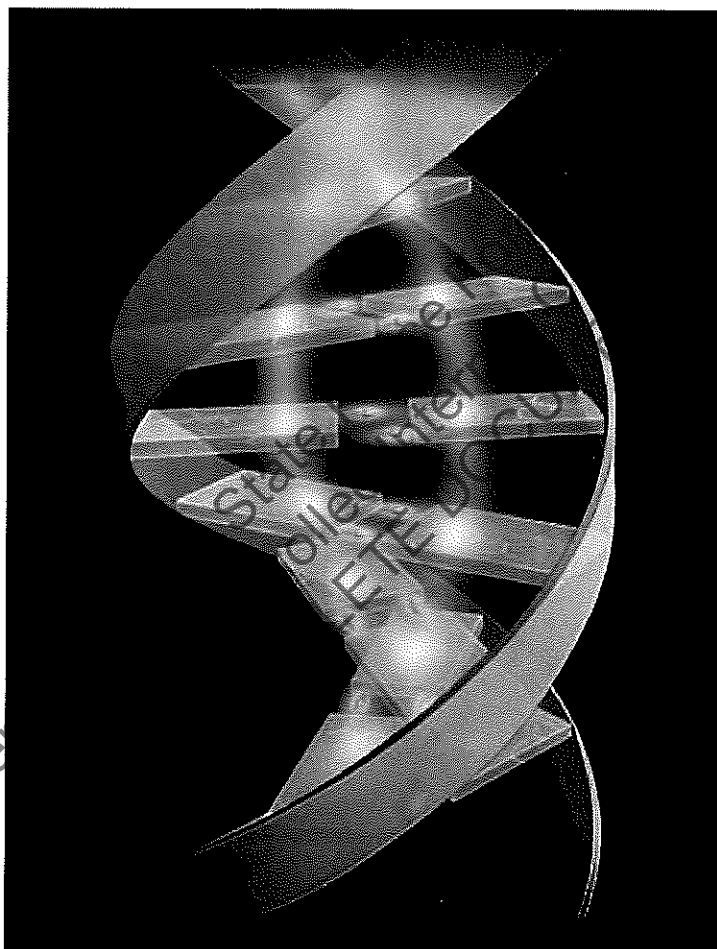


*ISP FORENSIC BIOLOGY
CASEWORK ANALYTICAL METHODS
MANUAL*



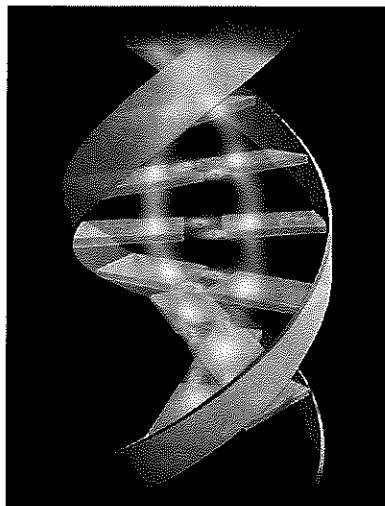
Revision 9

8/17/09

Issuing Authority: Quality Manager

Forensic Biology Casework Analytical Methods Manual

Revision #9



APPROVED
August 10, 2009

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WJG

Forensic Biology Casework Analytical Methods Manual
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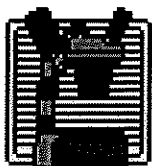
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**EXAMINATION OF BLOODSTAINED EVIDENCE****1.0 BACKGROUND:**

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.

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

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

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:

