

- Phenolphthalein Working Solution
- 3% Hydrogen Peroxide
- Sterile/Nanopure H₂O
- Cotton Swabs or Filter Paper

4.0 Procedure

4.1 Positive (known bloodstain) and negative control samples are processed, prior to testing any forensic samples (on the day of testing), to ensure the working solution reagents are functioning properly.

4.2 Cotton swabs or a folded piece of filter paper are used to collect the suspected blood onto the tip. A swab may be moistened with sterile/nanopure H₂O if necessary.

4.3 To the swab or filter paper with the suspected blood, add 1-2 drops of phenolphthalein working solution. Wait 10-15 seconds to detect potential false positives.

4.4 Add 1-2 drops of 3% H₂O₂ and note appearance or absence of bright pink color. Color reaction should occur rapidly (≤ 1 minute).

4.5 Document result in case notes. Record positive (+), as indicated by the development of the above color change, or negative (-) as indicated by the absence of the color change. Analyst may use other descriptive word(s) as well (e.g., strong, weak, slow, etc.).

5.0 Comments

5.1 Direct testing of a small cutting/sample may also be performed.

5.2 Color changes occurring prior to the addition of 3% H₂O₂ are generally considered inconclusive.

5.3 Color changes occurring after 1 min. are generally considered negative.

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O-Tolidine Test for Blood (BI-105)

1.0 Background/References

1.1 Most screening tests for blood depend on the catalytic action of the heme group. To minimize false positives, the test is frequently performed as a multi-step test. A good overview is found in the first reference.

1.2 References

1.2.1 Gaensslen, R. Sourcebook in Forensic Serology, Immunology, and Biochemistry. (1983) U.S. Dept. of Justice, Washington, D.C., p. 101-105.

1.2.2 Cox, M., (1991) "A Study of the Sensitivity and Specificity of Four Presumptive Tests for Blood," Journal of Forensic Sciences, Vol. 36, No. 5, p. 1503-1511.

1.2.3 Culliford, BJ and Nicholl, LC (1964) "The Benzidine Test: A Critical Review", Journal of Forensic Sciences, 9:175-191.

2.0 Scope

2.1 To provide a method for the localization and presumptive identification of bloodstains.

3.0 Equipment/Reagents

- 0.3% Ortho-Tolidine Stock
- 3% Hydrogen Peroxide
- Sterile/Nanopure H₂O
- Cotton Swabs or Filter Paper

4.0 Procedure

4.1 Positive (known bloodstain) and negative control samples are processed, prior to testing any forensic samples (on the day of testing), to ensure the working stock reagents are functioning properly.

4.2 Cotton swabs or a folded piece of filter paper are used to collect the suspected blood onto the tip. A swab may be moistened with sterile/nanopure H₂O if necessary.

4.3 To the swab or filter paper with the suspected blood, add 1-2 drops of o-tolidine working solution. Wait 10-15 seconds to detect potential false positives.

4.4 Add 1-2 drops of 3% H₂O₂ and note appearance or absence of blue-green color. Color reaction should occur rapidly (≤ 1 minute).

4.5 Document result in case notes. Record positive (+) as indicated by the development of the above color change, or negative (-) as indicated by the absence of the color change. Analyst may use other descriptive word(s) as well (e.g., strong, weak, slow, etc.).

5.0 Comments

- 5.1 Direct testing of a small cutting/sample may also be performed.
- 5.2 Color changes occurring prior to the addition of 3% H₂O₂ are generally considered inconclusive.
- 5.3 Color changes occurring after 1 min. are generally considered negative.
- 5.4 O-tolidine is designated as a potential carcinogen and should be used with caution.

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Human Blood Identification Using ABACard® Hematrace® Test (BI-106)

1.0 Background/References

1.1 Items of evidence with unknown sources of blood are often submitted in forensic casework and it is useful to be able to determine whether the blood is of human origin. The basis of the ABACard® Hematrace® test is the immunological detection of human hemoglobin.

1.2 References

1.2.1 ABACard® HemaTrace® for the Forensic Identification of Human Blood technical information sheet, Abacus Diagnostics Inc., Rev: 07/13.

2.0 Scope

2.1 To provide a uniform and reliable method for confirming the presence of blood on evidentiary material.

3.0 Equipment/Reagents

- OneStep ABACard® Hematrace® Test Kit

4.0 Procedure

4.1 Label an ABACard® Hematrace® test device for each sample, including controls.

4.2 Add samples to the buffer provided (generally ~2mm x 2mm stain cutting or ~1/16 to ~1/8 of cotton swabs) and allow them to extract at room temperature for 5-30 minutes (longer, if necessary for aged stains).

4.3 Apply ~150µl (4 drops with provided dropper) of a sample extract to the 'S' well of its corresponding test device and incubate at room temperature for ≤ 10 minutes.

4.4 A positive result is indicated by the appearance (within 10 minutes) of a pink line in both the control 'C' and test 'T' areas. A negative result is indicated by the absence of a pink line (after 10 minutes) in the 'T' area of a test device. Results are inconclusive anytime a pink line fails to develop in the 'C' area.

5.0 Comments

5.1 Samples must be at room temperature for the test. If extracts have been stored in refrigerator/freezer, allow them to reach room temperature before proceeding.

5.2 Both positive (known human bloodstain) and negative (extraction buffer alone) controls are used.

5.3 Since the reaction time is dependent on hemoglobin concentration, as well as other sample-specific factors, it is necessary to wait the full 10-minute incubation before reporting a negative result. However, a positive reaction may occur in much less time.

- 5.4 As with any antigen-antibody reaction, false negatives (as the result of a "high dose hook effect") may be produced with concentrated samples. When negative results are obtained with very 'heavy' stains, the sample should be further diluted and the test repeated.
- 5.5 Other reagents may be used for extraction. For example, 3-5% Ammonia Hydroxide (aged stains), saline, 1XPBS or PCR-TE. The volume used for extraction may be reduced for sample conservation or dilute stains (e.g., 150µl).
- 5.6 Although most nonhuman species tested do not produce a positive result with the ABACard® Hematrace® test, some cross reactivity has been reported (e.g., other primates, weasel, ferret, skunk). Therefore, when reporting results, the statement 'indicated the presence of human blood' should be used, rather than 'detected' or 'identified'. In instances where species cross reactivity may be plausible, a statement indicating that 'members of the mustelidae family cannot be excluded' may also be used in the report.

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Biological Screening: Use of Alternate Light Source (ALS) (BI-110)

1.0 Background/References

1.1 There are numerous forensic applications for the use of alternate lighting. In forensic biology, it is generally used to aid in the visualization of physiological fluids and trace evidence such as fibers.

2.0 Scope

2.1 To provide a method for enhancing visualization/localization of physiological fluids and trace evidence (as necessary for preservation) on evidentiary items.

3.0 Equipment/Reagents

- Alternate Light Source
- Leeds LSV2 Spectral Vision ALS Imaging Tool
- Leeds Vision 4.0 Software
- Computer/Monitor
- Filtered Safety Goggles

4.0 Procedure

4.1 Selection of the wavelength of light for viewing will depend on the alternate light source used, its available outputs, and the substrate being viewed. A broadband source covering $\leq 530\text{nm}$ wavelengths is sufficient for biological examination but will not eliminate potential background fluorescence as well as the use of a discrete wavelength band. Optimum visualization of physiological fluids and fibers is typically achieved at $\sim 450\text{nm}$ and $\sim 485\text{nm}$, respectively; however, it is often necessary to test each of the wavelengths ($450\text{nm} - 540\text{nm}$) and choose the most appropriate based on the background fluorescence encountered with the particular substrate. The following table illustrates the appropriate safety goggles to be used with various source outputs.

<u>WAVELENGTHS</u>	<u>SAFETY GOGGLES</u>
< 400 (UV)	Yellow/UV safe
< 530 broadband	Orange
400-450 discrete	Yellow
450-540 discrete	Orange
540-700 discrete	Red
700-1100 discrete >700 broadband	Red or IR safe

- 4.2 Follow manufacturer's operating instructions for specific details on equipment operation.
- 4.3 Examine evidence under optimum discrete wavelengths where possible and under appropriate broadband output when discrete wavelengths are not available.
- 4.4 Mark any fluorescent areas/potential biological stains, as appropriate and necessary, for ease of location under normal room lighting conditions.
- 4.5 Proceed to appropriate screening procedure(s) for any possible biological stains located (see BI-114; BI-122; BI-126; BI-128)

5.0 Comments

- 5.1 Failure to use safety goggles, or use of incorrect goggles could result in permanent eye damage. Avoid looking into the wand or shining on other individuals. Read any manufacturer's safety guidelines provided with the equipment.
- 5.2 Ultraviolet light may cause burns, so caution should be exercised to avoid direct and/or prolonged exposure to unprotected skin. Read any manufacturer's safety guidelines provided with the equipment.
- 5.3 The alternate light source equipment can generate heat and potentially cause burns to skin and other materials. Read any manufacturer's safety guidelines provided with the equipment.
- 5.4 Stains may be documented by still photos or using the Leeds imaging tool.
- 5.5 Photographs captured with the Leeds imaging tool may be annotated through the Leeds Vision 4.0 software.

Biological Screening: Use of Infra Red and UV Lighting (BI-111)

1.0 Background/References

1.1 In forensic biology, IR and UV lighting/photography may be used to aid in the visualization of physiological fluids, typically blood, on dark substrates that would normally make it difficult to see the suspected stain.

2.0 Scope

2.1 To provide a method for enhancing visualization/localization of bloodstains on evidentiary items.

3.0 Equipment/Reagents

- Leeds LSV2 Spectral Vision ALS Imaging Tool
- Leeds Vision 4.0 Software
- Computer/Monitor
- Sony 'Night Shot' video camera

4.0 Procedure

4.1 Follow manufacturer's operating instructions for specific details on equipment operation.

4.2 Examine evidence using the 'Infrared', 'Blood Absorption', or 'Blood Absorption 2' settings on the Leeds system or the 'Night Shot' setting on the Sony video camera. Stains will typically appear dark against a lighter background, under appropriate settings, when observed through the Leeds software or Sony camera viewfinder.

4.3 Mark any potential bloodstains, as appropriate and necessary, for ease of location under normal room lighting conditions.

4.4 Proceed to appropriate screening procedure(s) for any possible bloodstains located (see BI-104; BI-105)

5.0 Comments

5.1 Stains may be documented by still photos, using the Leeds imaging tool, or using the Sony video camera.

5.2 Photographs captured with the Leeds imaging tool may be annotated through the Leeds Vision 4.0 software.

5.3 While the 'Infrared' setting of the Leeds system typically provides sufficient contrast for suspected bloodstains, it may be necessary to examine items using each of the approved settings to determine which will provide the greatest contrast and visualization.

Brentamine Test for Acid Phosphatase (BI-114)

1.0 Background/References

1.1 Acid phosphatase is an enzyme found in elevated amounts in human semen, independent of the presence of spermatozoa. Various tests have been used for its detection. Though none of these tests are prostate-specific, at the limits of their detection, they are a good indicator of the presence of semen.

1.2 References

1.2.1 Gaensslen, R. Sourcebook in Forensic Serology, Immunology, and Biochemistry. (1983) U.S. Dept. of Justice, Washington, D.C., p 155-166.

1.2.2 Biology Methods Manual, Metropolitan Police Forensic Science Laboratory, p.3-16 through 3-19.

2.0 Scope

2.1 To provide a method to aid in location and presumptively indicate the presence of semen stains.

3.0 Equipment/Reagents

- Brentamine Solution A
- Brentamine Solution B
- Sterile/Nanopure H₂O
- Cotton Swabs or Filter Paper

4.0 Procedure

4.1 Prepare Brentamine Working Solution: Mix 500µl solution A and one drop solution B with 4.5ml of water. This solution should be prepared fresh each day it is used and should be stored at 4°C when not in use.

4.2 Positive (known semen stain) and negative (moistened swab or filter paper) control samples are processed, prior to testing any forensic samples (on the day of testing), to ensure the working solution is functioning properly.

4.3 Lightly rub a suspected semen stain with a pre-moistened cotton swab or press a moistened piece of filter paper against the stain.

4.4 Add Brentamine Working Solution to the swab or filter paper and observe for the appearance or absence of a pink to purple color change.

4.5 To avoid false positives, the results should be recorded as positive(+), as indicated by the development of the above color change, or negative(-), as indicated by the absence of the color change, within 1 minute of the addition of the Brentamine Reagent. Additional comments (e.g., strong, weak, slow, etc.) may also be helpful to record.

5.0 Comments

- 5.1 Positive reactions, though generally weak, may be obtained on anal/rectal and some vaginal swabs in absence of any semen.
- 5.2 The test may also be performed using 10-20 μ l of a sample extract or directly onto a small cutting.
- 5.3 This test may also be used for mapping large, possible semen stains via a moistened paper transfer method. A sheet(s) of moistened filter paper is pressed against the item of evidence. Marks are made on the paper to indicate the edges of the evidence for orientation of any subsequent color reaction. The paper has Brentamine Working Solution applied and analyzed as above.
- 5.4 Fast Blue B is a possible carcinogen and should be handled cautiously.

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Sample Extraction for Semen Identification (BI-116)

1.0 Background/References

1.1 The identification of semen is a multi-step process for which it is necessary to generate extracts of putative semen stains for use in the identification tests.

2.0 Scope

2.1 To provide a method of generating suitable extracts from evidentiary material for the performance of both presumptive (as needed) and confirmatory testing for the presence of semen, as well as other forensic analyses.

3.0 Equipment/Reagents

- Small (e.g., 12x75mm) tubes or 1.5/0.5ml microfuge tubes
- Pipette(s) and filter barrier tips
- Sonicator
- Centrifuge

4.0 Procedure

4.1 Label tubes with identifying information.

4.2 Take a sample (~2-5 mm² portion of stain or ~1/8 each of one or two cotton swabs), transfer to the appropriately labeled tube and extract in a minimal volume (50-100µl) of dH₂O at RT for ≥ 20 minutes.

4.3 At this point, agitation, vortexing, brief sonication and/or piggyback centrifugation may be used to assist in removing sperm/cellular material from the substrate.

4.4 Mix/resuspend the sample for use in microscopic examination (BI-118) and/or p30 detection (BI-120). Alternatively, the supernatant may be removed, without disturbing the pellet, for additional testing [e.g. AP screening (BI-114), p30, etc.] prior to resuspension.

5.0 Comments

5.1 Other reagents may be substituted for dH₂O (e.g., 1XPBS, PCR-TE, saline) in 4.2.

5.2 The sample sizes and extraction volumes are those typically used and are recommendations. The scientist has the discretion to increase or decrease the sample size and corresponding extraction volume as case circumstances dictate.

5.3 While the primary use of this liquid extract is for semen identification testing, these extracts may be used for other screening tests as well (e.g., saliva, urine, feces).

5.4 The sample may optionally be extracted in dH₂O directly on the microscope slide at the analyst's discretion. However, the quantity of sperm observed may be diminished and no sample will remain for further testing (e.g. p30) when using this method.

5.5 Extracts should be stored at 4°C (short term) or -20°C (longer term) and are discarded upon completion of case review.

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Semen Identification: Microscopic Examination (BI-118)

1.0 Background/References

1.1 The visual identification of spermatozoa is a means of positively identifying human semen. Human sperm have a distinctive size and morphology and, with differential staining, such as the "Xmas Tree" method, can be readily identified.

1.2 References

1.2.1 Gaensslen, R. Sourcebook in Forensic Serology, Immunology, and Biochemistry. (1983) U.S. Dept. of Justice, Washington, D.C., pp. 150-152.

2.0 Scope

2.1 To provide a confirmatory test for the identification of semen in cases where spermatozoa are present.

3.0 Equipment/Reagents

- Xmas Tree Stain Solution A
- Xmas Tree Stain Solution B
- $\geq 95\%$ Ethanol
- Glass Microscope Slide(s)
- Cover Slip(s)
- Mounting Medium
- Heat Source
- Microscope (Magnification $\sim 200X-400X$)

4.0 Procedure

4.1 The sample extract is mixed well and $\sim 5-10\mu\text{l}$, or $\sim 10\%$ deposited on a microscope slide and allowed to dry (this process may be expedited by use of a slide warmer or oven at $\sim 37^\circ\text{C}$).

4.2 Heat-fix the sample extract to the slide by slowly passing over a flame (for example, alcohol lamp or Bunsen burner).

4.3 Cover the heat-fixed sample extract with Xmas Tree Stain Solution A and allow staining for ≥ 15 minutes at RT.

4.4 Remove the stain with a gentle stream of dH_2O and cover the stained area briefly ($\sim 15-20$ seconds) with Xmas Tree Stain Solution B. Remove this stain with a stream of EtOH (95% or Absolute).

4.5 Allow the slide to dry and apply mounting medium or dH_2O and a coverslip prior to microscopic examination.

4.6 Scan the slide on $\geq 200X$ magnification. Sperm heads will retain the red stain, while the tails, if present, will appear green. Use 400X magnification if necessary to verify sperm morphology.

4.7 Documentation in notes should include the following:

4.7.2 An estimate of the number of sperm seen per field (e.g., 12/slide; 0-1/200X; 3-5/200X; 5-10/200X; $>10/200X$; or 1+ - 4+ etc.). The scientist may also note the condition of the sperm seen (e.g. heads only, mostly heads, some intact, etc.).

4.7.3 The presence of any epithelial cells (e-cell) and their number (e.g., rare, occasional, few, moderate, many, or 1+ - 4+). The scientist may also note e-cell descriptions [e.g. nucleated (NEC or nuc.) or anucleated (ANEC or A-nuc.)] and whether or not there are large squamous epithelial cells present.

4.7.4 If the situation arises in which there are only one to three sperm heads, a single intact sperm, or a few sperm heads of questionable morphology, a photograph of at least one sperm shall be taken and included in the note packet (see BI-119).

4.7.5 For ease of re-location, the position of sperm in cases where 3 or less have been identified may be documented in the case notes.

4.7.6 It is also good, if possible, to note the presence of significant amounts of bacteria, yeast or white blood cells.

5.0 Comments

5.1 Stains purchased commercially have expiration dates, while those prepared 'in-house' are generally stable for ≈ 6 months at RT. After this period, stains should be discarded or checked with a positive (known sperm) slide before use.

Digital Imaging (BI-119)

1.0 Background/References

1.1 Digital photography is frequently used to document the presence of spermatozoa identified through microscopy, the general appearance of evidence items or individual stains, and/or to aid in the documentation of tests performed during analysis. Foray Technologies' Authenticated Digital Asset Management System (ADAMS) – Digital Workplace allows for images to be imported, stored, and printed for inclusion in the case file. Digital Workplace, in combination with Adobe Photoshop, tracks the image history, including any modifications (e.g. brightness/contrast/color balance adjustments, annotations) made. A variety of reports may be printed to include the selected image(s) and associated case/history information as appropriate.

1.2 References

1.2.1 ADAMS User Manual

1.2.2 Adobe Photoshop User Guide

2.0 Scope

2.1 To provide a means of printing digital images for the case file and storing the associated image files.

3.0 Equipment/Reagents

- Imaging Computer with Monitor
- ADAMS and Photoshop Software
- Microscope
- Digital Microscope Camera
- Digital Hand Held Camera

4.0 Procedure

4.1 IMAGE ACQUISITION

4.1.1 Login to the imaging computer and double click the Digital Workplace icon on the desktop.

4.1.2 Click File>Acquire>From Folder. Browse to the location of the image(s) and select those to be imported (note: images should be transferred from the camera to the computer and renamed with a descriptor such as item number, prior to importing). Select 'Open'.

4.1.3 Enter case information into the "Acquisition Info" dialogue box. Case numbers are to be entered as 'Case #'. Select 'OK'. The software will assign a unique identification number to each image, in addition to the imported file name.

4.2 IMAGE PROCESSING

4.2.1 Images may be viewed in various formats using the 'View' option on the toolbar. If applicable, image descriptions/notes may be added under the 'asset information' view.