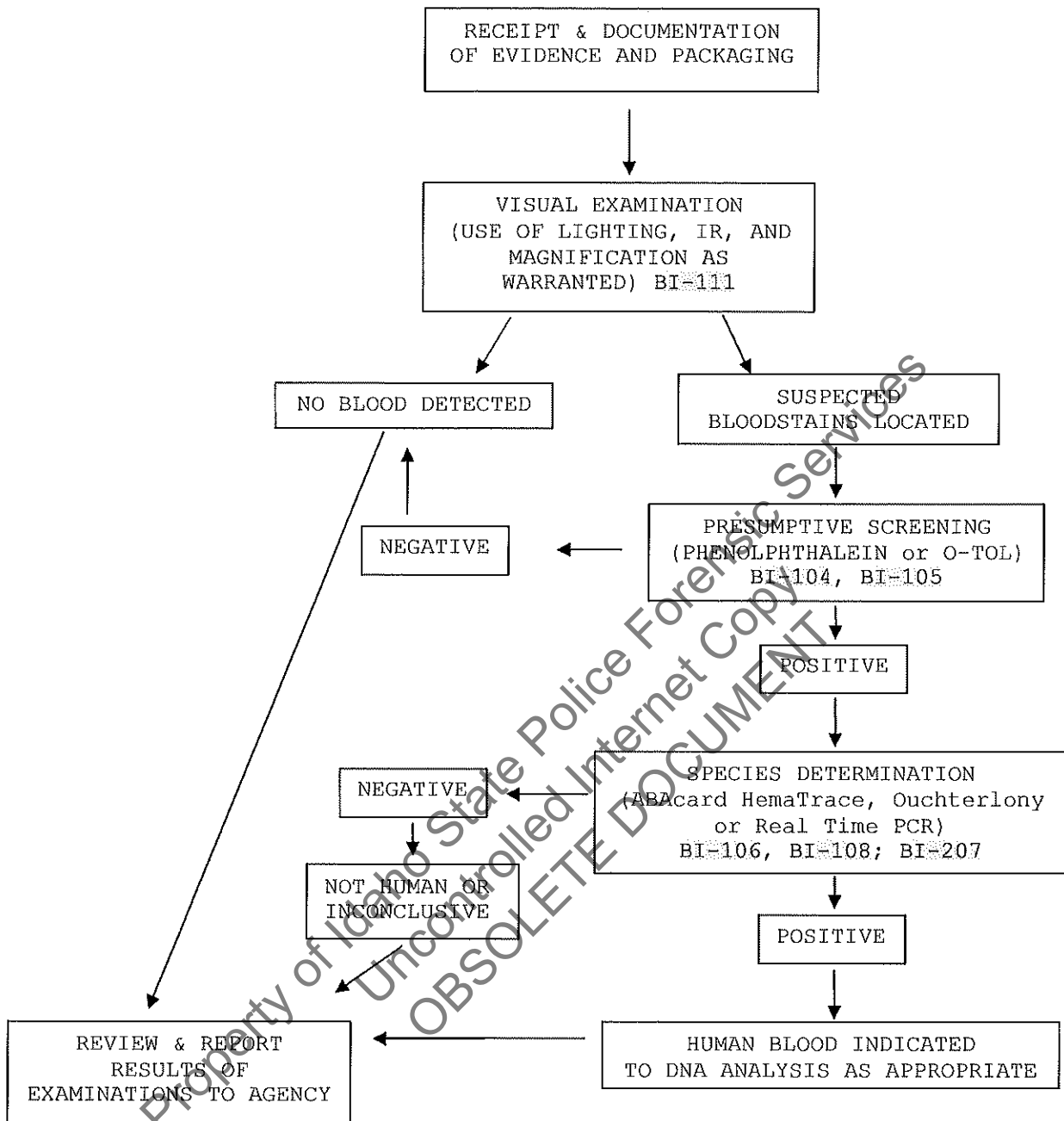
To provide uniform processing of evidentiary material for the presence of blood.

3.0 EQUIPMENT/REAGENTS:

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.

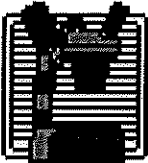
4.0 PROCEDURE:

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

1.0 BACKGROUND:

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.