4.2.2 If necessary, images may be processed using Photoshop. Processing may include adjustment of brightness, contrast, and/or color balance as well as cropping or adding annotations. Select the image(s) to be processed and click the Photoshop (Ps) icon on the toolbar.

4.2.3 Make the necessary adjustments in the Photoshop window and close when complete. Choose 'Ok' in the dialogue boxes that appear. The 'processed' image(s) will appear in the Digital Workplace view, in addition to the original image. The processing history may be viewed in the 'asset details report' or the 'asset information' view.

4.3 REPORT

4.3.1 Highlight image(s) to be printed, click File>Reports, select report type and click "Ok" in the printer dialogue box.

4.3.1.1 Select "Asset Detail Report" for an image with descriptions and notes. Choose the desired information to display in the "Asset Details Report Options" dialogue box.

4.3.1.2 Select "Contact Sheet" to display multiple images per page.

4.3.1.3 A single large image may also be printed directly by choosing File>Print.

4.3.1.4 Images/reports will either be printed to file or saved as a .pdf and attached to the case file in ILIMS.

4.3.2 Exit Digital Workplace and log off of the imaging computer when done.

5.0 Comments

5.1 Weekly system backups will be conducted by CJIS personnel.

5.2 Digital imaging software other than Photoshop may be used for image processing as long as the alternate software has been configured to run from within Digital Workplace. Image processing will occur through Digital Workplace so that the processing history is tracked.

Identification of Semen by P-30 Detection (ABAcard®) (BI-120)

1.0 Background/References

1.1 P-30 is a protein and its presence in semen is independent of the presence of spermatozoa. Immunological detection of p30 is commonly used as a confirmatory test for the presence of semen.

1.2 References

1.2.1 Sensabaugh, G. F. "Isolation and Characterization of a Semen-Specific Protein from Human Seminal Plasma: A Potential New Marker for Semen Identification." (1978) Journal of Forensic Sciences, 23(1): 106-115.

1.2.2 Spear, T. F. and Khoskebari, N. "The Evaluation of the ABAcard® p30 Test for the Identification of Semen." (2000) Crime Scene, 26(1): 9-12.

1.2.3 ABAcard® p30 Test for the Forensic Identification of Semen technical information sheet, Abacus Diagnostics Inc., Rev: 05/11.

2.0 Scope

2.1 This procedure is to be used as a confirmatory test for the presence of human semen in instances where a positive AP result was obtained but no spermatozoa were seen upon microscopic examination of the sample extract.

3.0 Equipment/Reagents

- OneStep ABAcard® p30 Test Kit
- Pipette(s) and filter barrier tips

4.0 Procedure

4.1 Label an ABAcard® p30 test device for each sample, including controls.

4.2 Add 10µl of each sample (see BI-116), positive control (known semen stain extract or Seri™ semen standard [10ng; 10µl of a 1:100 dilution]), and negative control (extraction buffer) to ~190µl (4 drops) of the extraction buffer provided. Mix thoroughly.

4.3 Transfer each extract (~200µl) to the 'S' well of the appropriately labeled test device and incubate at RT for 10 minutes.

4.4 A positive result is indicated by the appearance (within 10 minutes) of a pink line in both the control 'C' and test 'T' areas. A negative result is indicated by the absence of a pink line (after 10 minutes) in the 'T' area of a test device. Results are inconclusive anytime a pink line fails to develop in the 'C' area.

5.0 Comments

5.1 Samples must be at room temperature for the test.

- 5.2 Other reagents may be substituted for the extraction buffer (e.g., saline, 1XPBS, PCR-TE, dH₂O) in 4.2 if necessary.
- 5.3 Since the reaction time is dependent on p30 concentration, as well as other sample-specific factors, it is necessary to wait the full 10-minute incubation before reporting a negative result. However, a positive reaction may occur in much less time.
- 5.4 As with any antigen/antibody interaction, excess antigen may lead to a 'high dose hook effect' resulting in false negatives when the p30 concentration is very high. This effect should be considered when examination and presumptive tests have indicated the likelihood of the presence of semen. In those instances, the sample should be diluted and the test repeated.

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Amylase Test (Phadebas) (BI-122)

1.0 Background/References

1.1 Amylase is an enzyme that is present in high concentrations in saliva relative to other body fluids and its detection is indicative of the presence of this body fluid. This method for the detection of amylase consists of a tablet of water-insoluble starch, cross-linked to Cibacron Blue dye that is hydrolyzed to water-soluble blue fragments in the presence of alpha-amylase and detected by blue color development of the solution.

1.2 References

- 1.2.1 Gaensslen, R. Sourcebook in Forensic Serology, Immunology, and Biochemistry. (1983) U.S. Dept. of Justice, Washington, D.C., p 184-187.
- 1.2.2 Auvdel, Michael J. "Amylase Levels in Semen and Saliva Stains" (1986) Journal of Forensic Sciences, 31 (2) 426-431.
- 1.2.3 Keating, S.M. and Higgs, D.F. "The detection of amylase on swabs from sexual assault cases", (1994) Journal of the Forensic Science Society, 34: 89-93.
- 1.2.4 G.M. Willott, "An Improved Test for the Detection of Salivary Amylase in Stains," Journal of the Forensic Science Society, 14, pp. 341-344 (1974).
- 1.2.5 Phadebas Amylase Test directions for use, Pharmacia AB, Uppsala, Sweden, 1994 and Magle AB, Lund, Sweden, 2007.

2.0 Scope

2.1 To provide a presumptive screening test for the presence of saliva on evidentiary items.

3.0 Equipment/Reagents

- Phadebas Tablets
- 0.5N NaOH
- Sterile/Nanopure H₂O
- 12x75mm tubes
- Corks for tubes or parafilm™ or equivalent
- Pipette(s) and filter barrier tips

4.0 Procedure

4.1 Stain samples (~2-5mm²; 1/8-1/4 swab; 20µl extract) and controls [20µl dH₂O is used for negative control and either neat saliva or a saliva stain (≤2mm² cutting) as a positive control] are placed into appropriately labeled tubes.

4.2 Add 1ml dH₂O and 1/4 Phadebas tablet to each tube, cover tube, mix well (e.g. vortex) and incubate at 37°C for 30 minutes.

- 4.3 At RT, remove cork, add 250µl 0.5N NaOH to each tube, cover tube, mix well by inversion and spin for 5 minutes at low speed (<5,000 rpm).
- 4.4 Examine tubes and record the color of the supernatant. The intensity of the blue color, if present, may be graded as light, medium, dark, or 1+-4+. For reporting, see 5.1.

5.0 Comments

- 5.1 If the blue color of a sample is as dark as or darker than that of the 1:500 control, it is an indication of an elevated level of amylase and is reported as such. If the blue color of a sample is lighter than the 1:500 control, there is an indication that amylase is present; however, there is no demonstration of an elevated level. A sample that demonstrates absence of blue color consistent with the negative control is reported as 'did not indicate the presence of amylase'. Note: negative samples (like the control) may have a very slight blue tint and not appear perfectly clear.
- 5.2 A negative result is not necessarily the total absence of saliva, and therefore, DNA testing should not be abandoned because of the absence of detectable amylase activity depending on case circumstances.
- 5.3 This test is not human specific, there may be reactive amylases in plants and non-human animals.

Detection of Urine (Urease) (BI-126)

1.0 Background/References

1.1 Urea is a normal metabolite found in high concentration in urine. The urease reagent reacts with the urea present in a urine stain and releases ammonia, which may be detected with litmus paper.

1.2 References

1.2.1 Gaensslen, R. Sourcebook in Forensic Serology, Immunology, and Biochemistry. (1983) U.S. Dept. of Justice, Washington, D.C., p. 191-195.

1.2.2 Metropolitan Police Forensic Science Laboratory Biology Methods Manual, 1978, Section 4.

2.0 Scope

2.1 To provide a presumptive test for the presence of urine on relevant evidentiary material.

3.0 Equipment/Reagents

- Urease Reagent
- Sterile/Nanopure H₂O
- Small Corks (to fit 12x75mm test tubes)
- 12x75mm test tubes
- Red Litmus Paper

4.0 Procedure

4.1 Cut out ~2.0cm² piece of suspected urine stain and controls, cut them into small pieces and place them into appropriately labeled 12x75mm test tubes.

4.2 Add 3-4 drops of dH₂O and 6-7 drops of Urease Reagent to each test tube.

4.3 Cut a slit into the bottom of each cork and insert a small piece of red litmus paper into the slit.

4.4 Place one of the corks (with litmus paper) into each tube; do not allow the litmus paper to come into contact with the liquid.

4.5 Incubate the tubes for 30 minutes at 37°C.

4.6 Note and document any change in the color of the litmus paper that occurs within the incubation time. A positive reaction (+) is recorded when the red litmus paper turns blue. When there is no color change noted, a negative (-) result is recorded.

5.0 Comments

5.1 Controls include positive (known urine stain) and negative (dH₂O blank) and a substrate control where appropriate and available.

5.2 The Urease Test is one of many presumptive tests for urine; a confirmatory test for the identification of urine in a dried stain is not available.

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Detection of Fecal Material (BI-130)

1.0 Background/References

1.1 Edelman's Test is a presumptive test for the presence of fecal material and is based on the detection of urobilinogen which is found in high concentration in feces. Urobilinogen, which is oxidized to urobilin, is soluble in alcohol and, in the presence of neutral alcoholic salts, will form a green fluorescent complex with zinc.

1.2 References

- 1.2.1 Gaensslen, R. Sourcebook in Forensic Serology, Immunology, and Biochemistry. (1983) U.S. Dept. of Justice, Washington, D.C., p. 191-195.
- 1.2.2 Metropolitan Police Forensic Science Laboratory Biology Methods Manual, 1978, Section 4.

2.0 Scope

2.1 To provide a presumptive test for the presence of feces on relevant evidentiary material.

3.0 Equipment/Reagents

- 10% (w/v) Mercuric Chloride Solution
- 10% (w/v) Zinc Chloride Solution
- Amyl (Isopentyl) Alcohol
- Sterile/Nanopure H₂O
- 12x75mm test tubes
- Alternate Light Source

4.0 Procedure

- 4.1 Cut out ~0.5 cm² piece of suspected fecal stain and controls and place them into appropriately labeled 12x75mm test tubes.
- 4.2 Extract samples in ~3 drops of dH₂O for 15-30 minutes at RT.
- 4.3 Remove the material and add ~3 drops of 10% Zinc Chloride Solution to the extract.
- 4.4 Add 5 drops of Amyl Alcohol to the extract and vortex.
- 4.5 Spin sample for 5 minutes on low (~2000 rpm) in the serological centrifuge and transfer the upper phase to a new 12x75mm tube.
- 4.6 To the upper phase, add 3 drops of 10% Mercuric Chloride Solution and observe any color change under both white and long wave UV light.
- 4.7 A positive reaction is recorded when green fluorescence is visible under long wave UV light. Absence of green fluorescence under long wave UV light is recorded as a negative reaction. Under white light, the solution may become rose-pink if urobilin is present.

5.0 Comments

- 5.1 Controls include positive (known fecal stain) and negative (dH₂O blank) and a substrate control where appropriate and available.
- 5.2 The Edelman's Urobilinogen Test is one of many presumptive tests for feces; there are no confirmatory tests available for the identification of fecal material.
- 5.3 The production of a green fluorescent complex is indicative of feces from humans and other carnivores. Due to the presence of chlorophyll, the feces of herbivores will produce an orange-pink fluorescence in this test. Test results giving this orange-pink fluorescence will be recorded as inconclusive.

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Detection of Male DNA on Sexual Assault Kit Evidence (BI-132)

1.0 Background/References

1.1 Traditional serological methods of screening sexual assault kits are labor and time intensive. The majority of sexual assaults involve a female victim and male suspect(s). The detection of male DNA in such cases can be probative regardless of the biological source. DNA quantitation using Real-Time PCR with the Plexor® HY kit is a sensitive and efficient method for screening sexual assault kits for the presence of male DNA.

1.2 References

1.2.1 SwabSolution™ Kit Technical Manual (revision 09/16), Promega Corporation.

1.2.2 Koehler, D.J., "Male-Screening at Texas DPS" (PowerPoint presentation), August 2015.

2.0 Scope

2.1 To provide a protocol for screening sexual assault kits for the presence of male DNA.

3.0 Equipment/Reagents

3.1 Equipment:

- Disposable razor blades
- 1.5ml microcentrifuge tubes (1.5ml tubes)
- Pipettes and filter barrier tips
- Vortex mixer
- Microcentrifuge
- 70°C heat block/oven
- 96-well reaction plate/base
- Optical adhesive cover
- ABI 7500/computer
- ABI 7500 SDS software
- Plexor® Analysis software

3.2 Reagents:

- SwabSolution™ Reagent
- 1M Dithiothreitol (DTT)
- PCR TE (TE, 10mM Tris-HCl; 0.1mM EDTA, pH 8.0)
- 5x AmpSolution™ Reagent
- Plexor® HY kit

4.0 Procedure

NOTE: All orifice swabs, and body swabs suspected of containing a body fluid (or as appropriate per case scenario), from sexual assault kits with a female victim and male suspect(s) will be screened for the presence of male DNA utilizing this method. Traditional serological testing for detection of specific biological fluids will only be performed in such cases upon request from the prosecuting or defense attorney, or upon receipt of a court order.

4.1 LYSATE PREPARATION:

4.1.1 For each sub-item in the sexual assault kit that consists of orifice or body swabs from the same location, suspected to contain semen, cut $\sim 1/8$ of each swab into a single 1.5ml microcentrifuge tube. No more than four $\sim 1/8$ swab cuttings should be cut into a single tube. If a sub-item consists of more than four swabs from a single location the cuttings should be split into an additional tube(s). If an additional tube(s) is required a designator will be used to distinguish between the samples with the tubes and swab sticks being labeled accordingly (i.e. 1.2A/1.2B or 1.2-1/1.2-2). If the sub-item consists of fewer than four total swabs a slightly larger cutting may be taken from each swab as long the total amount of swab material in a single tube does not exceed $\sim 1/2$ swab when added together (i.e. if there are three swabs up to an $\sim 1/6$ cutting may be taken from each).

NOTE: It is assumed that the swabs comprising a sub-item from a single location were collected in a uniform manner with an approximate even distribution of biological material.

4.1.2 For each sub-item in the sexual assault kit that consists of orifice or body swabs from the same location, not suspected to contain semen, it is generally recommended to cut the equivalent of ~ 1 swab into a single 1.5ml microcentrifuge tube (i.e. $\sim 1/2$ of two swabs, $\sim 1/4$ of four swabs, etc.). 4.1.1 cutting sizes may also be followed for these samples if needed.

4.1.3 Add the following to each tube:

200 μ l SwabSolution™ Reagent

10 μ l 1M DTT

NOTE: A reagent blank tube will be created at this step.

4.1.4 Mix thoroughly and incubate at 70°C for ~ 1 hour.

4.1.5 Following incubation briefly mix and spin down each tube to ensure the contents are at the bottom of the tube. It is not necessary to remove swab cutting(s) from the tube. (Optional: Samples may be centrifuged at high speed ($\geq 10,000$ rpm) for 3-5 minutes to force swab cutting(s) to tube bottom).

NOTE: The prepared lysates may be stored at 4°C prior to quantitation if necessary. Lysates may be stored at 4°C for at least one week (and possibly up to 4 years according to the manufacturer) following preparation without impacting quantitation results. These lysates are considered in-progress work product and will be discarded following approval of the final case report except as noted in section 5.5 below, with the exception of lysates containing ~1 swab (4.1.2). These lysates are also considered work product but will be returned to the agency if not sent for further purification, following the same procedure for DNA extracts (See “DNA Extracts” section 4.8 in BI-200 below).

4.1.6 Proceed to 4.2.

4.2 DNA QUANTITATION:

4.2.1 Proceed to DNA Quantitation: Real-Time PCR (BI-207) incorporating the following modifications:

- Fill out the Y-Screen worksheets template (Form 132-BI).
- Reaction components and volumes for each sample will be:

Plexor® HY 2X Master Mix	10µl
Plexor® HY 20X Primer/IPC Mix	1µl
5X AmpSolution™ Reagent	4µl
Water	3µl

5.0 Comments

5.1 The “IPC Status” and “Curves Status” flags in the Plexor® Analysis software are important quality indicators. “Check IPC” and/or “Check Melts” flags may be indications that the quantitation results are not completely accurate for a particular sample. In these instances it may be appropriate to deem the results of the sample “inconclusive.” Results indicating very low levels of male DNA (i.e. <0.001ng/µl) should be interpreted with extra caution and these quality indicators should be carefully inspected. A very low level male DNA quantitation result associated with a “Check Melts” flag may be due to non-specific amplification or very low levels of true male DNA. Due to the lysates being the result of a “crude lysis” procedure without subsequent purification of the DNA it is possible that inhibition may be observed more frequently than for purified DNA extracts. The AmpSolution™ Reagent optimizes the reaction to help overcome the effects of the “crude lysis” procedure but it is still important to carefully evaluate the IPC results. The melt curve is the best way to assess whether or not a very low level quantitation result is actually DNA signal.

- 5.2 The screening analyst will forward portions of samples on for DNA analysis where male DNA was detected or with an “inconclusive” result, based upon case circumstances, orifice or body swab location, and/or quantitation results. Typically if the Auto:Y ratio is 250 or greater, and the sample is not suspected to contain semen such that a differential extraction would not be performed, the screening analyst will not forward the sample for subsequent STR analysis.
- 5.3 The screening analyst will indicate in the case notes whether or not semen is suspected for all sub-items with a positive or “inconclusive” result for male DNA in order to provide guidance to the DNA analyst as to whether or not a differential extraction may need to be performed. The determination as to whether or not semen is suspected will be based upon case circumstances, orifice or body swab location, and/or quantitation results. If there is insufficient information available to assist in this determination the screening analyst may elect to perform presumptive testing for acid phosphatase to aid in the determination. The results of any such acid phosphatase testing are required to be included in the final case report.
- 5.4 The Auto:Y ratio and/or quantity of male DNA detected during the quantitation of the “crude lysate” may not be fully representative of the results expected from a purified DNA extract that would be generated during STR DNA analysis when a differential extraction is to be performed. Therefore, obtaining a high Auto:Y ratio result from the “crude lysate” will not always be reason to forego subsequent STR DNA analysis.
- 5.5 The whole swab crude lysates for non-semen containing samples should be purified on the EZ1 rather than taking additional sample cuttings for extraction. A corresponding reagent blank will need to be purified; therefore, it may be advisable to include an additional reagent blank with Y-Screen batches. If crude lysates are to be purified on the EZ1, they should be stored refrigerated (short term) or frozen (long term). If EZ1 purification is to be performed, transfer the lysate to an EZ1 sample tube and proceed to step 4.8 of BI-200.
- 5.6 Orifice swabs contained in the kit that have no male DNA detected during the y-screen process may be used as a victim reference sample if a blood card/liquid blood was not provided. This includes oral, anal, and vaginal swabs.

Extraction Protocols for PCR DNA Typing Tests (BI-200)

1.0 Background/References

1.1 Many methods exist to obtain DNA, suitable for amplification, from a variety of sources. Caution must be exercised when selecting an appropriate extraction method, taking sample quantity into account.

1.2 References

- 1.2.1 Comey, CT et al. "DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis." J For Sci, Vol. 39, 1994, pp. 1254-1269.
- 1.2.2 Hochmeister, MN et al. "Typing of Deoxyribonucleic Acid (DNA) Extracted from Compact Bone from Human Remains." J For Sci, Vol. 36, 1991, pp. 1649-1661.
- 1.2.3 Yang, DY et al. "Technical Note: Improved DNA Extraction From Ancient Bones Using Silica-Based Spin Columns." Am J of Phys Anthropology, Vol 104:539-543, 1998, 539-543.