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

2.0 SCOPE:

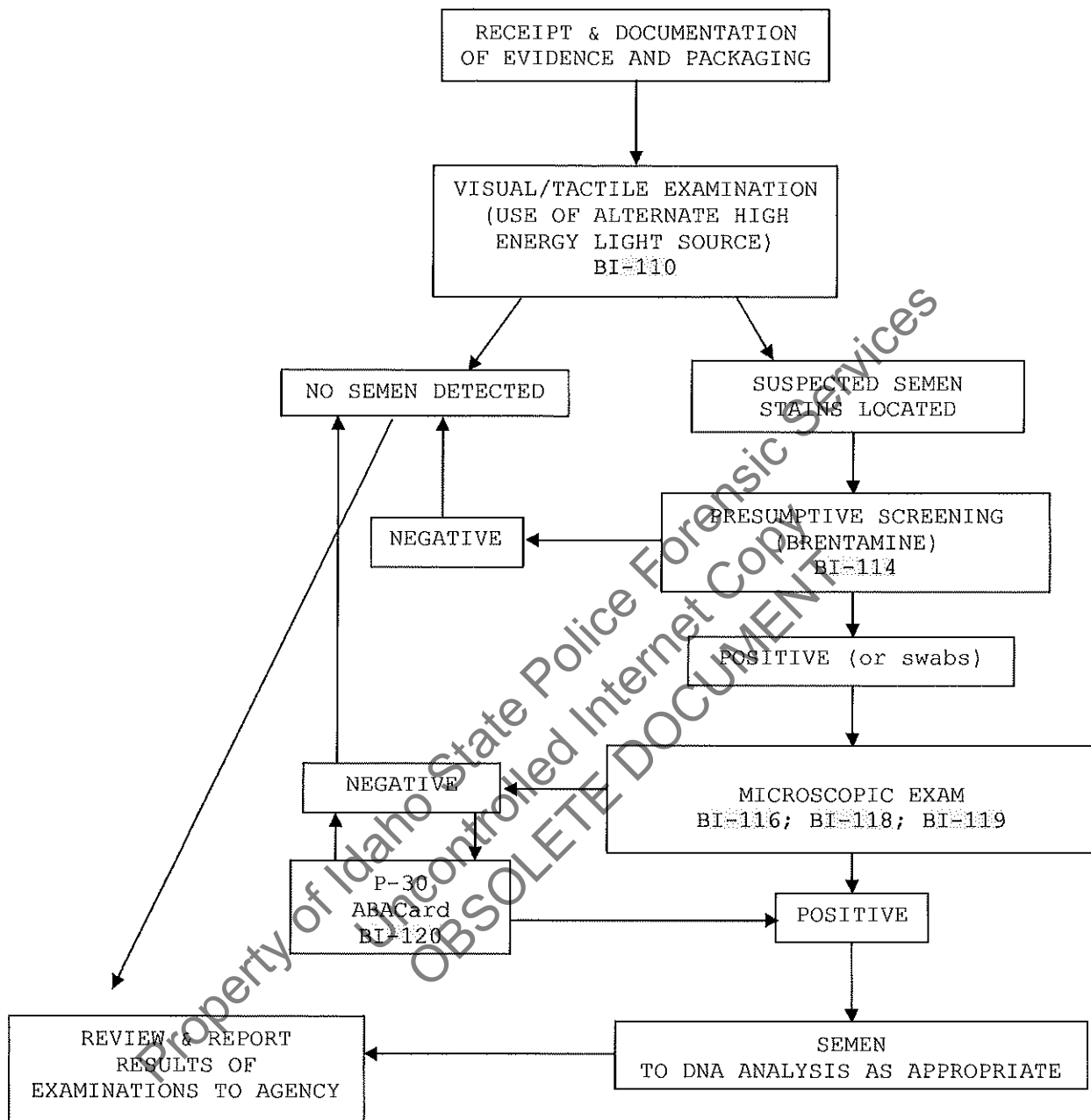
To provide uniform processing of evidentiary material for the presence of semen.

3.0 EQUIPMENT/REAGENTS:

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:

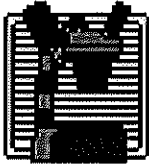
See Flow Chart on following page.



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.



EXAMINATION OF EVIDENCE FOR BODY FLUIDS

1.0 BACKGROUND:

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.

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

2.0 SCOPE:

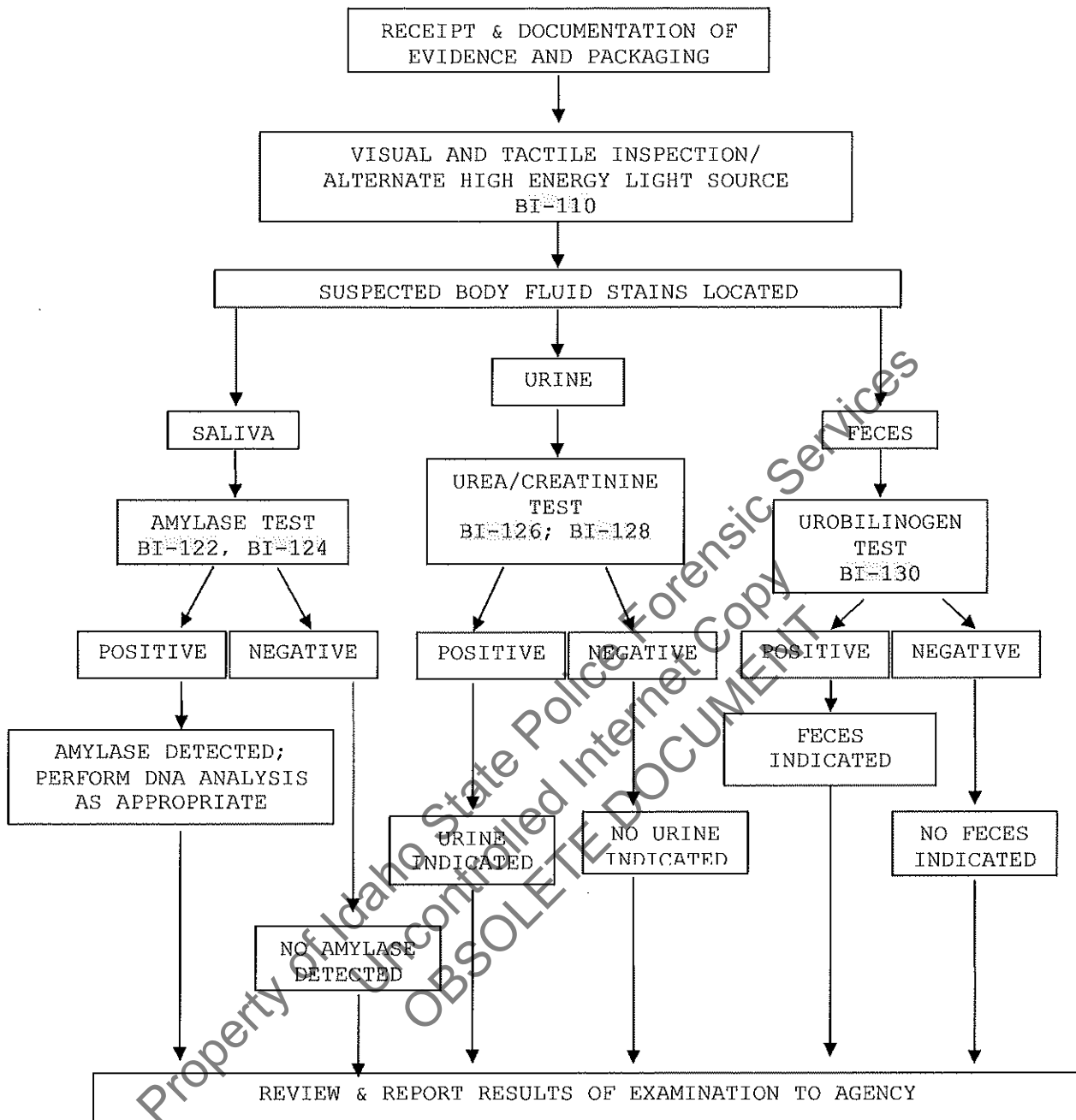
To provide uniform processing of evidentiary material for the presence of saliva, urine or feces.

3.0 EQUIPMENT/REAGENTS:

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:

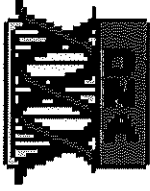
See Flow Chart on following page.



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

1.0 BACKGROUND:

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.

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. 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. Additionally, DNA testing may establish identity, but does not establish timeframe or consent. Sexual assault cases in which consent, rather than identity, is the issue will not be analyzed for DNA.

2.0 SCOPE:

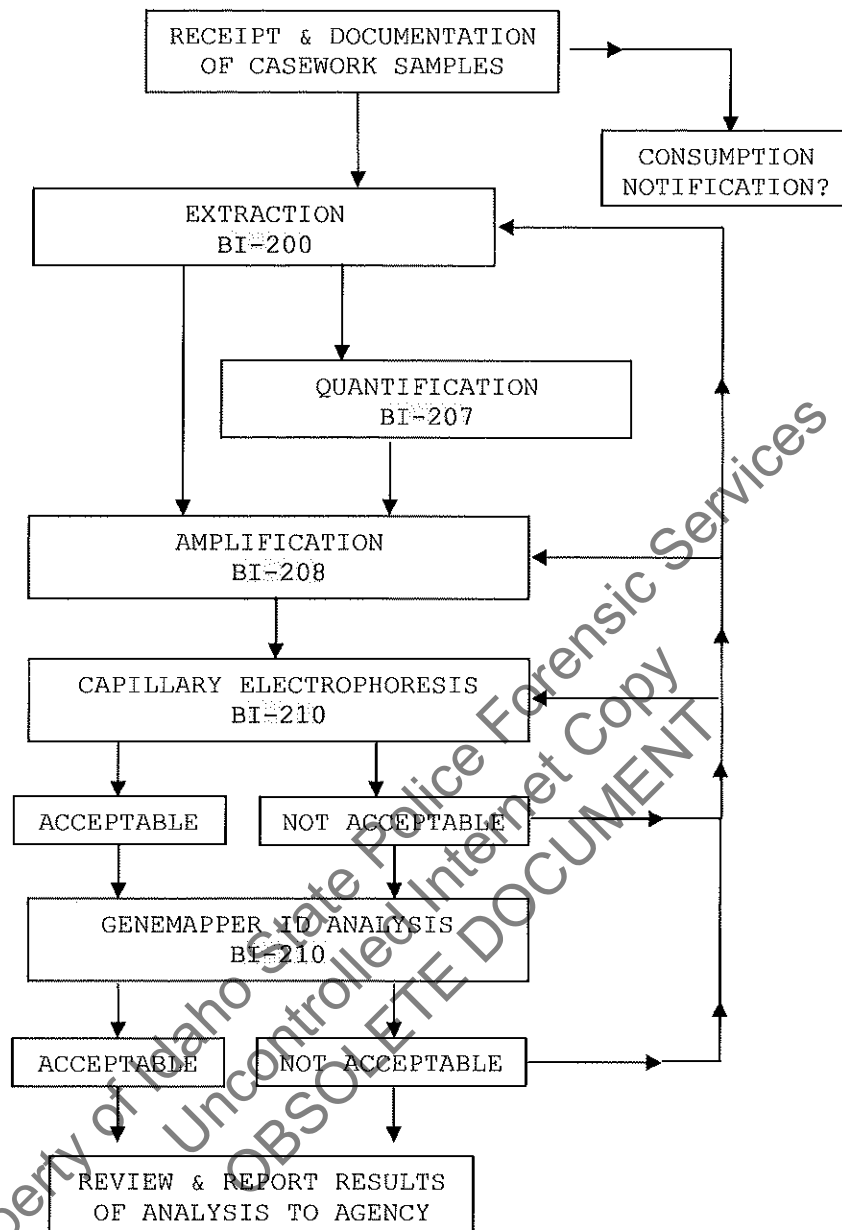
To provide uniform processing of DNA samples to achieve high quality data and consistent interpretation.