2.0 Scope

2.1 To provide appropriate protocols for the extraction of DNA suitable for PCR amplification and subsequent analyses.

3.0 Equipment/Reagents

3.1 Equipment:

- Qiagen BioRobot® EZ1 or EZ1 Advanced XL
- Qiagen EZ1 Investigator Kit and card
- Microcentrifuge
- 15/50ml conical tubes
- 56/95°C heat block/oven
- 1.5ml microcentrifuge tubes (1.5ml tubes)
- 2.0ml screw cap tubes (non-skirted base)
- Spin baskets
- MicroAmp tubes
- Pipettes and filter barrier tips
- Coarse Sandpaper, Blender, Hammer, Chisel, Drill or Dremel

3.2 Reagents:

- Stain Extraction Buffer (SEB)
- PCR TE (TE, 10mM Tris-HCl; 0.1mM EDTA, pH 8.0)
- Proteinase K (ProK, 20 mg/mL)
- 1M Dithiothreitol (DTT)

- Ethanol (EtOH)
- Phosphate Buffered Saline (PBS)
- Ethyl Ether
- Xylene
- 10% SDS
- Buffer G2

4.0 Procedure

NOTE: When analyzing individual cases, outside of the batch process, questioned and known reference samples must be extracted separately. If samples are extracted on the same day, questioned samples should be set up first.

The sample sizes listed below are the typical recommended amounts. Evidence samples vary in quantity and condition so sample sizes may be adjusted accordingly. The size of sample used may be case dependent and may be based upon the results of screening tests, visual appearance, and total sample amount. The analyst should make an effort to retain sufficient sample for replicate testing if possible; however, those samples of limited size/quality may need to be consumed (See BI-QA 5.2.2).

Sample cuttings will generally be taken by the screening analyst and placed into the appropriate tubes for DNA processing. Cutting sizes for extracted samples are mentioned below, see the direct amplification section for associated cutting sizes for those sample types.

4.1 WHOLE BLOOD SAMPLES (EZ1 EXTRACTION):

4.1.1 Place ~3µl – 10µl whole blood into an EZ1 sample tube provided in the EZ1 Investigator kit. Bring the volume up to 200µl with Stain Extraction Buffer.

4.1.2 Proceed to 4.6.

4.2 BLOOD/SALIVA/FTA/NON-SEMEN (TISSUE, EPITHELIAL CELLS) SAMPLES (EZ1 EXTRACTION):

4.2.1 Place one of the following samples into an EZ1 sample tube: ~3mm² – 1cm² cutting/portion or swabbing of samples on cloth or porous materials (includes cigarette butts, gum, and envelope flaps/stamps), ~1/8 – 1/2 (~equivalent of previous sample size) cutting/portion of each cotton swab containing sample, up to one whole swab (samples deposited on non-porous objects may need to be collected onto a swab with a small amount of sterile deionized water, TE, or SEB and the swab cut for testing. Record lot numbers of TE or SEB if used), or ~3mm² – 5mm² portion of tissue.

Note: samples requiring large volume or necessitating substrate removal may be placed into a spin tube for extraction.

4.2.2 Add the following to the tube:

190µl SEB

10µl Pro K

Note: Large and/or absorbent substrate cuttings may require additional SEB, up to 490µl.

Note: In some instances, 1M DTT may be a beneficial addition to a non-differential sample. In such instances, the following will be added to the tube:

180µl SEB (Up to 480µl may be added for large and/or absorbent substrate cuttings)

10µl Pro K

10 µl 1M DTT

4.2.3 Mix and incubate at 56°C for a minimum of 15 minutes, up to overnight. A 15 minute digest at 56°C, immediately followed by a 5 minute digest at 95°C, may alternatively be performed.

4.2.4 Large cuttings/substrates (if applicable) may be removed by piggyback/spin basket centrifugation at high speed ($\geq 10,000$ rpm) for 3-5 minutes and discarded.

4.2.5 Proceed to 4.6 (note: if using a spin tube, the cap may be cut from the tube for placement in the EZ1).

4.3 EXTRACTION OF HAIR SAMPLES

Note: For removal of hair(s) mounted on a slide, see 4.7.

4.3.1 Examine the hair(s) under a compound light microscope and/or stereomicroscope. Note if there is the presence of cellular material at the root and the presence of any body fluid (e.g., blood or semen) or other visible contaminants on the hair shaft. Any hair(s) examined using a compound light microscope should be photographed and included in the note packet (see BI-119).

4.3.2 Once a suitable hair(s), preferably anagen, has been identified it may be washed to reduce surface dirt and contaminants. This may be accomplished by immersing the hair(s) in sterile, deionized water and gently swirling. Each hair to be analyzed should be washed separately in fresh water. Alternatively, the hair(s) may be placed in a 1.5ml tube containing 1ml 10% SDS and sonicated briefly. Again, each hair to be analyzed should be treated separately. If the presence of any body fluid is noted on the hair shaft, it may be removed for separate DNA analysis, if necessary, by soaking the hair in a minimal amount of sterile deionized water or PCR TE for 30 minutes. Process this extract as you would a bloodstain (see 4.2.1).

4.3.3 Even if the hair(s) was washed prior to proceeding to 4.3.4, it may still have cellular material on its surface that did not originate from the hair donor. Therefore, in addition to cutting off ~0.5 - 1.0cm of the root-end, a ~0.5 - 1.0cm cutting of the shaft adjacent to the root may be processed separately as a control. The remaining shaft may be retained for subsequent analyses (e.g., microscopic exam, mitochondrial DNA).

4.3.4 To the 1.5ml or EZ1 sample tube containing the hair sample, add:

180µl SEB

10µl 1M DTT

10µl ProK

4.3.5 Mix and incubate at 56°C for minimum of 6 hours (may be left overnight).

4.3.6 Proceed to 4.6 for EZ1 isolation.

4.4 EXTRACTION OF BONE AND TEETH

4.4.1 Obtain a fragment of bone and remove any tissue present, using ethyl ether (shake vigorously with a few milliliters of ether in a polypropylene tube) or by boiling briefly. For older bones, or those without adhering tissue, clean the outer surface by sanding. For teeth, begin with step 4.4.2.

4.4.2 Rinse the bone/tooth, in the same manner, with distilled water.

4.4.3 Similarly, rinse the bone/tooth with 95% ethanol. Finally, clean the bone/tooth with a sterile cotton swab soaked with ethanol to ensure it is free of dirt and/or other contaminants. Allow bone/tooth to air dry.

4.4.4 Crush bone/tooth into small pieces/powder with blender (a chisel or hammer may be used initially). Alternatively, a drill and bit may be used on large bones to create a fine powder. Transfer the powder and/or small pieces created to a 1.5ml tube.

4.4.5 To the tube, add:

500µl SEB

15µl ProK

Mix thoroughly and incubate at 56°C overnight.

4.4.6 Spin in a centrifuge at high speed ($\geq 10,000$ rpm) for 3-5 minutes, transfer 200-500µl of the supernatant to an EZ1 sample tube and proceed to 4.6.

Note: For aged bones, it may be necessary to process multiple samples

4.5 MANUAL EZ1 DIFFERENTIAL EXTRACTION OF SEMEN-CONTAINING SAMPLES:

Note: For removal of sample from mounted slide, see 4.7.

- 4.5.1 Place cutting/sample (the size of sample used will be case dependent and based upon microscopic exam or Y-screen results and total sample amount) into an EZ1 sample tube (the sample may optionally be placed in a spin tube for ease of piggyback centrifugation) and add ~150µl PBS. Agitate the substrate to loosen cellular material and place at 4°C for 1-4 hours (up to overnight).
- 4.5.2 Sonicate samples for ~20 minutes to loosen cellular material from the substrate and perform piggyback/spin basket centrifugation for 3-5 minutes. Without disturbing the pellet, remove all but ~10-50µl of the supernatant and discard.
- 4.5.3 To the remaining cell pellet and substrate (if present) add the following:
190µl SEB
10µl Pro K
Note: Large and/or absorbent substrate cuttings may require additional SEB, up to 490µl.
- 4.5.4 Mix and incubate at 56°C for 15 minutes to a maximum of 1 hour.
- 4.5.5 Label a new sample tube. Remove substrate (if present) by using piggyback/spin basket centrifugation and discard. A final centrifugation on high speed for ≥1 minute may be performed to further solidify the pellet.
- 4.5.6 Remove all but ~10-50µl of the supernatant, taking care not to disrupt the cell pellet in the bottom of the tube. Transfer this supernatant (epithelial cell fraction) to the new, labeled sample tube and store at 4°C or proceed directly to 4.6.
- 4.5.6.1 Optional: The purpose of a differential extraction is, typically, to obtain a sperm fraction that is void of any epithelial contribution. In instances in which there is an overwhelming proportion of epithelial cells to sperm that appear intact microscopically, steps 4.5.3-4.5.4 may, at the scientist's discretion, be repeated 1-2 times prior to proceeding to 4.5.7. These additional supernatants do not need to be retained.
- 4.5.7 Wash the sperm pellet as follows: Resuspend the pellet in 500µl PBS by vortexing briefly. Spin in a microcentrifuge for ~5 minutes at maximum speed (>10,000rpm). Remove all but ~10-50µl of the supernatant and discard it. Note: 1000µl PBS should be used for 500µl sample volumes.
- 4.5.8 Repeat 4.5.7 1-5 more time(s). In instances of low sperm amounts, additional washes are recommended. The final wash performed is to be done using sterile deionized water.
- 4.5.8.1 Optional: Resuspend the pellet and place 3-5µl on a slide for microscopic evaluation (See BI-118). If intact epithelial cells remain, the pellet should be redigested (4.5.3 – 4.5.8).

4.5.9 To the remaining sperm pellet solution add:

180µl SEB

10µl 1M DTT

10µl ProK

Note: up to 480µl SEB may be used

4.5.10 Mix and incubate at 56°C for a minimum of 15 minutes (may be left overnight).

4.5.11 Proceed to 4.6 (note: if using a spin tube, the cap may be cut from the tube for placement in the EZ1).

4.6 BIOROBOT EZ1 or EZ1 Advanced XL ISOLATION PROCEDURE

4.6.1 Insert the Investigator Card into the card slot on the BioRobot EZ1 (if not already in place) and turn the instrument on. Note: the card may be left in place when the instrument is turned off.

4.6.2 Press “Start” to display the protocols menu and choose one of the following protocols:

- Choose “1” for the “Trace” protocol if no substrate is present in the sample tube.
- Choose “2” for the “Tip Dance” (Trace TD) protocol if the substrate is present in the sample tube.
- Choose “3” (EZ1 Advanced XL) or “4” (EZ1) for the “Large Volume” protocol for 500ul sample volumes. Note: Step through the prompt regarding additional ‘MTL’ Buffer.

4.6.3 Press “2” to elute in TE.

4.6.4 Select either the 50µl (EZ1 option 1 or EZ1 Advanced XL option 2) or the 200µl (EZ1 option 3 or EZ1 Advanced XL option 4) elution volume from the menu. The 50µl elution may be preferable for FTA, dilute samples, or those suspected to be of low DNA concentration. The 200µl elution volume may be preferable for samples exhibiting potential inhibition.

Note: A 40µl elution volume option is only available on the EZ1 Advanced XL (option 1). This option may be selected and 40µl elution may be preferable for samples suspected to be of very low DNA concentration.

4.6.5 Press any key to proceed through the text displayed in the LCD, which guides you through the following steps to load the instrument.

4.6.6 Open the workstation door.

4.6.7 Examine the reagent cartridge(s) for the presence of precipitate. Invert each cartridge to mix the magnetic particles then tap the cartridge(s) to deposit the reagents to the bottom of their wells.

- 4.6.8 Insert the appropriate number of reagent cartridges (1-6 per EZ1 extraction run or 1-14 per EZ1 Advanced XL extraction run) into the cartridge rack, snapping them into place. Additional samples (beyond 6 on the EZ1 or beyond 14 on the EZ1 Advanced XL) can be accommodated in subsequent instrument runs. Place the loaded cartridge rack into the instrument, followed by the tip rack.
- 4.6.9 Load 1-6 (EZ1) or 1-14 (EZ1 Advanced XL) tip holders containing filter-tips into row 2 of the tip rack.
- 4.6.10 Load 1-6 (EZ1) or 1-14 (EZ1 Advanced XL) opened and appropriately labeled elution tubes into row 1 of the tip rack. Make sure that the tube order matches that of the sample tubes.
- 4.6.11 Load 1-6 (EZ1) or 1-14 (EZ1 Advanced XL) opened sample tubes from relevant step above into row 4 of the tip rack.
- 4.6.12 Close the workstation door.
- 4.6.13 Press “Start” to start the extraction protocol.
- 4.6.14 When the protocol ends, the LCD displays “Protocol finished.” To run another protocol, press “ESC” to return to the protocols menu. Otherwise, press “Stop” twice to return to the first screen of the LCD.
- 4.6.15 Open the workstation door. Remove and cap the elution tubes containing the purified DNA. Discard the cartridges, tip holders/tips, and sample tubes.
- 4.6.16 At the completion of all runs for the day, clean the piercing tool (option #3 from the ‘tools’ screen on the EZ1 or option #3 from the ‘man(ual)’ screen on the EZ1 Advanced XL), O-rings and tip adaptor, tip rack, cartridge rack, and interior of the instrument with 70% Ethanol, followed, optionally, by nanopure water.
- 4.6.17 Switch off the instrument, leaving the Investigator card in place.
- 4.6.18 Proceed to real time PCR (see BI-207) for quantitation of the purified DNA obtained in step 4.6.15.

4.7 REMOVING MATERIAL FROM SLIDES:

4.7.1 FREEZING:

- 4.7.1.1 Place slide in -20°C freezer for 3-5 minutes.
- 4.7.1.2 Wearing safety glasses, pry the cover slip off.
- 4.7.1.3 Add a drop of xylene to dissolve the mounting medium.
- 4.7.1.4 Remove the hair and soak in 10-20ml xylene for 2-3 minutes to remove residual mounting medium.

Note: Sperm-containing slides are rinsed with sterile deionized water at this point and a suitable volume (~100µl) of stain extraction buffer may be added directly to the slide. Incubate ~5 minutes at RT and then by pipetting up and down, wash the sample off of the slide and transfer to an EZ1 sample tube or a 1.5ml tube. Repeat 3-4 times and proceed to 4.5.3.

4.7.1.5 Rinse the hair briefly in absolute ethanol to remove the xylene and proceed to hair extraction under 4.3.

4.7.2 SOAKING IN XYLENE:

4.7.2.1 Soak the slide in xylene for several hours until the cover slip can be slid or pried from the slide.

Note: This will likely remove markings from the slide.

4.7.2.2 Remove the hair and soak in about 10-20ml xylene to remove the residual mounting medium.

4.7.2.3 Rinse the hair briefly in absolute ethanol to remove xylene and proceed to hair extraction under 4.3.

4.8 DNA EXTRACTS:

4.8.1 After a sample has been extracted and during subsequent analyses (i.e. quantification and amplification), the DNA extract may be stored at 4°C. For longer storage periods, the extract should be frozen at approximately -20°C. These extracts are in-progress work product during this stage(s).

4.8.2 Following DNA testing, any leftover DNA extracts will be put into a plastic zip bag and/or coin envelope and given a new item number in ILIMS. An ILIMS barcode will be printed and placed on the envelope containing the extracts. Individual tubes may also be sealed with parafilm or other sealant to prevent leakage and/or evaporation if desired. DNA extracts will be stored in the laboratory evidence freezer at approximately -20°C pending return to the submitting agency. Additionally, a 'store frozen' sticker should be placed on the outside of the envelope containing DNA extracts prior to returning them to the agency.

5.0 Comments

5.1 An appropriate reagent blank (for each type of extraction) will be carried through all extraction steps to check the purity of the reagents being used. There need only be one reagent blank per extraction batch, in which the same lots of reagents are used. Questioned and known extraction batches will require separate reagent blanks. It is not necessary to have a separate blank for each case that is extracted at the same time. When extracting a batch of samples with multiple EZ1 Robots, a single reagent blank may be used, providing the instruments are running concurrently. Additional reagent blanks will be incorporated when the sample number exceeds the capacity of available instruments (requiring subsequent runs), so that one blank is included with each run (Note: it may be preferable to extract additional reagent blanks with each evidence batch in order to accommodate any further testing that may be necessary).

- 5.2 Presoaking bloodstains with PBS may help to prevent inhibition of amplification by heme products, particularly when analyzing DNA obtained from samples of "heavy" bloodstains (e.g. control bloodstains).
- 5.3 These procedures represent the 'usual' protocol for a given material; however, any of these different extraction methods are suitable for all biological materials, though minor modifications may be necessary.
- 5.4 Extracts may be re-purified on the EZ1 if needed (i.e.: Inhibition indicated at quantification or data analysis). A corresponding reagent blank would need to be purified as well. Refer to above step 4.6 of BI-200.

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DNA Quantitation: Real-Time PCR (BI-207)

1.0 Background/References

1.1 DNA methodologies that employ the PCR, such as STR analysis, necessitate consistent quantitation of human DNA to obtain optimum data.

1.2 References

- 1.2.1 Krenke, B.E., et al, "Developmental Validation of a Real-Time PCR Assay for the simultaneous Quantification of Total Human and Male DNA," Forensic Science International: Genetics 3 (2008), pp 14-21 Vol. 3.
- 1.2.2 Crouse, C., et al, "Improving Efficiency of a Small Forensic DNA Laboratory: Validation of Robotic Assays and Evaluation of Microcapillary Array Device," Croat Med J 2005, Vol. 46, No. 4, pp. 563-577.
- 1.2.3 Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems, Technical Manual (revision 09/17), Promega Corporation.
- 1.2.4 7500/7500 Fast Real-Time PCR Systems Maintenance Guide, Applied Biosystems.

2.0 Scope

2.1 To provide a reliable method for the consistent quantitation of small amounts of human DNA isolated from forensic samples and to determine the amount of male DNA present compared to the amount of total human DNA.

3.0 Equipment/Reagents

- ABI 7500/Computer
- ABI 7500 SDS Software
- Plexor® Analysis Software
- Pipettes and Barrier Tips
- Plexor® HY Kit
- 96-well Reaction Plate/Base
- Optical Adhesive Cover
- Microcentrifuge Tubes
- Centrifuge (optional)
- PCR-TE

4.0 Procedure

4.1 PREPARATION OF DNA STANDARDS:

- 4.1.1 Label 8 sterile microcentrifuge tubes, or strip tubes A through H or 1 through 8.
- 4.1.2 Dispense 40µl of PCR-TE into tubes B-H (Std. 2-8).
- 4.1.3 Mix the Plexor HY Male Genomic DNA Standard thoroughly by vortexing approximately 5 seconds. Transfer 10µl to tube A (Std. 1/undiluted) and to tube B (Std. 2). Mix the dilution thoroughly.

4.1.4 Prepare Std. 3-7 via a serial dilution by mixing and subsequent 10µl transfers starting with tube B and ending with G. The dilution series consists of 50, 10, 2, 0.4, 0.08, 0.016, 0.0032ng/µl (or adjusted per kit QC), and a no-template control, respectively.

4.2 REACTION PREPARATION:

4.2.1 Determine the number of samples to be quantitated (including, at minimum, 2 sets of DNA standards).

4.2.2 Fill out the 7500 Load Sheet on the Casework worksheets template (Form 206-BI) or electronic equivalent in ILIMS. Print a copy for the case file if applicable.

4.2.3 Calculate the volume of reaction components needed, based upon the number of samples to be quantitated and adding additional reactions to compensate for loss and variability due to pipetting. The following are the volumes needed per reaction.

Plexor HY 2X Master Mix	10µl
Plexor HY 20X Primer/IPC Mix	1µl
Water	7µl

Note: The volume of reaction components necessary to prepare the Reaction Mix will be automatically calculated upon Load Sheet data entry.

4.2.4 Thaw the master mix and primer mix and vortex 3-5 seconds. Pulse-spin prior to opening the tube (do not overspin).

4.2.5 Place a 96-well reaction plate into a base, being careful not to touch the top or individual wells. Do not place the plate directly onto the counter or any surface other than its base or the ABI 7500 thermal block.

4.2.6 Prepare the Reaction Mix by pipetting the required volumes of water, primer and master mixes into an appropriately sized microcentrifuge tube. Mix thoroughly and pulse spin.

4.2.7 Carefully pipette 18µl of the PCR Reaction Mix into the bottom of each reaction well to be used.

4.2.8 Add 2µl of sample or standard to the appropriate reaction well.

4.2.9 Seal the reaction plate with an Optical Adhesive Cover. Proceed to 4.3.

4.3 RUNNING THE REACTION:

4.3.1 Turn on the 7500 computer and login with the appropriate user name and password. After the computer has completely started up, power on the 7500 instrument, allowing it to warm up at least ~30 seconds. Launch the ABI 7500 SDS Software.

4.3.2 Open the instrument tray by pushing on the tray door. Place the plate into the tray holder so that well A1 is in the upper-left corner and the notched corner of the plate is in the upper-right corner.

- 4.3.3 Close the instrument tray by gently pushing the right side of the tray door.
- 4.3.4 In the SDS software, log in as “Guest”. Select “Template” from the home screen and chose “PlexorHY”. Under Experiment Properties, name the plate in the Experiment Name field. Other required settings should be as follows: **Which Instrument:** 7500 (96 wells), **Type of experiment:** Quantitation – Standard Curve, **Reagents:** Other, ensure “Include Melt Curve” is checked, and **Ramp speed:** Standard (~2 hours to complete a run).
- 4.3.5 In the Plate Setup screen, select the “Assign Targets and Samples” tab. Highlight the wells that contain samples or standards and apply the autosomal, Y, IPC, and passive reference detectors. Do not include any unused wells. It is not necessary to name the samples but may be done if desired.
- Note: Detectors are created during the initial instrument set-up and/or kit usage. Refer to the Plexor HY System Technical Manual for instructions on creating detectors if needed.
- 4.3.6 Select the Run Method tab and review the thermal cycler conditions [Stage 1: 1 cycle, 95°C, 2:00 min.; Stage 2: 38 cycles, 95°C, 00:05 min, 60°C, 0:35 min.; Stage 3: 1 cycle, 95°C, 00:15 min, 60°C, 1:00 min, 95°C, 00:15 min; 20µl sample volume]
- 4.3.7 Save the plate document as a .eds file with the appropriate plate name.
- 4.3.8 Click Start Run to begin the run. When the run has completed, proceed to 4.4.
- Note: Choose ‘yes’ at the SYBR Green message prompt.

4.4 ANALYSIS AND RESULTS:

- 4.4.1 Open the plate document to be analyzed.
- 4.4.2 Select Export from the top toolbar.
- 4.4.3 In the Export Data window, select the following Export Properties: **Data to export:** Multicomponent Data, **File type:** .xls. Specify the appropriate export file name and location. Select Start Export.
- Note: if sample names were defined, these can be exported by selecting Sample Setup. “Results” may also be exported with Multicomponent Data.
- 4.4.4 Launch the Plexor Analysis Software and select “Set Passive Reference On Import” in the file menu.
- 4.4.5 Import the .xls file by selecting File>Import New Run. Enter the plate (assay) name, select Applied Biosystems 7500 SDS 2.0 and assign dyes to the autosomal (FL), Y (C0560), IPC (CR610), and Passive Reference (IC5) targets. Check the amplification and melt boxes for autosomal, Y, and IPC and check Passive Reference for the passive reference. Click ‘next’.
- 4.4.6 Enter the analyst’s initials under Operator Name and select ‘next’. Browse for the appropriate plate file and import it. Enter the number of amplification cycles (38) and the range of melt temperatures (60 to 95 for the default dissociation profile). Select “Finish”.

Note: for analyzing data exported with SDS v1.4 and prior, select “7500 SDS v1.4 and prior” for the instrument and change the Autosomal Dye name to “FLR”.

4.4.7 Select the ‘Sample IDs’ tab and enter the names for each well. Alternatively, the 7500 Load Sheet may be copied and pasted into the Sample IDs window using the Ctrl-T function.

Note: sample names (including standard and no-template controls) must be unique or the software will report concentration averages and an error will occur when importing data into the worksheets.

4.4.8 Define the unknown samples, no-template controls, and standards by highlighting the appropriate wells in the well selector pane of the ‘PCR Curves’ tab and clicking the corresponding icon in the toolbar.

4.4.9 Highlight the standard wells and click the ‘Create Dilution Series’ icon in the tool bar. Select ‘vertical series’ and ‘decreasing’. Enter 50 (or adjusted value from the kit QC) as the starting concentration and 5 as the dilution factor. Click ‘apply’.

4.4.10 Leave the standard wells highlighted and in the melt curves window, drag the target melt temperature line to the midpoint of the melt curves. Do this for the autosomal, Y, and IPC curves. The melt temperature (T_m) for each sample will be displayed in the table on the right.

- A ‘yes’ or ‘no’ in the T_m column indicates whether a sample has a melt temperature within the target range. A ‘no call’ indicates that the sample has a melt curve within the range but there is insufficient sample to cross the threshold. An increase in the T_m for unknowns may indicate the presence of impurities.

4.4.11 With the standard wells still highlighted, select the autosomal channel and click the ‘Add Standard Curve’ icon in the tool bar. Repeat for the Y channel. Select the ‘Standard Curves’ tab to view the standard curves and print the screens for the case file. Review the autosomal and Y data for inconsistencies from the following:

- An R^2 value of ≥ 0.990 indicates a close fit between the standard curve regression line and the individual CT data points of quantification standard reactions.
- An R^2 value of < 0.99 may be due to variability of the 0.0032ng/ μ l dilution. These may be omitted as necessary. If the R^2 value does not improve with removal of these standards, the Plexor HY System Technical Manual (section 7) may be referred to for troubleshooting guidelines. At least one of each of the other standards must be present.
- The slope should fall within the typical slope range of -3.2 to -4.0 for the autosomal target and -3.0 to -3.6 for the Y target. A slope of -3.3 indicates 100% amplification efficiency.

4.4.12 Under the 'PCR Curves' tab, highlight all of the samples, standards, and no-template controls (if any standards were omitted in 4.4.11, do not include them here). Select the 'Add Standard Curve' icon for the autosomal and Y channels. Click 'ok' to replace the existing standard curves.

Note: To view only the plot of the standards, right-click in the Curve panel and the "Display Only Standards" option will appear.

4.4.13 Open the forensics report by selecting Forensics>Set Normalization and IPC Parameters>OK. Check the IPC and Curves Status columns for potential problems and troubleshoot as necessary (viewing other report tabs if desired). Unknown samples with IPC Ct values several cycles higher than those of DNA standards with similar concentrations, is an indication that inhibition may have occurred.

4.4.14 Highlight and copy the forensics report. Paste the data into the '7500 Raw Data' tab of the casework worksheets or electronic equivalent in ILIMS. Open the '7500 Results' tab and review the imported data. Delete any unused wells from the sheet. Delete the standards and no-template controls and adjust values in the dilution scheme columns as needed (Note: does not apply for kit QC documentation). Print a copy of the results sheet for the case file if applicable.

4.4.15 Proceed to STR amplification (BI-208).

Note: When targeting the male component of a male/female mixture, STR analysis may be stopped if no male DNA is detected or if the Auto:Y ratio is 250 or greater.

5.0 Comments

5.1 After initial thawing, the Plexor HY Male Genomic DNA Standard will be stored at approximately 4°C.

5.2 DNA standards may be prepared using different volumes as long as the ratios are the same.

5.3 Refer to the Plexor HY System Technical Manual for additional user information and troubleshooting guidelines.

5.4 A template may also be created in the Plexor Analysis Software when running multiple plates with the same layout.

5.5 The Halogen Lamp may be checked manually to determine if replacement is needed. Place the Green Calibration Tray in the block. Select Instrument>Calibrate and set the exposure time to 4096ms, lamp control to Max, and select Filter A. Click Snapshot and observe results. Expected results should consist of red fluorescence displayed in all wells. Lack of fluorescence indicates the need for lamp replacement. The lamp status should be checked as well by selecting Instrument>Lamp Status/Replacement and viewing the condition.

5.6 In order to extend the life of the Halogen Lamp, the instrument should be turned off anytime it is not in use. Lamp life is approximately 2,000 hours.

STR Amplification: PowerPlex® Fusion 6C System (BI-208)

1.0 Background/References

1.1 DNA analyses have revolutionized forensic biology. The advent of PCR allowed scientists to begin analyzing evidentiary material present in minute quantities and degraded states. The identification of forensically significant STR loci allows scientists to combine the discrimination attainable with the older RFLP technology with the speed and sampling capabilities of other PCR-based methodologies. The PowerPlex® Fusion 6C System is a 27-locus system that includes all CODIS and ESS loci. The loci included are: Amelogenin, D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, SE33, D22S1045, DYS391, FGA, DYS576, and DYS570.

1.2 References

- 1.2.1 Butler, J. Forensic DNA Typing: Biology and Technology Behind STR Markers. (2001) Academic Press.
- 1.2.2 PowerPlex® Fusion 6C System Technical Manual, Promega Corporation
- 1.2.3 Hares, D. Selection and implementation of expanded CODIS core loci in the United States. Forensic Science International: Genetics, July 2015, Volume 17, pages 33-34.
- 1.2.4 Thermo Scientific Savant™ DNA 120, DNA120 OP SpeedVac™ Concentrators Instruction Manual. Thermo Fisher Scientific. 2008.

2.0 Scope

2.1 To provide a reliable method for consistent, high quality amplification of DNA from forensic samples ensuring the generation of suitable PCR product for capillary electrophoresis and analyses of these STR loci.

3.0 Equipment/Reagents

- 10% Bleach or Bleach Substitute
- Thermocycler
- Microcentrifuge
- MicroAmp tubes
- 96-well Reaction Plate/Base
- Strip caps
- Pipettes and filter barrier tips
- PowerPlex® Fusion 6C System Kit Contents
- SwabSolution™

4.0 Procedure

4.1 DNA TEMPLATE:

4.1.1 Based upon the quantity of DNA isolated and its initial concentration, the scientist should have all samples at an optimal concentration for amplification (e.g., 0.1ng/μl-0.2ng/μl). It is also convenient to have all samples that are to be amplified at the same time to be at the same concentration if possible for ease in the preparation of PCR Master Mix and reaction additions. The maximum amplification volume for low level or undetected samples is 15μl for PowerPlex Fusion 6C. For such samples, it may be appropriate to concentrate the sample prior to amplification using the DNA SpeedVac. The analyst may also choose to extract, quantitate, and combine additional sample prior to amplification as an alternative.

Note: If a sample is concentrated using the DNA SpeedVac, an equivalent volume of a corresponding reagent blank shall be similarly concentrated. For this reason it is advisable to include an additional reagent blank if it is suspected that DNA SpeedVac concentration will be performed.

4.1.2 The amount of DNA template added to an amplification reaction should be targeted at ~1.0-2.0ng but may be adjusted if necessary.

4.2 AMPLIFICATION SET-UP:

4.2.1 Determine the number of samples to be amplified.

4.2.2 Thaw the PowerPlex Fusion 6C 5X Master Mix, PowerPlex Fusion 6C 5X Primer Pair Mix, and Amplification Grade Water (optional).

4.2.3 Calculate the volume of reaction components needed based upon the number of samples (including extraction and amplification controls) to be amplified and adding additional reactions to compensate for loss and variability due to pipetting. Use 'Amp Worksheet' tab of the Fusion 6C Casework DNA Worksheets (Form 206-BI) or electronic equivalent in ILIMS to record thermal cycler positions and to automatically calculate necessary volumes. The following is a list of the 'fixed' amounts to be added for a 25μl reaction.

PowerPlex Fusion 6C 5X Master Mix	5μl
PowerPlex Fusion 6C 5X Primer Mix	5μl
DNA Template + dH2O	15μl

Note: The amount of reaction mix added to each sample is dependent on the volume needed to add the DNA template.

4.2.4 Prepare the reaction mix by pipetting the required volumes of 5X master mix, 5X primer mix, and water (if needed) into an appropriately sized microcentrifuge tube. Mix thoroughly and pulse-spin.

- 4.2.5 Pipette the appropriate amount of reaction mix into each well of a 96 well plate or microAmp tube if processing cases individually. The negative amplification control should be the last sample processed.
- 4.2.6 If DNA concentrations were not the same, add appropriate volume of dH₂O as necessary.
- 4.2.7 Pipette each DNA sample into the appropriate well. Place strip caps on each column of samples following addition of all samples to that column. When using microAmp tubes, only the tube to which the DNA is being added should be opened at this time and only one DNA-containing tube should be open at any time. Use 2800M control DNA for the positive amplification control and dH₂O or PCR-TE for the negative amplification control. Again, making additions to the negative control last.
- 4.2.8 Ensure all strip caps or sample tubes are closed tightly. Remove lab coat and, touching only the rack/MicroAmp tray, transport the samples to the thermal cycler in the Amp/PostAmp room, using the other hand on the door knob.
- 4.2.9 Place the samples into the thermal cycler. Do not set racks down in this room. Remove/discard gloves and return any racks to the main biology lab.

4.3 THERMAL CYCLING PARAMETERS:

Select the pre-programmed 'fusion 6C' thermal cycling profile with the following conditions:

96°C for 1 minute, then:

96°C for 5 seconds,
60°C for 1 minute
for 29 cycles, then:

60°C for 10 minutes, then:

4°C soak

Note: The ramp speed should be set to 'Max' and the reaction volume 25µl.

4.4 DIRECT AMPLIFICATION:

4.4.1 SAMPLE CUTTING/LYSATE PREPARATION

4.4.1.1 Direct amplification may be performed on casework reference samples.

Place ~1/4 of a buccal swab or ~3mm² FTA® cutting (or similar) into a single 1.5ml microcentrifuge tube or an EZ1 sample tube.

4.4.1.2 Add the following to each tube, depending on the substrate:

300µl SwabSolution™ for buccal swabs

50µl SwabSolution™ for FTA® cutting (or similar)

Note: A reagent blank tube will be created at this step.

4.4.1.3 Mix thoroughly and incubate at 70°C for ~30 minutes.

4.4.1.4 Following incubation briefly mix and spin down each tube to ensure the contents are at the bottom of the tube. It is not necessary to remove swab cutting(s) from the tube.

4.4.2 AMPLIFICATION SET UP OF DIRECT AMPLIFICATION SAMPLES

4.4.2.1 The method for direct amplification samples is the same as Section 4.2 above, with the following exceptions:

Reagent Component Volumes:

PowerPlex Fusion 6C 5X Master Mix	2.5µl
PowerPlex Fusion 6C 5X Primer Mix	2.5µl
5X AmpSolution™	2.5µl
Amplification Grade Water	3.0µl
SwabSolution™ Lysate	2.0µl

Note: Positive controls for direct amplification should be targeting ~2.5ng

Thermal Cycling Parameters:

96°C for 1 minute, then:

96°C for 5 seconds

60°C for 1 minute

for 26 cycles, then:

60°C for 10 minutes

4°C soak

Note: The ramp speed should be set to 'Max' and the reaction volume 13µl.

4.5 AMPLIFIED DNA PRODUCT:

- 4.5.1 After cycling has concluded remove samples from thermal cycler. Samples should be run on the Genetic Analyzer as soon as possible after amplification. Prior to capillary electrophoresis and/or before analysis is completed the samples may be stored at approximately 4°C. For longer storage periods, samples should be frozen at approximately -20°C. Amplified product is ONLY stored in the Amp/PostAmp room.
- 4.5.2 At a point in time after STR analysis is completed (i.e., case has been reviewed and report approved), the amplified product will be disposed of in a biohazard container in the amp/post-amp room. As needed, this container will be sealed and transported directly out of the laboratory. The container will be placed into a second biohazard bag, sealed and disposed of with other biohazardous material.

5.0 Comments

- 5.1 Clean surfaces with freshly made 10% bleach solution or bleach substitute prior to set-up.
- 5.2 Wear gloves at all times during amplification set-up.
- 5.3 Mix all reagents thoroughly (e.g., vortex) and do not centrifuge prior to dispensing.
- 5.4 After initial thawing, the 2800M DNA standard and any dilutions will be stored at approximately 4°C.

STR Typing: Capillary Electrophoresis and Data Analysis (BI-210)

1.0 Background/References

1.1 Any eukaryotic genome is interspersed with repeated DNA sequences that are typically classified by the length of the core repeat sequence, and the range of contiguous repeats typically seen or the overall length of the repeat region. STR (Short Tandem Repeat) loci are scattered throughout the genome occurring every 10,000 nucleotides or so, and have core repeat units of 2-6 base pairs (bp) in length with overall lengths of less than 400 bp.

STR loci examined for human identification purposes were selected for the following characteristics: 1) high discriminating power (generally >0.9) with observed heterozygosity of >70%, 2) loci on separate chromosomes to avoid linkage, 3) ability to obtain robust, quality, reproducible data when multiplex amplification is performed, 4) low stutter, 5) low mutation rate and 6) small allele sizes (<500 bp) for enhancement of analysis of degraded samples.

By 1997, as the result of a community-wide forensic science effort, the following 13 STR loci, all tetranucleotide repeats, were selected as the basis for NDIS, the CODIS (COmbined DNA Index System) National Database: D3S1358, TH01, D21S11, D18S51, D5S818, D13S317, D7S820, D16S539, CSF1PO, vWA, D8S1179, TPOX, FGA. When all 13 CODIS core loci were examined, the average random match probability was found to be <1 in 1×10^{12} among unrelated individuals, offering the promise of individualization. In 2017 the CODIS core loci expand from 13 to 20 with the addition of: D1S1656, D2S441, D10S1248, D2S1338, D12S391, D19S433, and D22S1045.

In addition to the 20 core CODIS loci, the PowerPlex Fusion 6C multiplex includes a gender identification locus (Amelogenin), Y-STR loci (DYS391, DYS576, and DYS570), two pentanucleotide repeat STR loci (Penta D and Penta E), and SE33. STR typing (with amplified products generated from this kit, separated by capillary electrophoresis on the 3500 Genetic Analyzer using data collection and analysis software employed in developing the genetic profiles) will be used to produce STR profiles from evidentiary material for case comparisons and applicable entry into CODIS.

1.2 References

- 1.2.1 Butler, J. Forensic DNA Typing: Biology and Technology Behind STR Markers. (2001) Academic Press.
- 1.2.2 PowerPlex® Fusion 6C System Technical Manual
- 1.2.3 PowerPlex® 6C Matrix Standard Technical Manual
- 1.2.4 ABI 3500/3500xl Genetic Analyzer User Guide

2.0 Scope

2.1 To provide a reliable method for generating STR genetic profiles from forensic casework and offender DNA database samples.

3.0 Equipment/Reagents

- 3500 Genetic Analyzer with Data Collection Software
- GeneMapper™ IDX Software
- Computer
- Heating Block (or 9700 Thermal Cycler)
- Capillary Arrays
- Syringe
- Pipettes and Filter Barrier Tips
- 96 Well Reaction Plates and Septa
- Buffer Reservoirs and Septa
- POP4 Polymer
- Genetic Analyzer Buffer (Anode and Cathode)
- PowerPlex® Fusion 6C Kit Contents
- PowerPlex® 6C Matrix Standards
- Deionized Formamide
- Nanopure Water

4.0 Procedure

4.1 AMPLIFIED FRAGMENT DETECTION USING THE 3500

Note: Prior to using the ABI 3500 Genetic Analyzer for samples, a spectral calibration (matrix standards) must be run to achieve proper color separation of the dyes used for the amplification primers, allelic ladders and size standard. To prepare a matrix, six standards are run under the same capillary electrophoresis conditions that will be used for samples and allelic ladders. Use the 6C Matrix Standard set, which includes the FL-6C, JOE-6C, TMR-6C, CXR-6C, TOM-6C and WEN dyes in a single tube for the blue, green, yellow, red, purple, and orange matrix standards, respectively. This is performed when necessary due to performance, or after any instrument maintenance/repair that involves adjustment/replacement of the CCD camera or laser, or a new capillary is installed.