3.0 EQUIPMENT/REAGENTS:

As listed in individual analytical procedures.

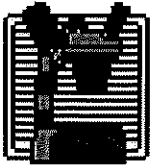
4.0 PROCEDURE:

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).



BI-100

PROCESSING LIQUID BLOOD

1.0 BACKGROUND:

Most known reference standards in forensic casework are 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, any post-mortem blood samples associated with the case are to be checked out and bloodstains made for preservation.

2.0 SCOPE:

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 1 ml pipet with sterile 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 ~1ml of blood into pipet. Carefully spot, at minimum, 1 drop (~50ul) blood onto each circle.
- 4.4 Allow bloodstain card to air-dry completely before packaging.
- 4.5 Place dried stain card into an envelope (~3¼" x 5½"). Seal envelope with evidence tape on flap and label with initials and date across seal. Label front of coin envelope with Case Number and Item Number minimally.
- 4.6 Make Case DNA Packet (See BI-102) and place bloodstain sample inside.

5.0 COMMENTS:

- 5.1 Exercise caution and wear appropriate protective gear when preparing bloodstains (e.g., gloves, labcoat, 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.



DNA PACKETS

1.0 BACKGROUND:

It has become increasingly important to retain evidence for possible future analyses and to secure samples for nonprobative casework analyses that are necessary for the validation of any new technology. Therefore, where possible, a DNA packet is created for each case that is submitted for analysis to Forensic Biology and for which evidence exists for retention (e.g. reference sample(s) and/or positive biological screening results).

2.0 SCOPE:

To provide a method to ensure adequate sample retention for sample re-analyses and new protocol/technology development.

3.0 EQUIPMENT/REAGENTS:

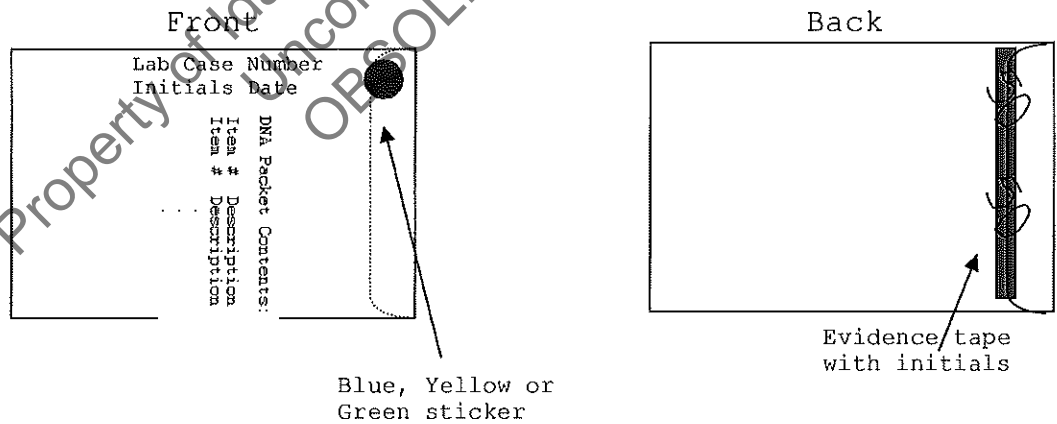
Coin Envelopes (~3 $\frac{1}{4}$ " x 5 $\frac{1}{2}$ ", and other sizes as needed)
DNA Packet Envelope (~6 $\frac{1}{2}$ " x 9 $\frac{1}{4}$ " manila envelope)
Blue, Green, and Yellow Circular Stickers

4.0 PROCEDURE:

4.1 Cuttings/swabs containing previously identified biological evidence, as well as known reference bloodstain cards should be packaged in separate coin envelopes. Swabs packaged in separate envelopes within an outer container (sexual assault evidence collection kits, for example) do not need to be repackaged into a new coin envelope. Each envelope will be labeled with Case Number, Item Number, Date, Scientist's Initials and sealed with evidence tape.