Additionally, a Spatial Calibration must be performed prior to running any samples. The instrument uses images collected during the spatial calibration to establish a relationship between the signal emitted for each capillary, as well as the position where that signal falls and is detected by the CCD camera. This is performed any time a capillary is installed or replaced (including temporary removal of a capillary) or if the instrument is moved.

4.1.1 Turn on the computer but do not log in. Turn on the instrument. Wait until the instrument shows a steady green light, then log into the computer. Once a “Y” is showing for all applications in the bottom right menu, start the Data Collection Software. Refer to the ABI 3500/3500xl Genetic Analyzers Getting Started Guide for detailed instructions on instrument set-up (including creation of instrument protocols, results groups, and spatial calibration).

Shut down is performed in the opposite order (Data Collection software, 3500, then computer).

4.1.2 Create a new plate record:

4.1.2.1 For a spectral calibration plate, go to the Maintenance tab, select “Spectral”, and under the Calibration Run tab, choose the appropriate fields:

- Chemistry Standard: Matrix Standard
- Dye Set: Promega J6

4.1.2.2 For a sample plate fill out the ‘3500 Load Sheet’ tab of the casework worksheets (Form 207-BI) or electronic equivalent in ILIMS. Print a copy for the case record if applicable. Choose the ‘3500 Plate Document’ tab and ensure the information corresponds to the Load Sheet information entered.

Verify the information on the template is as follows:

- Plate Name: [Batch name, ..._REIN], ..._RESET, etc.]
- Application Type: HID
- Capillary Length (cm): 36
- Polymer: POP4
- Number of Wells: 96
- Owner Name: [Initials]
- Sample Name:
 - Allelic Ladder: LADDER_[well]
 - Controls: POS_CTRL, NEG_CTRL, QRB (questioned reagent blank), KRB (known reagent blank), RB...F2 (sperm reagent blank), RB...F1 (epithelial cell reagent blank), etc. Note: the batch date and analyst initials will also be added to the reagent blank names as identification for future analyses.
 - Case Samples: Y9999-9999_XZZ..., where:
 - Y = Letter for Lab (M, C, or P)
 - 9999-9999= Lab Case Number
 - X= Specimen Type (Q=Questioned; V=Victim; S=Suspect; E=Elimination; M=Mother; F=Alleged Father; C=Child; FB=Paternal uncle; FS=Paternal Aunt; FM=Paternal Grandmother; FF=Paternal Grandfather, etc.)

- ZZ...= numbers and letters that designate case Item (including 'F2' for sperm cell and 'F1' for epithelial cell at end of number to delineate fraction). (e.g., M2001-0112_Q1AF1)
- Sample Type: Sample Categories of 'Sample', 'Allelic Ladder', 'Positive Control', or 'Negative Control', may optionally be typed in.
 - Note: If the ladders are not at least designated here or in the software, a warning will occur stating you do not have any ladders on the plate.
- Results Group 1: Enter the appropriate results group.
- Instrument Protocol 1: Enter the appropriate instrument protocol (i.e., Fusion6C_15sec, Fusion6C_10sec)
PowerPlex Fusion 6C specific assay and protocol settings for sample plates are as follows:

- Application Type: HID
- QC Protocol: ILS 500
- Capillary Length: 36 cm
- Polymer: POP4
- Dye Set: Promega_J6
- Run Module: HID36_POP4
- Inj. Voltage (kV): 1.2
- Oven Temp (°C): 60
- Run Time (sec): 1500
- Run Voltage (kV): 13
- PreRun Tim (sec): 180
- Prerun Voltage (kV): 15
- Inj. Time (sec): 15/10
- Inj. Voltage (kV): 1.2
- Data Delay (sec): 1

- 15 seconds is the default injection time, 10 seconds may be used for overblown samples run with direct amplification.
- Run time may be increased, as needed, to account for temperature fluctuations affecting the run.

4.1.2.3 Perform a 'Save As' of the plate document to disc (i.e. USB drive) for subsequent transfer to the ABI 3500. The document must be saved as a .txt file (tab delimited).

4.1.2.4 To import the previously saved plate document, click "Workflow" from the dashboard, "Assign Plate Contents", then "Import". Browse to locate the saved .txt file and choose 'OK'.

- 4.1.2.5 Review the information to ensure that it is correct or make changes as necessary.
- 4.1.2.6 To duplicate an injection (a replicate injection that uses the same instrument protocol as the original injection) select Create Injection List > Preview Run > select the injection to duplicate > Duplicate Injection button.
- 4.1.2.7 Reinjections using a different protocol (ie: a lower injection time) can be added in the Monitor Run screen after the run has begun. Both reinjections and duplications may be added at any point in the run, prior to the last injection, if the scientist notices that a sample would benefit from reinjection (e.g., repeat because of bad injection or to vary injection times [from 10-15 seconds]). Additional Results Groups and Instrument Protocols may also be filled in on the original Load Sheet template prior to importing.
- 4.1.3 The scientist may choose to preheat the oven so that the instrument will be ready to run. It is recommended to start the preheat at least 30 minutes before the run if the instrument is cold. From the dashboard screen, ensure the temperature is set to 60 °C, then click the Start Pre-Heat button. Note: The oven will automatically turn off after 2 hours of instrument inactivity.
- 4.1.4 Prepare samples for capillary electrophoresis:
- 4.1.4.1 For amplified products (including controls), typically 1µl – 1.5µl of sample is added to 10µl of ILS Master Mix (made by adding 0.5µl WEN Internal Lane Standard 500 (WEN ILS500) size standard/sample; 9.5µl deionized formamide/sample and adding additional sample quantities) that has been dispensed into the wells of a pre-labeled plate. For Allelic Ladders add 1µl Ladder to 10µl Master Mix. Note: The master mix may be altered by adding 0.25µl WEN ILS500 size standard to 9.75µl deionized formamide if ILS peaks are too high.
- 4.1.4.2 The 6C Matrix Mix is diluted by adding 10µl to one tube of Matrix Dilution Buffer. 10µl of the diluted 6C Matrix Mix is then added to 500µl of deionized formamide (without size standard). Load 15µl of the 6C Matrix Mix with formamide into each of eight wells on the pre-labeled plate, which will include each of the eight capillaries (e.g. wells A1 through H1).
Note: At the first use, thaw the 6C Matrix Mix and Matrix Dilution Buffer completely. After the first use, store the reagents at 2-10°C, protected from light. The diluted 6C Matrix Mix can be stored for up to 1 week at 2-10°C.
- 4.1.5 Following sample addition, place a plate septa on the plate. The plate septa may be cut to cover only those well columns being used on smaller plate runs. Note: Optional heat denature for ~3 minutes at 95°C for amplified product ONLY. Do not heat denature the matrix standard.
- 4.1.6 Place the sample plate into the plate base and secure the plate retainer clip on top, making sure that no gray is visible through the holes.

4.1.7 Place the plate assembly in the instrument and close the doors. From the Assign Plates for Run screen, click Link Plate for Run.

4.1.8 Verify the correct scheduling of the run in the 'Preview Run' screen. Select a run and confirm that the corresponding wells highlighted in the plate diagram are correct for that run. Make adjustments to the plate record if necessary.

4.1.9 Click Start Run. Monitor electrophoresis in the Monitor Run screen. Each injection (set of eight samples) will take ~40 minutes.

4.1.10 After completion of the spectral calibration run, evaluate the spectral and set the active calibration. Confirm that Dye Set J6 is selected. Click on individual wells in the plate diagram to see results for each of the eight capillaries. For each capillary, verify that six peaks are present in the spectral profile (lower pane), that the order of the peaks are, from left to right, blue-green-yellow-red-purple-orange, and that the peaks are regular in appearance. Next verify that six peaks are present in the raw data profile (upper pane), that the order of the peaks are, from left to right, orange-red-yellow-green-blue-purple.

Note: One adjacent-capillary borrowing event is allowed for an 8-capillary instrument. A passing capillary will be colored green in the plate diagram, borrowed will be yellow.

Rerun the spectral calibration as necessary until a passing spectral run is achieved.

4.2 DATA ANALYSIS: GENEMAPPER® IDX (GMIDX)

4.2.1 Data analysis is NOT performed on the instrument computers. Transfer the run folder (including the sample sheet) to an analysis computer using a portable USB drive. After analysis and review are complete, a copy of the run folder and GMIDX project will be stored on the ISFIS network drive. The Run Folder on the instrument computer may be deleted at this point. Case-specific CDs will be made for discovery upon request.

Note: prior to data analysis, the appropriate panels and bins must be imported into GeneMapper® IDX. Additionally, previously run 310 Macintosh data must first be converted to PC files using the 'Mac to Win' conversion program.

4.2.2 Set up the analysis methods for GMIDX analysis as follows (analysis methods are created and stored in the 'Analysis Methods' tab in 'GeneMapper Manager'):

- 'General' Tab: Name the analysis method so that it reflects what the method is [e.g. Fusion6C – CW Direct Amp].

- ‘Allele’ Tab: Choose the appropriate bin set. For extracted samples, choose ‘Use marker-specific stutter ratio and distance if available’. All values should be 0. Note: questioned samples should be exported for STRmix without the stutter filters applied. For direct amplification samples, choose ‘Use marker-specific stutter ratio and distance if available’. ‘Global Cut-off Value’ should be 0.2, ‘Global Minus Stutter Distance’ should be from 3.25 to 4.75 for the Tetra marker repeat type.
- ‘Peak Detector’ Tab: Advanced Peak Detection Algorithm, light smoothing, Local Southern size calling method with baseline window of 51 pts, min. peak half width = 2, polynomial degree = 3, peak window size = 15, and slope thresholds = 0.
 - Analysis range may be set to either full or partial and is empirically determined for each run and/or instrument. When using partial range, the start and stop points are determined by a review of the raw data and choosing points that will not include the primer peaks but will cover the size range of 60 to ≥ 500 bases.
 - Peak Amplitude Threshold was empirically established as 70 RFU in all colors for extracted samples, 75 RFU in all colors for direct amplification samples. Peaks below these thresholds are deemed inconclusive for their respective sample types.
- ‘Peak Quality’ Tab: The minimum peak height ratio for Heterozygote Balance should be set at 0.7. Set the max peak width to 1.5 bp and pull-up ratio to 0.05. For extracted samples, the signal level minimum peak heights should be set to 400.0 for Homozygous, 70.0 for Heterozygous. For direct amplification samples, the signal level minimum peak heights should be set to 250.0 for Homozygous, 75.0 for Heterozygous. Allele number may be set according to analyst preference and sample type.
- ‘Quality Flags’ Tab: The quality flags are only used as a tool to aid in data analysis and review (i.e. to assist in calling attention to potential artifacts or data quality concerns). These flag settings may be adjusted according to analyst preference and sample quality.

4.2.3 Create and store a size standard for GMIDX analysis, under the ‘Size Standards’ tab in ‘GeneMapper Manager’. Name the size standard so that it reflects what the standard is [e.g. WEN_ILS500 (60-500)].

Data analysis will be performed using the ‘Basic or Advanced’ size standard. The size standard consists of the following peaks: 60, 65, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, and 500.

4.2.4 Create a GeneMapper® IDX Project:

- 4.2.4.1 From the GMIDX main menu, select File/Add Samples to project. Highlight the appropriate run folder in the pop-up window and click 'Add to List'. Once the run folder has been copied to the column on the right, click 'Add' to populate the project with the samples in the run folder.
- 4.2.4.2 In the Samples table, for each sample, select the sample type, analysis method, panel, and size standard from the pull-down lists if not auto-populated based on the 3500 import file. Ladders must be assigned the sample type of 'Allelic Ladder' for the analysis to occur. In order to use the control concordance quality flag, all controls must be marked appropriately as either 'Positive Control', or 'Negative Control'. All others may be marked as 'Sample'.
- 4.2.4.3 Save the project as the batch # (and any other descriptors that may be necessary). Optionally, samples may be added to the project individually, rather than the entire run folder. Note: the analyzed project will be exported to the batch folder at the completion of analysis.
- 4.2.4.4 Analyze the samples by clicking the green Analyze button. If the project has not already been saved, a prompt will appear to enter a project name before analysis will commence.
- 4.2.5 Evaluate GeneMapper® IDX Data:
- 4.2.5.1 The Raw Data may be reviewed to determine analysis start/stop points, or to identify baseline problems, off-scale data, excessive 'spikes' or other anomalies that may interfere with data analysis and require re-injection or other corrective measures. Expand the run folder located in the navigation pane on the left. Highlight the sample(s) of interest to view the associated sample information, raw data and EPT data. Minimize or highlight the run folder to return to the main project window.
- 4.2.5.2 Check the 'SQ' (sizing quality) for all samples. A green square indicates that the sample has passed the sizing criteria and need not be manually examined. Examine the size standard of each sample with yellow and/or red 'SQ' to confirm correct assignment of fragment sizes. Highlight the sample(s) of interest and click the Size Match Editor button. If necessary, adjust the peak assignments by right clicking on a peak and deleting, adding, and/or changing values. If all peaks are correctly labeled but the quality score is below 1.0 (may be checked by choosing Tools/Check Sizing Quality), click the 'Override SQ' button to set the SQ to 1.0, if desired. Once all edits have been made, click 'OK' to save the changes and close the Size Match Editor (clicking 'Apply' saves the changes but leaves the Size Match Editor open). These samples are ready for reanalysis in the project window.
- 4.2.5.3 Examine the blue, green, yellow, red, and purple allelic ladders. Check that correct allelic assignments were made.

Note: GMIDX automatically averages all valid ladders in a run for genotyping. Genotypes are assigned by comparing the sizing of unknown alleles from samples with the sizing of known alleles contained within the averaged allelic ladders of each locus. A ladder(s) may be omitted from analysis by deleting it from the main project window prior to analysis or by changing its sample type to "Sample".

- 4.2.5.4 Data may be examined in various combinations of colors and/or tables to identify bleed-through, spikes, stutter, -A, off-ladder variants, etc. Sample Plots viewed from the 'Samples' tab/window, allows all loci in a given color(s) to be viewed simultaneously. The Sample Plots view from the 'Genotypes' tab/window; however, allows loci to be viewed individually (more than one locus can be viewed at the same time by adjusting the number of panes displayed).
- 4.2.5.5 GeneMapper® IDX includes a series of quality flags (PQVs) to alert the analyst of potential sample quality concerns. A green square indicates that sample data has passed all of the quality checks, but yellow or red indicate that the data has a problem with one or more of the quality checks. A yellow or red flag does not necessarily mean that the data is bad or unusable and the flags are not to be relied on solely. The analyst may choose to use the PQVs, in combination with manual data examination to aid in the identification of bleed-through, spikes, stutter, off-ladder variants, -A, etc. Once the data has been evaluated and deemed acceptable, the analyst may choose to override the yellow or red Genotype Quality (GQ) flag by right clicking on the flag in the Genotypes Sample Plots view. Note: overriding the GQ flag will cause all other flags to turn from the original color to gray.
- 4.2.5.6 All negative controls (including reagent blanks) should be examined to verify that each displays a relatively flat baseline in blue, green, yellow, red, and purple.
- 4.2.5.7 Review all samples (including positive controls) for the above listed 'artifacts' and evaluate: peak height and shape, matrix/spectral quality, and individual sample profiles. Compare each sample with the allelic ladder(s) and examine for off-ladder or microvariants, signals that were too low to be genotyped and assignment of genotypes to stutter peaks (or minor peaks that may have been subtracted as 'stutter', etc).
- 4.2.5.8 Edit peaks as necessary, by right clicking on the peak label and selecting 'add allele', 'delete allele' or 'rename allele'. The allele should be labeled, at minimum with the allele call, however the analyst may select up to four allele labels, including peak height and size, from the 'Plot Settings Editor' window. Note: labels added to artifact peaks, such as spike, pull-up, etc. will appear in the Genotypes table as an additional allele.

- 4.2.5.9 Samples demonstrating an off-ladder (< or > smallest or largest ladder allele, respectively), tri-allele, or microvariant (alleles with incomplete repeats) allele(s) should be re-analyzed for verification where necessary (e.g., evidentiary profile in no-suspect case). Microvariants will be labeled and reported as "X.Y" (where X is the number of complete repeats and Y is the number of base pairs of the incomplete repeat). Off-ladder will be reported as > or < the largest or smallest ladder allele, respectively. Note: the nomenclature for upload to NDIS may necessitate a change in allele designation. STRmix requires only numeric values and off-ladder alleles reported as > or < should be assigned the numeric value for the actual extrapolated allele call (e.g., 26 instead of >25) prior to export.
- 4.2.5.10 GMIDX automatically flags off-scale (camera saturation) data. This data may still be acceptable if it is limited to a few or a single peak and the overall data for that sample is of good quality.
- 4.2.5.11 An allele/genotypes table should be exported as a text file for import into ILIMS. The table may also be exported as a .cmf file for CODIS import. To create a .cmf file, the specimen category must be assigned and the export fields set in the 'CODIS Export Manager' under tools in the main menu. Tables for STRmix should be exported as text files for questioned (allele, size, height) and knowns (allele, size) with 12 alleles per locus.
- 4.2.5.12 'Samples Plots', consisting of only the individual case samples, will be printed as pdf for case notes. It is not necessary to include the internal lane standard dye channel.

5.0 Comments

- 5.1 The 3500 Data Collection Software does not allow the entry of spaces in titles, sample names, etc. An alternative character (e.g., underscore or dash) must be used in place of spaces when entering information.
- 5.2 If data is rejected and will not be used for reporting purposes, a note will be made in the associated worksheets/case file. If a reinjection is performed after data analysis, a re-setup of the CE plate is made, or a re-amplification is performed, notes will be made accordingly to indicate the rework performed. The new data will be reported unless otherwise noted in the case file. A sample set up for multiple injections upfront does not constitute rework.
- 5.3 If an unresolved peak is observed, a note should be made on the electropherogram after printing as to the allele call, approximate size and height. This locus will need to be ignored for interpretation.

STR Interpretation Guidelines and Statistical Analyses (BI-212)

1.0 Background/References

1.1 Following generation of an STR profile the results must be interpreted by a forensic scientist. This analytical method provides general interpretation guidelines for interpreting samples in forensic DNA casework. These guidelines are designed in order to ensure that conclusions are supported by reliable data, derived using a scientific approach, are objective, and 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 but may not account for all scenarios that will be encountered. In those scenarios the forensic scientist and DNA technical lead will determine the course of action that is scientifically most appropriate.

Software programs are available to the forensic scientist as tools to aide in their interpretation. 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. DNA analysis is a comparative test and STRmix™ provides statistical weight to comparisons by calculating Likelihood Ratios (LRs). The PopStats software from the FBI is used for statistical calculations in paternity/kinship cases.

As part of the interpretation and write up process, analyst will ensure CODIS profiles meet eligibility requirements as defined by the FBI.

1.2 References

1.2.1 STRmix Operations and User's Manuals

1.2.2 ISP Forensic Services STRmix and Fusion 6C Validations

1.2.3 D. Taylor, J.-A. Bright and J.S. Buckleton. The interpretation of single source and mixed DNA profiles. *Forensic Science International: Genetics*, 2013 7(5): 516-528.

1.2.4 J.-A. Bright, D. Taylor, J.M. Curran and J.S. Buckleton. Developing allelic and stutter peak height models for a continuous method of DNA interpretation. *Forensic Science International: Genetics*, 2013. 7(2): 296-304.

1.2.5 J.-A. Bright, D. Taylor, J.M. Curran and J.S. Buckleton. Degradation of forensic DNA profiles. *Australian Journal of Forensic Sciences*, 2013. 45(4): 445-449.

1.2.6 D. Taylor. Using continuous DNA interpretation methods to revisit likelihood ratio behaviour. *Forensic Science International: Genetics*, 2014. 11: 144-153.

- 1.2.7 J.-A. Bright, D. Taylor, J.M. Curran and J.S. Buckleton. Searching mixed DNA profiles directly against profile databases. *Forensic Science International: Genetics*, 2014. 9: 102-110.1.2.8
- 1.2.8 J.-A. Bright, J.M. Curran and J.S. Buckleton. The effect of the uncertainty in the number of contributors to mixed DNA profiles on profile interpretation. *Forensic Science International: Genetics*, 2014. 12: 208-214.
- 1.2.9 J.-A. Bright, I.W. Evett, D. Taylor, J.M. Curran and J.S. Buckleton. A series of recommended tests when validating probabilistic DNA profile interpretation software. *Forensic Science International: Genetics*, 2015. 14: 125-131.
- 1.2.10 J.-A. Bright, K.E. Stevenson, J.M. Curran and J.S. Buckleton. The variability in likelihood ratios due to different mechanisms. *Forensic Science International: Genetics*, 2015. 14: 187-190.
- 1.2.11 D. Taylor, J.-A. Bright and J.S. Buckleton. Considering relatives when assessing the evidential strength of mixed DNA profiles. *Forensic Science International: Genetics*, 2014. 13: 259-263.

2.0 Scope

- 2.1 To provide a reliable and consistent method for interpreting STR genetic profiles from forensic casework and performing statistical analyses.

3.0 Equipment/Reagents

- GeneMapper™ IDX Software
- Computer
- STRmix™ Software
- PopStats Software

4.0 Procedure

4.1 CONTROLS

4.1.1 The purpose of a REAGENT BLANK (RB) is to determine if the reagents used for DNA extraction/isolation were contaminated with human DNA and as a method for monitoring facility decontamination. In GeneMapper® IDX, peaks above threshold should only appear in the WEN (orange dye) lane, corresponding to the WEN ILS500 size standard. Electropherograms for the blue, green, yellow, red, and purple dyes should show a relatively flat baseline throughout the range (discounting primer signal, fluorescent 'spikes' or WEN bleed-through). If detectable signal, with characteristic 'peak' shape is visible in the electropherogram of a reagent blank and does not disappear upon re-injection, results for all associated samples may be deemed inconclusive (close examination at 70 RFU is performed on all samples to examine for presence of any alleles seen in the RB). Data may be deemed acceptable if contamination is 'isolated' to the RB. The reagent blank should be treated the same as the least concentrated DNA sample within the associated batch, in terms of volume and amount amplified, injection time/amount, and analysis threshold. If more than one reagent blank is processed with a batch of samples, the blank with the highest signal detected at quantitation (if applicable) will be amplified. The remaining blank(s) need not be carried through. Additionally, the reagent blank will be reamplified with samples from the set if any of the amplifications conditions are more sensitive than the original.

4.1.2 The purpose of the POSITIVE AMPLIFICATION CONTROL (2800M DNA supplied with the Fusion 6C kit) is to assess the amplification process, ensuring that adequate sample amplified simultaneously would produce an appropriate signal. All expected alleles (see below) must be detected, using standard parameters or all of the samples associated with amplification may be deemed inconclusive. Data may be deemed acceptable if all alleles are present [though some (generally 4 or fewer) are below analytical threshold] AND relative peak heights of all other samples appear as expected (i.e. the problem is confined to the 2800M sample).

LOCUS	GENOTYPE	LOCUS	GENOTYPE	LOCUS	GENOTYPE
AMEL	X,Y	D3S1358	17,18	D1S1656	12,13
D2S441	10,14	D10S1248	13,15	D13S317	9,11
Penta E	7,14	D16S539	9,13	D18S51	16,18
D2S1338	22,25	CSF1PO	12,12	Penta D	12,13
TH01	6,9.3	vWA	16,19	D21S11	29,31.2
D7S820	8,11	D5S818	12,12	TPOX	11,11
D8S1179	14,15	D12S391	18,23	D19S433	13,14
SE33	15,16	D22S1045	16,16	DYS391	10
FGA	20,23	DYS576	18	DYS570	17

4.1.3 The purpose of the NEGATIVE AMPLIFICATION CONTROL is to determine if any human DNA contamination occurred in the process of amplification set-up (or beyond that point) and as another method of monitoring facility decontamination. In the GeneMapper® IDX electropherograms, peaks above threshold should only appear in the WEN (orange dye) lane, corresponding to the WEN ILS500 size standard. Electropherograms for the blue, green, yellow, red, and purple dyes should show a relatively flat baseline throughout the range (discounting primer signal, fluorescent 'spikes' or WEN bleed-through). If detectable signal, with characteristic 'peak' shape, is visible in the electropherogram of a negative amplification control and does not disappear upon re-injection, results for all of the samples associated with that amplification will be examined for the presence of the same peak(s). The negative amplification control should be treated the same as the positive amplification control within the associated batch, in terms of volume and amount amplified, injection time/amount, and analysis threshold.

If extraneous peaks appear only in this control, the data for other samples associated with that amplification need not be deemed inconclusive. This occurrence should be documented and the scientist's determination (and basis for it) documented in the case file.

4.1.4 After evaluation of the data, the Profile Comparison tool in GMIDX may be used to assist with contamination evaluation. From the GMIDX main screen, select Tools > Profile Comparison.

4.1.4.1. Export the Sample Concordance tab results.

4.1.4.2. Select the Sample Comparison tab and "Compare Profiles" at 80% Match Threshold. Export the results. If there are no profiles that meet the 80% threshold, a popup will occur that states "No matches found that meet the selected percent match threshold" after the "Compare Profiles" button is selected. If this occurs, a screenshot may be taken (be sure to include the plate name!) to show the comparisons were attempted. Note: The popup that states "There is no data to export" does not indicate that the comparisons were attempted and therefore should not be included.

4.1.4.3. Evaluate the exported comparisons for potential contamination.

4.2 RFU THRESHOLD:

4.2.1 A threshold of 70 RFU will be used for data analysis of all extracted samples, 75 RFU for direct amplification samples. If necessary, go back in the process as follows: repeat injection, perform re-analysis (i.e., changing amount of amplified product added for fragment analysis), re-amplification (increase DNA template), or re-extraction.

4.2.2 Peaks below the analysis threshold (based on data obtained and signal/baseline) will not be interpreted.

4.2.3 Peaks marked as off-scale in GeneMapper® IDX (indicating camera saturation) will not be interpreted if multiple peaks are affected and if it causes excessive artifacts (i.e. split peaks, increased stutter, pull-up, etc.) which interfere with data interpretation. If the overall quality of the data is not acceptable, the sample must be diluted, reinjected, reanalyzed (decrease the amount of amplified product added) or reamplified (decrease DNA template) as deemed appropriate by the scientist. Note: Peaks that exceed the saturation threshold of 30,000 RFU in STRmix are treated qualitatively.

4.2.4 Multiplex amplification kits are designed so that heterozygous loci in single-source samples generally demonstrate relatively balanced peak heights [typically >70% peak height ratio (phr)]. Some samples, although single-source, may at times demonstrate greater imbalance due to degradation, stochastic effects, primer binding site mutations, preferential amplification, etc. Peak height ratios for these loci (<70% phr) will be flagged in GeneMapper® IDX.

4.3 EXTRA PEAKS (NON-MIXTURES)

4.3.1 PCR amplification of STR loci typically produces a minor product peak one core repeat unit shorter than the main allele peak (n-3 for trinucleotide loci, n-4 for tetranucleotide loci, and n-5 for pentanucleotide loci). This minor peak is referred to as the stutter peak. Percent stutter generally increases with allele length and does not change significantly with the quantity of input DNA (peak heights within ~70-4500 RFU); however, may be increased in low-copy samples. The measurement of percent stutter may be unnaturally high for main peaks that are off-scale or due to problems with spectral performance and can be corrected by diluting (or reamplifying less DNA) the sample and/or applying a new spectral. n+3/n+4/n+5 and n-6/n-8/n-10 stutter may also be seen. Marker specific stutter filters are applied in GeneMapper® IDX. Validation data is available to aid in distinguishing between true alleles and stutter peaks. For Fusion 6C, STRmix™ is able to model -1 repeat stutter (ie: n-4), +1 repeat stutter (ie: n+4), and -2 repeat stutter (ie: n-8) for all STR loci, and -1/2 repeat stutter (n-2) for D1S1656 and SE33.

4.3.2 Electronic or fluorescent spikes are random events that produce generally spike-shaped peaks in most or all dye colors at the same location (equivalent bp size) within a single injection. Peak heights usually vary between dye colors for a given spike. These anomalies are generally not reproducible and will typically be eliminated upon reinjection. If the spike is above the analysis threshold and falls within an allelic range that could interfere with either computer analysis or scientist's analysis, the scientist will label the spike in the GeneMapper® IDX software so that it appears on the printed electropherograms.

- 4.3.3 Dye “blobs” are anomalies that typically occur in the same approximate location in multiple injections and do not always disappear upon reinjection. Blobs generally look like broad or irregular peaks and may occur in a single color or multiple colors at the same approximate location but can vary in height. The blob should be labeled on the electropherogram (in GMIDX) if it falls within a diagnostic region and is of significant size to potentially interfere with analysis.
- 4.3.4 Bleed-through or pull-up peaks are a result of the spectral not correcting for all of the spectral overlap and may be increased due to off-scale peaks. These pull-up peaks are in the same location (same bp size) as peaks in another color(s) and are easily recognized. The presence of bleed-through should be labeled on the corresponding electropherogram (in GMIDX) if it falls within a diagnostic region and is of significant size to potentially interfere with analysis. If excessive bleed-through occurs in a color other than orange, and is not due to off-scale data, a new spectral may need to be performed.
- 4.3.5 Taq Polymerase can catalyze the addition of a single nucleotide (predominantly adenosine) to the 3' ends of double stranded PCR product, resulting in product one base pair longer than the actual target sequence (+A). Amplification parameters include a final extension time, so that the reaction is driven to full A addition (i.e. all product is +A). Split-peaks may occur as a result of incomplete A addition and appear as a single allele represented by two peaks one base pair apart (-A and +A). This can occur when the amount of template DNA is too great (overloaded sample). In this instance, Taq is unable to add the A nucleotide to the entire amount of product generated in the time allotted. These samples will typically contain off-scale data as well. Split peaks can be alleviated by incubating samples at 60°C for an additional 10 minutes, followed by dilution prior to reinjection. It may be necessary to re-amplify the sample with less template DNA.

4.4 MIXTURES

- 4.4.1 If, after the elimination of possible stutter and/or bleed-through, a profile shows at least 3 peaks at 2 or more loci, this is strong evidence of a mixture. A profile showing 3 or more peaks at a single locus and additional peaks below the analysis threshold at other loci is also indicative of a mixture.
- 4.4.2 Loci that demonstrate only two alleles but have a heterozygous ratio of <70% may also be indicative of a mixture. However, if data are obtained from multiple loci, a scientist should expect to see this or other mixture indications (> 2 alleles) at additional loci.
- 4.4.3 Mixture assessment, in terms of determining the presence of a mixture (# of potential contributors) is performed prior to examining the reference profiles.

4.5 Manual Interpretation

- 4.5.1 Comparison of probative and non-probative (as defined in Biology QA Manual section 10.3.7) reference samples to either a full or partial single source profile (or a fully deduced profile originating from a mixture) may be performed manually. If all of the alleles detected in the questioned sample are also detected in the probative reference sample, peak heights/peak balance conform to expectations, and any missing alleles are reasonably explainable, then the comparison may be deemed a match and a LR statistic will be calculated in STRmix. When the above criteria are met for comparison to a non-probative reference sample a qualitative statement, such as “consistent with”, may be used without a supporting statistic. If there are differences in alleles between a questioned sample and a reference sample that are not reasonably explainable, then an exclusionary statement may be used.
- 4.5.2 Mixtures may be interpreted manually in order to determine whether or not a non-probative reference sample is a potential contributor to the mixture. If all of the alleles present in a component of the mixture can be attributed to a non-probative reference sample, peak heights/peak balance conform to expectations for the component, and any missing alleles are reasonably explainable, then a qualitative statement may be used indicating that the non-probative reference sample is a potential contributor to the mixture without a supporting statistic. If all contributors to a mixture have been attributed to non-probative reference samples, then exclusionary statements may be used for probative reference samples without performing STRmix analysis.
- 4.5.3 If the complexity of a given profile is such that it is too difficult to interpret manually, then STRmix may be used to aid the interpretation even if it is a profile that is permissible to interpret manually as described above.
- 4.5.4 STRmix analysis may be conditioned upon a reference sample that was manually determined to be a potential contributor to a mixture if conditioning is appropriate.
- 4.5.5 Following deconvolution of a profile in STRmix, if the weighting for a single (fully deconvoluted) genotype is >99% at one or more loci, these loci may be utilized to manually exclude individuals not possessing the >99% genotype(s). If there is only a single locus where this is the case the analyst needs to inspect the data closely similarly to if a single locus yielded a LR of 0 as in step 4.6.2.5.
- 4.5.6 In some circumstances it may be beneficial to make inferences based on evidence to evidence comparisons. In such circumstances, only a qualitative statement (e.g., consistent with) shall be made with respect to reporting the comparison.

4.5.7 In cases where a probative single source profile (or a discernable profile originating from a mixture) is developed but a LR would not be calculated due to lack of reference samples for comparison (e.g., unknown suspect, or manual elimination of the available reference samples), the profile may be used for CODIS entry purposes without STRmix deconvolution.

4.6 STRmix Analysis

4.6.1 Suitable mixture profiles will be deconvoluted in STRmix prior to making conclusions about comparisons with probative known reference samples.

Note: Results that are too complex (5 or more contributors) or contain minimal data (typically results at fewer than 6 loci) should not be interpreted.

4.6.1.1 Prior to exporting data from GeneMapper® IDX questioned samples need to be exported with stutter filters turned off. Known samples must be exported with stutter filtered. +1 repeat stutter should be left labeled when exporting Fusion or Fusion 6C questioned sample data but should be deleted if exporting PP16 or PP16HS data. All -2 repeat stutter and -1/2 repeat stutter at D1S1656 and SE33 should be left labeled for Fusion 6C data. All other artifacts must be deleted prior to export. Questioned samples must be exported with allele, size, and height. Known samples must be exported with allele and size but may also include height. The export tables will typically contain 12 alleles per locus (if a profile contains more than 12 alleles, including stutter, then the number of alleles will need to be increased in both the export table and STRmix settings).

4.6.1.2 Launch the STRmix software and select “Interpretation” to begin analysis of a sample. Note: “Batch Mode” may be used if multiple samples are to be processed at once.

4.6.1.3 Enter the Case Number and Sample ID.

The Case Notes section may be utilized to document assumptions made; however, entering data into the number of contributors and hypotheses fields is sufficient documentation for these assumptions and comparisons. Assumptions may also, optionally, be documented on the corresponding electropherogram.

4.6.1.4 Enter the number of contributors to the profile. Note: The number of contributors will be based on the entire profile and will be the number that best explains the data. If the scientist is unable to determine the number of contributors to a profile it may be appropriate to interpret the profile using alternate numbers of contributors (e.g., 3 and 4) and report both statistics. In some scenarios where the number of contributors cannot be confidently determined it may be most appropriate to not interpret the profile.

Select the appropriate DNA kit.

MCMC accepts and # burn-in accepts default to 500,000 and 100,000 respectively. Note: Some complex mixtures may benefit from increasing the iteration #s 10-fold. This shall only be done following consultation with the DNA technical lead.

The “Other Settings” button may be used to adjust a default setting only for the run being performed. Any changes will appear in bold in the results. The default settings were determined either empirically via internal validation or are the manufacturer recommendations and shall not be changed without consulting the DNA technical lead with the exception of the “Ignore Loci” button which may occasionally need to be used as in scenarios where a tri-allelic locus is detected.

Select “Next” to proceed with analysis.

4.6.1.5 Under Evidence Profile Data select “+” and then “Select”. Navigate to the table exported from GeneMapper® IDX for questioned samples, highlight the appropriate sample, select “Confirm”. Note: It is also possible to use a STRmix input file or to create a file for the electropherogram (EPG). See the STRmix Manuals if this is necessary.

At this point a reference EPG will only be added if the interpretation is being conditioned upon the presence of a known contributor or if it is a single source profile and manual comparison has already included an individual. If this is the case a reference EPG can be added following the same method as above. After adding a reference EPG for conditioning, check the “Contributor to: Hd” box in order to assign the contributor to both Hp and Hd. If needed, check the “Perform Database Search” check box. Note: Suitable mixture profiles with an unknown contributor(s) will be compared to the default database which is set as the ISP staff elimination database. Single source profiles only need to be compared against the elimination database if the source is unknown and this comparison may be performed either in STRmix or CODIS. This is a quality assurance measure to check for possible sample contamination. The STRmix search will return candidate matches where the LR exceeds a certain threshold. The default setting for this threshold has been set to 10,000. Ultimately the forensic scientist will assess the search results and determine if contamination is or is not likely.

Select “Start” to begin the deconvolution.

4.6.1.6 The interpretation will be generated after deconvolution is complete.

At this point it is appropriate for the forensic scientist to inspect the report to verify that the analysis is appropriate. Some important aspects to check include: mixture proportions, run information diagnostics, locus efficiencies, and intuitiveness of the weighting. In general, mixture proportions and weighting will be consistent with the expectations of the analyst based on their manual review of the profile. Locus efficiencies are expected to approximately mimic the appearance of the electropherogram. Further information on interpreting run information diagnostics can be found in the STRmix Manuals.

Mixtures assumed to be from two individuals, contributors with less than 70 rfu for the STRmix deconvolution template should be considered trace contributors and not considered for comparisons. For mixtures assumed to be from three or four contributors, contributors with less than 100 rfu for the STRmix deconvolution template should be considered trace contributors and not considered for comparisons. Manual interpretation may be utilized when determining whether to condition on the donor of an intimate body swab prior to deconvolution (For example, a victim may be conditioned on her own vaginal swab without first determining the template amount via STRmix and remain conditioned on if the deconvolution does not meet the guideline template rfu values above). Exceptions to these guidelines may be made after consultation with the technical leader.

Contributor percentage, degradation, total allele count, number of minor contributors, number of obligate alleles, shared mixture proportions, assumed contributors, overall profile quality, and close relatedness of persons of interest (POIs) should all be considered when deeming one or more contributors in a mixture interpretable. If significant ambiguity exists with one or more contributors in a profile, which makes it impossible to intuitively evaluate the STRmix deconvolution or future comparisons, analysts should deem that portion of the profile uninterpretable. If no portion of the data can be evaluated intuitively, the entire profile should be deemed uninterpretable.

Close the .PDF file, then select “Finish” in the STRmix software.

4.6.2 Following initial deconvolution of suitable questioned mixture profiles, probative comparisons to known reference samples from POIs will be individually compared to the profile and LRs calculated using the “Investigation” > “LR from Previous” buttons within STRmix. Note: STRmix analysis may not be necessary for comparisons to single source profiles or those deemed non-probative in accordance with Biology QA Manual section 10.3.7.

Each POI shall be compared separately using the propositions:

Hp: POI and N-1 unknown contributors

Hd: N unknown contributors

Where N is the total number of contributors to the profile.

Or

Hp: Known, POI, and N-2 unknown contributors

Hd: Known and N-1 unknown contributors

Where known represents contributors whose presence the interpretation is conditioned upon.

If more than one POI results in a LR supporting inclusion, then it may be appropriate to calculate a LR under alternate propositions incorporating multiple POIs. This should only be done when supported by the data and case circumstances. The most relevant LR(s) will be reported and all LR reports will be retained in the case file. In this scenario the propositions used are:

Hp: POIs and N-X unknown contributors

Hd: N unknown contributors

Where X is the number of POIs in Hp.