4.2 All sealed envelopes will be placed inside a larger manila envelope (DNA Packet Envelope) and labeled as below.

- 4.3 The DNA packet itself need not be sealed until biological screening of the case is completed and all samples are believed to have been collected.
- 4.4 DNA Packets for crimes without a statute of limitations (i.e., Homicides, and Sexual Assaults where DNA evidence exists, including references for criminal paternity testing, and nonsuspect/database cases) will be identified by placement of a blue circular sticker on the outside of the DNA Packet (see below). Likewise, cases that have negative biological screens (so that the DNA Packet will consist solely of the reference bloodstains, except criminal paternity cases) will be identified by the presence of a yellow circular sticker. Green stickers will be placed on the DNA Packets of all other cases.
- 4.5 Once sealed, the DNA Packet will be taken to a FES and entered as an additional item of evidence to allow for tracking in the ETS. The storage location will have a barcode.
- 4.6 DNA Packets will be stored at $\leq -20^{\circ}\text{C}$ as space allows, and then, if necessary, either returned to the submitting agency, or placed in room temperature storage after any requested DNA analyses have been performed. However, prior to return to a submitting agency, the Biology/DNA Supervisor should be notified to ensure maintenance on site is no longer necessary.



- 4.7 Following DNA testing, any leftover DNA extracts will be put into a plastic ziplock bag or coin envelope and placed in the DNA Packet. Individual tubes may also be sealed with parafilm or other sealant to prevent leakage and/or evaporation if desired.

5.0 COMMENTS:

- 5.1 The DNA Packet is NOT meant to contain "items of evidence" but rather biological samples that have been removed from items of evidence. Not every item or every stain on every item should be included in a DNA Packet. The person performing the biological screening should use discretion and prioritize sample collection contacting a DNA Analyst or the Biology Program Manager if necessary.
- 5.2 Given the small sample necessary for DNA testing, discretion should be used in determining the size of the stain cutting. Rarely, if ever, should a cutting exceed the dimensions of the coin envelope.
- 5.3 On **RARE** occasions when it is deemed necessary to have more stains collected in a given case than will fit into a single DNA Packet Envelope, multiple packets will be made. The first packet's barcode will consist of the case number followed by DNA. Subsequent packets will receive barcodes consisting of the case number followed by DNA2, DNA3, etc.

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PHENOLPHTHALEIN TEST FOR BLOOD

1.0 BACKGROUND:

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.

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

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:

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 (sterile/nanopure H₂O) control samples are processed, prior to testing any forensic samples (on the day of testing), to ensure the working solution reagents are functioning properly.

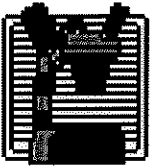
4.2 Cotton swabs or a folded piece of filter paper are used to collect the suspected blood onto the tip. A swab may be moistened with sterile/nanopure H₂O if necessary.

- 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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BI-105

O-TOLIDINE TEST FOR BLOOD

1.0 BACKGROUND:

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.

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

Burdett, PE (October 1976) "Presumptive Tests for Blood - A Comparative Survey", *CRE Report*, No. 201.

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

2.0 SCOPE:

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

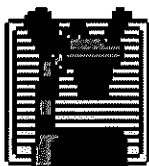
4.0 PROCEDURE:

4.1 Positive (known bloodstain) and negative (sterile/nanopure H₂O) 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 (\leq 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.

**HUMAN BLOOD IDENTIFICATION USING ABACARD® HEMATRACE® TEST****1.0 BACKGROUND:**

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.

2.0 SCOPE:

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 extraction tubes for identification.

4.2 Using the buffer provided, allow samples (generally ~2mm x 2mm stain cutting) to extract at room temperature for 5-30 minutes (longer, if necessary for aged stains).

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

4.4 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.

4.5 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 crossreactivity 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 crossreactivity may be plausible, a statement indicating that 'members of the mustelidae family cannot be excluded' may also be used in the report.



SPECIES IDENTIFICATION: OUCHTERLONY DOUBLE DIFFUSION

1.0 BACKGROUND:

Methods commonly used to identify the species of origin of a biological sample are immunological in nature. The Ouchterlony Double Diffusion technique was first described in 1949 and involves the diffusion of antibody (Ab) and antigen (Ag) in an agarose gel. The formation and detection of a precipitin line (as the result of Ab-Ag complex formation) is used to determine the species of origin of a particular sample.