Or

Hp: Known, POIs, and N-1-X unknown contributors

Hd: Known and N-1 unknown contributors

4.6.2.1 From the STRmix main screen select “Investigation”, then “LR from Previous” and navigate to the “results” file for the initial deconvolution of the sample of interest.

- 4.6.2.2 In the Sample ID section the item # of the known sample being compared may be added to the end of the questioned item Sample ID. Select “Next”.
- 4.6.2.3 Under Reference Profile Data select “+” and then “Add”. Navigate to the table exported from GeneMapper® IDX for known samples, highlight the appropriate sample, select “Confirm”, and select “Add”. Note: It is also possible to use a STRmix input file or to create a file for the EPG. See the STRmix Manuals if this is necessary.

Select “Start”.

- 4.6.2.4 Populations using the NIST allele frequency data from African American, Caucasian, and Hispanic populations will be the default. Theta values of 0.01 are to be used for each population. Additional population data may also be used when available and relevant to a particular case. For small, isolated populations (e.g., Native Americans) a θ value of 0.03 will be used.
- 4.6.2.5 A “LR from Previous Report” will be generated.

This report will contain a number of different LR calculations for the POI and shall be included in the case file. The LR value reported will be the lowest Total LR from the three population groups incorporating the 99.0% 1-sided lower HPD.

Comparisons in which STRmix assigns a POI to a contributor previously deemed uninterpretable, will be reported as an exclusion to the comparable portion of the profile. The report will be regenerated and the LR will be removed from the report.

If a single locus results in an LR of 0 while all other loci have LRs > 0 the forensic scientist needs to inspect the data closely.

The STRmix internal validation data shows that the LR ranges below support the associated conclusion:

PP16 or PP16HS Data

LR > 1,000 supports inclusion

0.001 < LR < 1,000 is inconclusive

LR < 0.001 supports exclusion

Fusion Data

LR > 100 supports inclusion

0.01 < LR < 100 is inconclusive

LR < 0.01 supports exclusion

Fusion 6C Data

The level of support is based on SWGDAM recommendation 1.2 from the SWGDAM recommendations for Genotyping Results Reported as Likelihood Ratios. This recommendation states “...A qualitative statement that conveys the degree of support indicated by the likelihood ratio may be reported in addition to the numerical value for the likelihood ratio...”.

The scale of verbal qualifiers from the SWGDAM document are as follows:

<i>LR for H_p Support and $1/LR$ for H_a Support</i>	Verbal Qualifier
1	Uninformative
2 – 99	Limited Support
100 – 9,999	Moderate Support
10,000 – 999,999	Strong Support
$\geq 1,000,000$	Very Strong Support

Close the .PDF file, then select “Finish” in the STRmix software.

4.6.2.6 Repeat the “LR from Previous” process for each known reference sample where the comparison is probative. After analysis and review are complete, a copy of the STRmix files for each case will be stored on the ISPFS network drive.

4.7 Major and Minor Contributors

4.7.1 LR statistical calculations performed by STRmix take into account contributor order through incorporation of the factor of $N!$ calculation. Therefore, the LR statistic applies to the mixture as a whole rather than a component (major or minor) of the mixture. Nevertheless, determination of major and minor contributors may provide useful qualitative information or be useful for CODIS searches. In addition to the criteria below, the mixture proportions and weights within STRmix may be useful in determining major and minor contributors.

4.7.2 In a probable 2-person mixture (no more than 4 alleles at any given locus) it may be possible to determine a major versus minor contributor at some or all loci. A major profile is one in which a distinct, predominant DNA profile is present (as determined by number of peaks, relative peak heights, and peak balance). A minor contributor is the less predominant DNA profile in the mixture.

4.7.3 For loci where distinct major/minor genotypes are discernible (this will occur rarely in a mix of more than two individuals' DNA), both genotypes may be reported.

4.7.4 Given that heterozygous peak ratios are not 100% (complete balance), caution must be exercised in determining "shared alleles", as a scientist does not know (a priori) which allele of a heterozygous individual may be predominant (i.e., the "highest RFU peak" in the 3-peak mixture may not be the shared allele). Calculations to determine the relative peak height ratios of 3-peak loci may be performed to assist in this determination.

4.8 Conclusions

4.8.1 PP16/PP16HS and Fusion: In general, an LR > 1 supports Hp (inclusion) and an LR < 1 supports Hd (exclusion). An LR = 1 is typically viewed as uninformative. STRmix validation data shows that occasionally a known non-contributor can result in an LR > 1 and a true contributor an LR < 1. The range of LRs for which this may occur was observed to be between 0.001 and 1,000 for PP16 or PP16HS data and between 0.01 and 100 for Fusion data during internal validation. For this reason, STRmix comparisons in which the LR is between 0.001 and 1,000 for PP16 or PP16HS data and between 0.01 and 100 for Fusion data will be reported as inconclusive. LRs > 1,000 for PP16 or PP16HS data and > 100 for Fusion data will be reported as an inclusion. LRs < 0.001 for PP16 or PP16HS data and < 0.01 for Fusion data will be reported as an exclusion. The numerical LR only needs to be reported for inclusions and inconclusive results.

Fusion 6C: The verbal scale above (4.6.2.5) will be used for Fusion 6C data. As LRs increase in magnitude, the scale reflects stronger degrees of support. LRs occur on a continuum. The categories recommended here have been chosen in part based on the observation that adventitious support for a proposition is most commonly observed within the Limited Support category and generally not expected within the Very Strong Support category. Adventitious support is described as an LR > 1 for an individual whose DNA is not present in the sample or an LR < 1 for an individual whose DNA is present in the sample. While incidents of adventitious support in the limited support category represent the rare minority of observations, they reduce but do not eliminate confidence in the association of this person to the DNA sample.

When an LR <1 is obtained, the inverse statistic will be reported, up to 1/1000. LRs <1/1000 will be reported as exclusions without reporting the LR, though it will be maintained in the case file.

The quality of the DNA profile and strength of the inclusion will be reflected in the magnitude of the LR. Note: See Section 10.3.7 of the Biology QA Manual for additional information on reporting of conclusions and statistics.

4.9 Paternity Statistics

4.9.1 For a paternity calculation, the LR corresponds to the PI (Paternity Index).

The PE (Probability of Exclusion) represents the probability that a randomly selected individual would possess one or more alleles inconsistent with paternity.

4.9.2 In addition to the LR and PE used in paternity, the probability of paternity may be used. However, given that this statistic requires non-genetic information (i.e., the prior odds of paternity), the prior odds used (e.g., 50%) should be explicitly stated (See Biology QA Manual, section 10.3.7 for reporting of statistical frequencies).

5.0 Comments

5.1 Within PopStats the frequency for a heterozygous profile is determined by the equation $f(pq) = 2pq$ and the frequency for a homozygous profile is determined by the equation $f(pp) = p^2 + p(1-p)\theta$. However, STRmix uses the Balding and Nichols model also known as NRC II recommendation 4.2.

5.2 Further information about STRmix settings are detailed in the ISP Forensic Services STRmix and Fusion 6C Validations.

5.3 More detailed information on the biological models, minimum allele frequency, and mathematical calculations underlying the STRmix software can be found in the STRmix Manuals.

5.4 Amelogenin, DYS391, DYS576, and DYS570 are not utilized in statistical calculations; however, these loci should be compared manually and the results should be consistent with the conclusions drawn.

5.5 Kinship statistics other than paternity may be calculated in PopStats when needed.

5.6 Legacy data may, on rare occasions, require reinterpretation for the purposes of STRmix deconvolution and/or comparison to known reference samples. If this occurs, a qualified analyst will refer to the interpretation guidelines in place at the time of the original analysis and compare to that currently in place. Any changes in interpretation will be documented in the case notes. (Note: to be qualified, an analyst and technical reviewer must first either receive training and competency testing, or reestablish technical skills/knowledge through the review of the previous validation(s) and analytical method(s), and be approved by the Technical Lead for a specified period of time)

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Y-STR Analysis: PowerPlex® Y23 System (BI-214)

1.0 Background/References

1.1 Short tandem repeat (STR) loci found on the Y chromosome are referred to as Y-STRs. The ability to perform testing that is specific for male DNA is a valuable tool in forensic DNA testing, particularly in scenarios where an abundance of female DNA is present in a sample containing a significantly lesser amount of male DNA that is unable to be detected using traditional autosomal STR testing. The method for performing Y-STR testing is similar to that for autosomal STRs, with the main differences occurring at the interpretation and statistical calculation steps. The Y chromosome is inherited through the paternal lineage; therefore, male relatives are expected to have the same Y-STR haplotype. Due to the Y-STR loci being linked together on the Y chromosome a “counting method” is employed for statistical calculations. This paternal inheritance and Y-STR linkage imparts a much lower power of discrimination to Y-STR testing as compared to autosomal STR testing. The PowerPlex® Y23 System allows for the co-amplification of the following 23 Y-STR loci in a 5-color multiplex: DYS576, DYS389I, DYS448, DYS389II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385a/b, DYS456 and Y-GATA-H4. Y-STR results are not useful for CODIS entry and require a known reference sample(s) from the male(s) of interest for comparison.

1.2 References

- 1.2.1 PowerPlex® Y23 System Technical Manual, Promega Corporation
- 1.2.2 PowerPlex® 5C Matrix Standard Technical Manual
- 1.2.3 ABI 3130/3130xl Genetic Analyzer Getting Started Guide
- 1.2.4 ABI 3130/3130xl Genetic Analyzer Maintenance Troubleshooting and Reference Guide
- 1.2.5 GeneMapper™ IDX Software User Guide
- 1.2.6 ISP Forensic Services Y23 Validation
- 1.2.7 SWGDAM Guidelines for Y-Chromosome STR Typing (2014)
- 1.2.8 CADOJ Y-Mix Tool Instructions

2.0 Scope

2.1 To provide a reliable method for Y-STR amplification, capillary electrophoresis, data analysis, interpretation, statistical calculations, and reporting.

3.0 Equipment/Reagents

- 10% Bleach or Bleach Substitute
- Thermocycler
- Microcentrifuge

- MicroAmp tubes
- Pipettes and filter barrier tips
- PowerPlex® Y23 System Kit Contents
- 3130 Genetic Analyzer with Data Collection Software
- GeneMapper™ IDX Software
- Computer
- Heating Block (or 9700 Thermal Cycler)
- Capillary Arrays
- Syringe
- 96 Well Reaction Plates and Septa
- Buffer Reservoirs and Septa
- POP4 Polymer
- Genetic Analyzer Buffer
- PowerPlex® 5C Matrix Standards
- Deionized Formamide
- Stabilizer Reagent
- Nanopure Water
- CAD0J Y-Mix Tool Microsoft® Excel Program

4.0 Procedure

4.1 AMPLIFICATION USING THE POWERPLEX® Y23 SYSTEM

4.1.1 The optimal amount of male DNA input for PowerPlex Y23 amplification is ~1.0-2.0ng but may be adjusted if necessary. The maximum volume of DNA extract that can be added to the amplification is 17.5µl. Samples may need to be diluted and/or concentrated in order to normalize male DNA concentrations. Artifacts have been observed when amplifying excessive amounts of female DNA.

Note: A corresponding reagent blank will be amplified using the most sensitive amplification conditions of any associated sample.

4.1.2 The method for PowerPlex Y23 amplification is the same as PowerPlex Fusion 6C (BI-208) with the following exceptions:

Reagent Component Volumes

PowerPlex Y23 5X Master Mix	5.0µl
PowerPlex Y23 10X Primer Mix	2.5µl
DNA Template + dH2O	17.5µl

'Y23' Thermal Cycling Parameters

96°C for 2 minutes, then:

94°C for 10 seconds,
61°C for 1 minute
72°C for 30 seconds
for 30 cycles, then:

60°C for 20 minutes, then:

4°C soak

Note: The ramp speed should be set to 'Max' and the reaction volume 25µl.

4.2 AMPLIFIED FRAGMENT DETECTION USING THE 3130

Note: Prior to using the ABI 3130 Genetic Analyzer for samples, a spectral calibration (matrix standards) must be run to achieve proper color separation of the dyes used for the amplification primers, allelic ladders and size standard. To prepare a matrix, five standards are run under the same capillary electrophoresis conditions that will be used for samples and allelic ladders. Use the 5C Matrix Standard set, which includes the Fluorescein Matrix, JOE Matrix, TMR-ET Matrix, CXR-ET Matrix, and WEN Matrix in a single tube for the blue, green, yellow, red, and orange matrix standards, respectively. This is performed when necessary due to performance, or after any instrument maintenance/repair that involves adjustment/replacement of the CCD camera or laser.

Additionally, a Spatial Calibration must be performed prior to running any samples. The instrument uses images collected during the spatial calibration to establish a relationship between the signal emitted for each capillary, as well as the position where that signal falls and is detected by the CCD camera. This is performed any time a capillary is installed or replaced (including temporary removal of a capillary) or if the instrument is moved.

4.2.1 Turn on the computer, turn on the instrument, start Data Collection Software and wait for green squares to appear for all applications on the service console. Expand the necessary subfolders on the left tree pane of Data Collection. Refer to the ABI 3130/3130xl Genetic Analyzers Getting Started Guide for detailed instructions on instrument set-up (including creation of instrument protocols, results groups, and spatial calibration).

Shut down is performed in the opposite order (Data Collection software, 3130, then computer). The Data Collection Software must be closed by choosing 'Stop All' and waiting for all red symbols to appear before closing. Never use the 'X' to close while green or yellow symbols are displayed.

4.2.2 Create a new plate record:

4.2.2.1 For a spectral calibration plate, expand the tree pane of the Data Collection Software and click 'Plate Manager,' under 'ga3130'. Choose 'New', and fill in the dialog boxes, with 'Spectral Calibration' as the application. Fill in the applicable dialog boxes on the Spectral Calibration Editor as follows (clicking 'OK' when complete to save):

- Sample Name: date_Spectral
- Priority: May optionally be changed to a number
- Instrument Protocol 1: Choose the Promega_G5_WEN instrument protocol from the drop down menu

PowerPlex Fusion (same dye set as used for PowerPlex Y23) specific run module and protocol settings for Spectral Calibration are as follows:

- Module Type: Spectral
- Template: Spect36_POP4
- Inj. kV: 1.2
- Data Delay Time: 400
- Run Time (seconds): 700
- Protocol Type: Spectral
- DyeSet: G5
- Array Length: 36
- Chemistry: Matrix Standard
- Lower condition bound: 4.0
- Upper condition bound: 12.0
- Inj.Secs: 12

4.2.2.2 For a sample plate fill out the '3130 Load Sheet' tab of the casework worksheets (Form 206-BI) or electronic equivalent in ILIMS. Print a copy for the case record if applicable. Choose the '3130 Plate Document' tab and ensure the information corresponds to the Load Sheet information entered. Verify the information on the template is as follows:

- Container Type: 96-Well
- Application Type: regular
- GeneMapper: GeneMapper_Generic_Instance
- Sample Name:
 - Allelic Ladder: LADDER

- Controls: POS [or (+), etc.], NEG [or (-), etc.], QRB (questioned reagent blank), KRB (known reagent blank), RB...F2 (sperm reagent blank), RB...F1 (epithelial cell reagent blank), etc. Note: the batch date and analyst initials will also be added to the reagent blank names as identification for future analyses.
- Case Samples: Y9999-9999_XZZ..., where:

- Y = Letter for Lab (M, C or P)
- 9999-9999= Lab Case Number
- X= Specimen Type (Q=Questioned; V=Victim; S=Suspect; E=Elimination; M=Mother; F=Alleged Father; C=Child; FB=Paternal uncle; FS=Paternal Aunt; FM=Paternal Grandmother; FF=Paternal Grandfather, etc.)
- ZZ...= numbers and letters that designate case Item (including 'F2' for sperm cell and 'F1' for epithelial cell at end of number to delineate fraction). (e.g., M2001-0112_Q1AF1)

- Priority: May optionally be changed to a number
- Sample Type: Sample Categories of 'Sample', 'Allelic Ladder', 'Positive Control', or 'Negative Control', may optionally be typed in.
- Results Group 1: Enter the appropriate results group. These are typically denoted by the analyst initials and should automatically populate from the Load Sheet.
- Instrument Protocol 1: Enter the appropriate instrument protocol (i.e., Y23_5sec, Y23_3sec, or Y23_10sec).

PowerPlex Y23 specific run module and protocol settings for sample plates are as follows:

- Module Type: Regular
- Template: HIDFragmentAnalysis36_POP4
- Inj. kV: 3
- Run Time (seconds): 1800
- Protocol Type: Regular
- DyeSet: G5
- Inj.Secs: 3-10
 - 5 secs – Generally produces good results for Allelic Ladders and samples $\geq 1.0\text{ng}$ [injection times may be adjusted (3-10 seconds per analyst's discretion)].

- Run time may be increased to account for temperature fluctuations affecting the run as needed.

4.2.2.3 Delete any unused wells. Perform a 'Save As' of the plate document to disc (i.e. USB drive) for subsequent transfer to the ABI 3130. The document must be saved as a .txt file (tab delimited).

4.2.2.4 Import the previously saved plate document by selecting 'Import' on the 'Plate Manager' window. Browse to locate the saved .txt file and choose 'OK'. 4.2.2.5 Open the imported plate record by highlighting it and clicking 'Edit'. Review the information in the GeneMapper Plate Editor to ensure that it is correct or make changes as necessary. Click 'OK' when complete to save the plate record. 4.2.2.6 To perform more than one run of a sample (e.g. multiple injection times) select Edit/Add/Sample Run in the GeneMapper Plate Editor window. This will add additional Results Group and Instrument Protocol columns to the end of the plate record. These additional runs may be added at any point in the run, prior to the last injection, if the scientist notices that a sample would benefit from re-injection (e.g., repeat because of bad injection or to vary injection times [from 3-10 seconds]). Additional Results Groups and Instrument Protocols may also be filled in on the original Load Sheet template prior to importing.

4.2.3 In the manual control window, the scientist may choose to set the oven to 60°C so that it will be ready to run. Choose Oven in the 'Send Defined Command for' drop down menu box. In the 'Command Name' box, choose Turn On/Off oven, with a 'Value' of 'On' and click 'Send Command'. Next, in the 'Command Name' box, choose Set oven temperature, with a 'Value' of 60.0 and click 'Send command'. Note: once the oven has been turned on and the temperature set, the oven will only preheat for 45 minutes before shutting itself off.

4.2.4 Prepare samples for capillary electrophoresis:

4.2.4.1 Sample Preparation

Deionized Formamide	9.5µl
WEN ILS 500 Y23	0.5µl
Stabilizer Reagent	0.5µl
Amplified Sample	1.0-1.5µl
(or PowerPlex Y23 Allelic Ladder)	

Note: Stabilizer Reagent is a necessary custom component not included with the PowerPlex Y23 kit which must be requested separately from Promega and stored refrigerated upon receipt. The samples will be heat denatured for 3 minutes at 95°C, but do not require a subsequent snap cool step.

The master mix may be altered by adding 0.25µl WEN ILS500 size standard to 9.75µl deionized formamide if ILS peaks are too high.

4.2.4.2 The 5C Matrix Mix is diluted by adding 10µl to one tube of Matrix Dilution Buffer. 10µl of the diluted 5C Matrix Mix is then added to 500µl of deionized formamide (without size standard). Load 15µl of the 5C Matrix Mix with formamide into each of four wells on the pre-labeled plate, which will include each of the four capillaries (e.g. wells A1 through D1). Note: Do not heat denature the 5C Matrix Mix with formamide. At the first use, thaw the 5C Matrix Mix and Matrix Dilution Buffer completely. After the first use, store the reagents at 2-10°C, protected from light. The diluted 5C Matrix Mix can be stored for up to 1 week at 2-10°C.

4.2.5 Following sample addition, place a plate septa on the plate and heat denature for ~3 minutes at 95°C. Note: the plate septa may be cut to cover only those well columns being used on smaller plate runs.

4.2.6 Place the sample plate into the plate base and secure the plate retainer clip on top, making sure that no gray is visible through the holes.