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

Saferstein, R. *Forensic Science Handbook* (1982) pp.284-297.

2.0 SCOPE:

In forensic biology, it is usually the determination of whether a bloodstain is of human origin that is of concern. That determination will generally be made using the ABACard® Hematrace® test. However, there may be instances where it is important to determine what nonhuman species was the source of a given sample or whether a nonblood sample is of human origin. In those situations this method may be used and is limited only by the availability of specific antisera and positive control materials (this method may also be used in place of the ABACard® Hematrace® test for the identification of human blood).

3.0 EQUIPMENT/REAGENTS:

3% Ammonium Hydroxide (for aged stains)
Antisera
Agarose, E25 or Sigma Type I
Glass Microscope Slide(s) (5 x 7.5 cm)
GelBond® (cut to 5 x 7.5 cm)
Agarose Punch or equivalent (e.g., pipet and vacuum)

1M NaCl
2% Coomassie Blue Stain and Destain solutions
filter paper

4.0 PROCEDURE:

- 4.1 Extract a small sample (e.g., 2mm² bloodstain) in ~50 μ l dH₂O (or 3% Ammonium Hydroxide for aged bloodstains). Bloodstain extracts should be somewhat dilute and straw-colored in appearance. Extraction time and dH₂O volume will vary depending on stain concentration in order to achieve the desired straw color supernatant.
- 4.2 In order for the agarose to sufficiently adhere to a microscope slide, GelBond® must be adhered to the slide and the agarose gel formed on top of it. Cut GelBond® to the approximate size of your microscope slide and adhere hydrophobic side to slide with a few drops of dH₂O.
- 4.3 Prepare a 1% agarose gel by boiling 0.8 g agarose in 8 ml dH₂O. Carefully pour agarose gel onto hydrophilic side of the GelBond®. Allow solidification of gel.
- 4.4 Using a pre-made Ouchterlony punch or pipet/pipet tip with vacuum, create a pattern of Ag wells around a central Ab well as depicted below (~3mm between Ab and Ag wells) in the solidified agarose.



- 4.5 Pipet appropriate antisera into central well(s) and sample extract(s) (include a positive control of interest and an extraction reagent blank; substrate control where appropriate) into surrounding well(s).
- 4.6 Allow immunodiffusion to take place overnight, at room temperature, in a moisture chamber (enclosed vesicle with dH₂O-moistened paper towel, filter paper, or sponge).

4.7 Precipitin bands at this stage are best viewed with strong backlighting against a dark background. The immunodiffusion gel should be soaked, dried and stained for enhanced visualization.

4.8 Staining

- 4.8.1 Soak immunodiffusion gel in 1M NaCl for ≥ 6 hours (may be left overnight) to remove uncomplexed proteins.
- 4.8.2 Rinse the gel in dH₂O for ~5 minutes; dampen two pieces of filter paper with dH₂O and place on top of gel, followed by a stack of paper towels to serve as a wick. Place a weight on top of the paper towels to 'press' the gel for ≥ 30 minutes. Remove the weight, paper towels, and filter paper and dry the gel in an oven at 56°C-65°C for ≥ 20 minutes.
- 4.8.3 Immerse gel in Stain Solution for 10-15 minutes.
- 4.8.4 Destain until background is clear and blue precipitate bands can easily be seen.
- 4.8.5 Rinse with dH₂O and allow to dry.

5.0 COMMENTS:

- 5.1 A clear, distinct precipitin band between the antisera well and sample well is a positive test result. Extraction blanks should be negative (i.e. no precipitin band present).
- 5.2 "Spurs" may be seen on precipitin bands produced from closely related species.
- 5.3 Note: the gel/GelBond will separate from the glass slide at some point, however, the gel should remain in contact with the GelBond.

**BIOLOGICAL SCREENING: USE OF ALTERNATE LIGHT SOURCE (ALS)****1.0 BACKGROUND:**

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:

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
Filtered Safety Goggles

4.0 PROCEDURE:

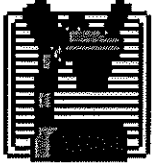
4.1 Selection of the wavelength of light for viewing will depend on the alternate light source used, its available outputs, and the substrate being viewed. A broadband source covering $\leq 530\text{nm}$ wavelengths is sufficient for biological examination but will not eliminate potential background 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 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.

Wavelengths	Safety Goggles
< 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-124; 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 wand can generate heat and potentially cause burns to skin and other materials. Read any manufacturer's safety guidelines provided with the equipment.