4.2.7 Place the plate assembly in the instrument and close the doors. The plate map on the 'Plate View' window, under 'Run Scheduler' will turn yellow when the plate is in place and has been detected by the instrument.

4.2.8 Prior to running the plate, confirm that dye set G5 is selected and the correct active calibration for dye set G5 is set in spectral viewer.

4.2.9 Locate the plate record in the 'Plate View' window and highlight it by clicking on it once. With the plate record highlighted, click the plate map to link the plate to that specific record. The plate map will turn from yellow to green when it is successfully linked. Verify the correct scheduling of the run in the 'Run View' window. Select a run and confirm that the corresponding wells highlighted in the plate diagram are correct for that run. Make adjustments to the plate record if necessary.

4.2.10 Click the green Run Instrument arrow button in the toolbar to start the run. Monitor electrophoresis by observing the run, view, array, or capillaries viewer window. Each injection (set of four samples) will take ~45 minutes. Note: to run a duplicate plate record, the plate may need to be unlinked prior to linking the duplicated record. This is done by highlighting the currently linked plate record and clicking 'unlink'.

4.2.11 After completion of the spectral calibration run, open the 'Spectral Viewer' window to evaluate the spectral and set the active calibration. Confirm that Dye Set G5 is selected. Click on individual wells in the plate diagram to see results for each of the four capillaries. For each capillary, verify that five peaks are present in the spectral profile (upper pane), that the order of the peaks are, from left to right, blue-green-yellow-red-orange, and that the peaks are regular in appearance. Next verify that five peaks are present in the raw data profile (lower pane), that the order of the peaks are, from left to right, orange-red-yellow-green-blue, and that the peak heights are above 750 RFU (1,000-4,000 RFU is ideal). If all four capillaries pass, then the calibration should be saved and set as the active calibration.

Note: All four capillaries must pass in order to accept a spectral calibration. A passing capillary will be colored green in the plate diagram. Additionally, capillary status may be viewed in the 'Event Log' under 'Instrument Status'. Rerun the spectral calibration as necessary until all four capillaries pass.

4.3 DATA ANALYSIS USING GENEMAPPER® IDX (GMIDX)

4.3.1 GMIDX analysis of PowerPlex Y23 data is the same as PowerPlex Fusion 6C (BI-210) with the following exceptions:

Panels and Bins

Panels: PowerPlexY23_Panels_v2.0

Bin Set: PowerPlexY23_Bins_v2.0

Note: Increase marker specific stutter ratio to 0.13 for DYS635 and 0.07 for DYS643. The default N-1 repeat stutter values are used at all other loci. Similar artifacts are observed in both autosomal and Y-STR DNA testing. In addition to N-1 and N+1 repeat stutter, it is possible to observe N-2 repeat stutter (DYS481) and n-2 bp/n+2 bp stutter (DYS19); however, GMIDX is not able to filter these types of stutter.

Analysis Method

Allele: Select to use marker-specific stutter ratio if available.

Plus stutter ratio values should be set to 0.07 for trinucleotide repeat loci and 0.04 for tetra, penta, and hexanucleotide repeat loci.

Peak Detector: Peak amplitude thresholds should be set at 50 RFU for all dye channels.

Note: The duplicate homozygous alleles option needs to be deselected. It is necessary to use the WEN ILS500 reagent that is included with the PowerPlex Y23 kit; however, the GMIDX size standard is the same as PowerPlex Fusion 6C [e.g. WEN_ILS500 (60-500)].

4.4 INTERPRETATION OF Y-STR RESULTS

- 4.4.1 Only single source and/or deduced single source Y-STR profiles will be interpreted. It may be possible to deduce a single source Y-STR profile, at all or some loci, in a mixture that is distinguishable into major and/or minor components. It may also be possible to deduce a single source Y-STR profile, at all or some loci, when the presence of a known contributor(s) (i.e. victim, consensual partner, etc.), whose DNA is reasonably expected to be present in a sample, can be assumed and the foreign alleles are attributable to a single individual.
- 4.4.2 Since the Y chromosome is haploid, a single allele is expected at each single copy Y-STR locus. Peaks detected with the PowerPlex Y23 kit must be above a 50 RFU analytical threshold in order to be considered allelic. The PowerPlex Y23 kit contains one multi-copy locus (DYS385 a/b) where two alleles are expected. The stochastic threshold for DYS385 a/b was determined to be 200 RFU and peak height ratios are generally >70% for samples with optimum amounts of input DNA. Duplications and deletions are rare genetic anomalies that may result in greater than or less than the expected number of alleles to be detected at a locus. A deletion causing a null allele may be differentiated from an undetected allele by considering the overall quality/peak heights of the profile and the location of the missing allele. It is permissible to use a null allele(s) in the Y-STR database search performed for statistical calculations. A sample is generally considered to be a mixture of more than one male if two or more alleles are present at two or more single-copy loci. An evaluation considering peak heights and repeat-unit differences at a given locus may be useful to differentiate between duplications and mixtures.

4.4.3 A mixture is considered distinguishable if it can be separated into major/minor components. In a 2-person male mixture it may be possible to deduce both the single source major and minor profile at all or some loci when the mixture ratio is greater than 2:1 (i.e. 67% & 33% contributors). For 3- and 4-person male mixtures it may be possible to deduce the profile of a major contributor at all or some loci when the major accounts for greater than 2/3 of the mixture (i.e. >67% contribution). Indistinguishable mixtures, minor contributors in 3- and 4-person male mixtures, mixtures of 5 or more males, and partial profiles with results at only a few loci will be considered uninterpretable. Caution should be exercised in distinguishing major/minor contributors when sub-optimal DNA input amounts were used resulting in a low level profile and/or at loci where peaks are of a similar height (i.e. near 50/50 in a 2-person mixture) even if the remainder of the profile is distinguishable.

4.4.4 Interpretation of questioned profiles, including deconvolution of mixtures, will occur prior to comparison with probative known reference profiles. Non-probative comparisons may be part of the interpretation process for questioned profiles to aid in determining number of contributors and whether or not a single source profile can be deduced from a mixture. A known and questioned profile comparison will be considered to be a match if all of the alleles detected in the questioned profile are consistent with alleles represented in the known profile. Reasonably explainable differences (i.e. allelic dropout, etc.) will not preclude declaration of a match. A non-match or exclusion occurs when unexplainable differences are present in the allele calls for the questioned and known profiles.

4.4.5 Assumptions used in the interpretation (i.e. assuming a known contributor) will be documented in the notes and clearly stated in the report, as appropriate.

4.4.6 The expected PowerPlex Y23 profile for the 2800M positive control is:

<u>LOCUS</u>	<u>HAPLOTYPE</u>
DYS576	18
DYS389I	14
DYS448	19
DYS389II	31
DYS19	14
DYS391	10
DYS481	22
DYS549	13
DYS533	12
DYS438	9
DYS437	14
DYS570	17

DYS635	21
DYS390	24
DYS439	12
DYS392	13
DYS643	10
DYS393	13
DYS458	17
DYS385a/b	13, 16
DYS456	17
Y-GATA-H4	11

Note: Autosomal interpretation guidelines related to interpretation of controls and artifacts are also applicable to Y-STRs (see BI-212).

4.5 Y-STR STATISTICAL CALCULATIONS USING CAD0J Y-MIX TOOL

4.5.1 After a Y-STR profile is interpreted to be from a single male or deconvoluted into a deduced single male profile component of a mixture, and determined to match a probative known reference sample, a statistical calculation will be performed. A statistical calculation is not required for a non-match/exclusion or a match to a non-probative known reference sample if only a qualitative statement is to be reported. In cases where both autosomal and Y-STR testing has been performed, although it may be possible to calculate a combined statistic, the general practice will be to report autosomal and Y-STR statistics separately.

4.5.2 The “counting method” with application of a 95% upper confidence interval (UCI) using the Clopper and Pearson approach as recommended by SWGDAM (2014) will be used for Y-STR statistical calculations. The United States national database (with subpopulations) from the Y-Chromosome Haplotype Reference Database (YHRD – <https://yhrd.org/>) will be the database used, and the CAD0J Y-Mix Tool will be the program used for performing the database search and calculations. The combined database consisting of African American, Asian, Caucasian, Hispanic, and Native American populations will be reported. Generally, the 95% UCI value reported will be in the format of “1 in X”.

4.5.3 Typically all suitable loci for a single source sample or all suitable loci where a single male contributor profile has been deduced from a mixture will be used in the statistical calculation. Upon consultation with the DNA Technical Lead it may be appropriate to calculate a statistic using a reduced number of loci on a case by case basis. There are fewer full PowerPlex Y23 haplotypes (~5,717) than PowerPlex Y haplotypes (~35,864) in YHRD; therefore, using fewer loci may actually result in a more discriminating statistic when using the counting method. In the event that a reduced locus search of the database was to be performed, any matches that would have been non-matches had more loci been used must be excluded for statistical purposes.

4.5.4 Open the current version of the CAD0J Y-Mix Tool in Microsoft Excel.

4.5.5 Verify the following settings:

Setting

Limit database to samples with all the loci entered above?	Yes
Treat this profile as a single source sample?	Yes
Desired UCI	95.0%
Use $(x+1)/(N+1)$?	No

4.5.6 Enter the single source/deduced single source alleles for each relevant locus of the Y-STR haplotype obtained from the evidentiary sample in the appropriate cell by either typing the value or selecting the allele from the drop-down list provided.

Note: Use caution when manually entering the Y-STR haplotype as loci are listed alphanumerically in the CAD0J Y-Mix Tool and are not in the PowerPlex Y23 order. It may be helpful to sort the results alphanumerically prior to entry to reduce the possibility of a transcription error.

4.5.7 Click on the "Compare the profile to the database." button to run the database search and calculate the relevant statistics.

4.5.8 Print or save an electronic copy of the results for the case file.

Note: There are not fields for sample identifier, analyst, or date so it is up to the analyst to document the relevant details associated with any printed or electronic copy of results.

4.6 Y-STR REPORTING STATEMENTS

4.6.1 Introductory statement:

Deoxyribonucleic Acid (DNA) Analysis, employing the Polymerase Chain Reaction (PCR), was used to generate a Y-Chromosome Short Tandem Repeat (Y-STR) profile from (a portion of) Items #s.

Note: The following note will appear in all reports in which Y-STR DNA testing was attempted.

Loci Examined: DYS576, DYS389 I, DYS448, DYS389 II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385, DYS456, and YGATAH4

Note: The following note should appear in reports where a differential extraction was performed.

A differential extraction was performed on samples with results from Fractions 1 and/or 2, which is intended to separate sperm cells from non-sperm cells. However microscopic confirmation of spermatozoa was not performed during this procedure. Fraction 2 should contain any sperm cells present in the sample. Results may not be listed in the report for non-probative fractions.

4.6.2 Profile match statement for single source:

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) from a single male or male lineage. "*Name*" and all other paternally related males are included as a possible source of the male DNA from this sample. The DNA profile (*or partial DNA profile*) was compared to data from the Y-Chromosome Haplotype Reference Database (YHRD). The number of times this DNA profile was observed in the database was "*x*" (*# of haplotypes with selected alleles*) in "*N*" (*# of haplotypes*) tested individuals. This corresponds to an estimated population frequency (upper 95% confidence interval) of 1 in ... for all populations.

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) from a single male or male lineage. "*Name*" is consistent with being a possible source of the male DNA from this sample.

Note: Qualitative statements such as "consistent with" without an associated statistic will only be used for non-probative associations.

4.6.3 Profile match statement for deduced single source:

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) consistent with originating from two males or male lineages. A major (*and/or minor*) DNA profile determined to be from a single male or male lineage was obtained from this mixture. "*Name*" and all other paternally related males are included as a possible source of the major (*or minor*) male DNA profile from this mixture. The major (*or minor*) DNA profile was compared to data from the Y-Chromosome Haplotype Reference Database (YHRD). The number of times this DNA profile was observed in the database was "*x*" (*# of haplotypes with selected alleles*) in "*N*" (*# of haplotypes*) tested individuals. This corresponds to an estimated population frequency (upper 95% confidence interval) of 1 in ... for all populations.

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) consistent with originating from *three (or four)* males or male lineages. A major DNA profile determined to be from a single male or male lineage was obtained from this mixture. "*Name*" and all other paternally related males are included as a possible source of the major male DNA profile from this mixture. The major DNA profile was compared to data from the Y-Chromosome Haplotype Reference Database (YHRD). The number of times this DNA profile was observed in the database was "*x*" (*# of haplotypes with selected alleles*) in "*N*" (*# of haplotypes*) tested individuals. This corresponds to an estimated population frequency (upper 95% confidence interval) of 1 in ... for all populations. No conclusions can be made regarding possible minor contributors to this mixture.

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) consistent with originating from two males or male lineages. "*Name*" is consistent with being a contributor to this mixture. Assuming "*Name*" is a contributor to this mixture, a foreign DNA profile determined to be from a single male or male lineage was able to be deduced. "*Name*" and all other paternally related males are included as a possible source of the foreign male DNA profile from this mixture. The foreign DNA profile was compared to data from the Y-Chromosome Haplotype Reference Database (YHRD). The number of times this DNA profile was observed in the database was "*x*" (*# of haplotypes with selected alleles*) in "*N*" (*# of haplotypes*) tested individuals. This corresponds to an estimated population frequency (upper 95% confidence interval) of 1 in ... for all populations.

4.6.4 Exclusionary statements:

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) from a single unknown male or unknown male lineage. "*Name*" is not the source of the male DNA on this item.

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) consistent with originating from *X (# of contributors)* males or male lineages. A major (*and/or minor*) DNA profile determined to be from a single unknown male or unknown male lineage was obtained from this mixture. "*Name*" is excluded as a possible source of the major (*or minor*) male DNA profile from this mixture.

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) consistent with originating from "X" (# of contributors) males or male lineages. "Name" is consistent with being a contributor to this mixture. Assuming "Name" is a contributor to this mixture, a foreign DNA profile determined to be from a single unknown male or unknown male lineage was able to be deduced. "Name" is excluded as a possible source of the foreign male DNA profile from this mixture.

4.6.5 Uninterpretable statements:

Male specific DNA testing performed on this item indicated a DNA profile (*or partial DNA profile*) consistent with originating from "X" (# of contributors) males or male lineages. Due to the number of contributors and complexity of this mixture, no conclusions can be made as to possible contributors to this mixture.

Due to insufficient quantity or degradation, male specific DNA testing performed on this item indicated only a partial DNA profile. Due to the low level results and limited data, no conclusions can be made.

4.6.6 No male DNA profile obtained statement:

Due to insufficient quantity or degradation, male specific DNA testing performed on this item resulted in no male DNA profile being obtained.

5.0 Comments

- 5.1 In general, Y-STR testing will only be performed on samples where male DNA was detected during quantitation. If the Auto:Y ratio is 250 or greater autosomal STR testing does not need to be attempted prior to Y-STR testing. Autosomal testing will generally be attempted prior to Y-STR testing when the Auto:Y ratio is less than 250.
- 5.2 There are a number of factors to consider prior to attempting Y-STR testing (i.e. availability of male reference samples for comparison, quantitation results, case scenario, results from other items in case, probative value, etc.); therefore, Y-STRs are considered complimentary to autosomal STR testing and may not be necessary in all cases.
- 5.3 These Y-STR interpretation, statistical calculation, and reporting guidelines represent the standard method used in most cases; however, they may not cover all scenarios encountered during forensic DNA casework. Situations not covered by the standard method will be handled on a case by case basis upon consultation with the DNA Technical Lead.

Driftcon FTC: Temperature Verification (BI-400)

1.0 Background/References

1.1 Successful DNA amplification is dependent on consistent thermal cycling parameters and achieving proper heating and cooling to facilitate the various steps necessary for PCR. It is necessary to employ a method of monitoring the thermal cycler performance and verify that the correct temperature has been achieved in order to have confidence in the amplification process. A variety of temperature probes have been developed to test the temperature of specific wells within the different thermal cycler instruments. However, most of these have proven to be cumbersome, time consuming, and not amenable to testing the newer real-time PCR instruments. The Driftcon FFC is able to test multiple temperatures within a short period of time and can be used on the thermal cyclers, as well as real-time instruments.

1.2 References

- 1.2.1 Driftcon Operations Manual, version 1.1
- 1.2.2 Driftcon Quick Start Guide, version 1.4

2.0 Scope

2.1 To provide a reliable method for verifying the performance of laboratory thermal cyclers, to include real-time PCR instruments.

3.0 Equipment/Reagents

- Computer with Driftcon Software
- Driftcon Hardware Module and Cables
- Driftcon FFC Fixture
- Driftcon Smart Card
- ABI 7500 FFC Adaptor
- Cork Leveler

4.0 Procedure

4.1 9700 THERMAL CYCLER VERIFICATION

- 4.1.1 Set up the Driftcon by connecting the hardware module to the computer and FFC fixture/probe plate with the provided cables.
- 4.1.2 Insert the smart card into the Driftcon hardware module until it stops.
- 4.1.3 Turn on the Driftcon computer. Open the Driftcon Software and login with the appropriate user name and password.
- 4.1.4 Turn on the 9700, place the fixture into the plate with the cable facing out, and select/start the 'Driftcon' protocol. Do Not close the 9700 lid as it will damage the fixture and void the warranty.

- 4.1.5 In the Driftcon software, choose the instrument to be tested. If the list of instruments does not appear automatically, click start and follow the wizard prompts.
- 4.1.6 Choose the Driftcon default protocol and make sure the steps match those in the 9700 protocol. Leave the humidity, pressure, and temperature blank. Enter notes as needed (these will appear on the final report).
- 4.1.7 Start the test by clicking the check mark in the lower right corner. Make sure the 9700 has heated completely and the protocol started before starting the test. The protocol will complete in ~25 min.
- 4.1.8 Once the run has completed, print the report and place it in the QC binder or electronic equivalent.
- 4.2 7500 REAL-TIME INSTRUMENT VERIFICATION
- 4.2.1 Set up the Driftcon by connecting the hardware module to the computer and FFC fixture/probe plate with the provided cables.
- 4.2.2 Insert the smart card into the Driftcon hardware module until it stops.
- 4.2.3 Turn on the Driftcon computer. Open the Driftcon Software and login with the appropriate user name and password.
- 4.2.4 Turn on the 7500 computer, login with the appropriate user name and password, and open the 7500 SDS software.
- 4.2.5 Turn on the 7500. Remove the tray from the plate loader and replace it with the FFC adaptor. Place the fixture into the adaptor with the cable facing out. Place the cork leveler on top of the fixture and close the plate loader.
- 4.2.6 In the 7500 SDS software, choose File > New and select Absolute Quantitation for Assay, 96-Well Clear for Container, and Driftcon for Template.
- 4.2.7 Save the plate document as a .sds file with the appropriate plate name and open the Instrument tab.
- 4.2.8 In the Driftcon software, choose the instrument to be tested. If the list of instruments does not appear automatically, click start and follow the wizard prompts.
- 4.2.9 Choose the Driftcon default protocol and make sure the steps match those in the 7500 protocol. Leave the humidity, pressure, and temperature blank. Enter notes as needed (these will appear on the final report).
- 4.2.10 Start the 7500 run and Driftcon test. Start the test by clicking the check mark in the lower right corner. Make sure the 7500 has heated completely and the protocol started before starting the test. The protocol will complete in ~25 min.
- 4.2.11 Once the run has completed, print the report and place it in the QC binder or electronic equivalent.

5.0 Comments

- 5.1 The second page of the report contains information regarding the number of measurements for each probe. The number should be approximately double the protocol time. If significantly higher or lower, the instrument may be heating too slowly or too quickly. The percentage should be 100% for each probe. If a percentage is lower than 100%, the probe may not have been in the well and measurements missed. Adjust the probes and run the protocol again.
- 5.2 Pass/better than specifications/fail data for each temperature may be found beginning on page four of the report. The measured value, along with the target specifications are shown in this section.

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