**BIOLOGICAL SCREENING: USE OF INFRA RED LIGHTING****1.0 BACKGROUND:**

In forensic biology, IR 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:

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

3.0 EQUIPMENT/REAGENTS:

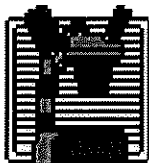
Digital Camera equipped with IR filter
'Night Shot' video camera

4.0 PROCEDURE:

- 4.1 Follow manufacturer's operating instructions for specific details on equipment operation.
- 4.2 Examine evidence using the 'Night Shot' setting on the video camera. Stains will appear dark against a lighter background, under IR, when observed through the 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 video camera or with a digital camera equipped with an IR filter.



BRENTAMINE TEST FOR ACID PHOSPHATASE

1.0 BACKGROUND:

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.

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

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

2.0 SCOPE:

To provide a method to presumptively identify the presence of semen and an aid to be used in locating 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 Stock: Mix 1 part solution A and one part solution B with 8 parts of water. This solution should be prepared fresh each day it is used.

- 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 stock reagents are 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 Stock 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 is sprayed with Brentamine Reagent and analyzed as above.
- 5.4 Fast Blue B is a possible carcinogen and should be handled cautiously.



SAMPLE EXTRACTION FOR SEMEN IDENTIFICATION

1.0 BACKGROUND:

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:

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
Centrifuge

4.0 PROCEDURE:

- 4.1 Label tubes with identifying information.
- 4.2 Take a sample (~3-5 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 (50µl - 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; BI-119) 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.

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**SEMEN IDENTIFICATION: MICROSCOPIC EXAMINATION****1.0 BACKGROUND:**

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.

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

2.0 SCOPE:

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
≥95% Ethanol
Glass Microscope Slide(s)
Cover Slip(s)
Mounting Medium
Microscope (Magnification ~200X-400X)
Mideo System

4.0 PROCEDURE:

4.1 The sample extract is mixed well and ~5-10 μ l, or ~10% deposited on a microscope slide and allowed to dry (this process may be expedited by use of a slide warmer or oven at ~37°C).

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

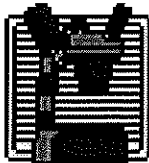
- 4.3 Cover the heat-fixed sample extract with Xmas Tree Stain Solution A and allow staining for ≥ 15 minutes at RT.
- 4.4 Remove the stain with a gentle stream of dH₂O and cover the stained area briefly (~15-20 seconds) with Xmas Tree Stain Solution B. Remove this stain with a stream of EtOH (95% or Absolute).
- 4.5 Allow the slide to dry and apply mounting medium or dH₂O and a cover-slip 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. The Mideo System may be used as an alternate scanning method, or as a means of documentation only (see BI-119).
- 4.7 Documentation in notes should include the following:
- 4.7.1 A description of the condition of the sperm seen (e.g. heads only, mostly heads, some intact, etc.).
- 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.). A representative photograph depicting the overall rating of the slide shall be taken and included in the note packet (see BI-119).
- 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 Anuc.)] 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 second qualified scientist must verify the identification. A photograph of the single 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 should 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 \approx 6 months at RT. After this period, stains should be discarded or checked with a positive (known sperm) slide before use.

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BI-119

SPERM DOCUMENTATION: MIDEO SYSTEM

1.0 BACKGROUND:

The presence of semen may be confirmed by microscopic identification of spermatozoa. The Mideo System allows microscopic images to be visualized on a computer screen, captured, stored and printed. The EZDoc software allows for tracking of images and any modifications. A report with a representative slide view or views of individual spermatozoa will be included in the case file when sperm are identified.

EZDoc Plus Administrator/User Manual.

2.0 SCOPE:

To provide a means of confirming and documenting the presence of semen in cases where spermatozoa are present; as well as other photographic documentation and/or storage.

3.0 EQUIPMENT/REAGENTS:

Imaging Computer with Flat Screen LCD
EZDoc Plus Case Image Management Software
BX-45 Ergonomic Microscope (Magnification ~200X-400X)
Digital Microscope Camera
Touch Screen and Controller
Printer

4.0 PROCEDURE:

4.1 SPERM SEARCH

Note: Refer to BI-118 for slide preparation.

4.1.1 Turn on the computer, monitor, microscope and touch screen controller.

- 4.1.2 Double click the EZDoc Plus icon on the desktop. Enter a User Name and Password, select a role (typically scientist), select a previously used Case ID or choose "New" to enter a new case, enter the Evidence ID, click "OK"; the window will display a live video image.
- 4.1.3 Choose "GO TO LOAD" on the controller screen and place microscope slide in the slide holder with frosted portion on the right. Choose "GO TO HOME" and the slide will move under the oculars.
- 4.1.4 Search the slide at 200X magnification using the joystick or select a pattern from the controller screen (use 400X magnification if necessary to verify sperm morphology). To start a pattern, select the 'Pattern' tab, 'Run' tab, then highlight a pattern and press "RUN".
- 4.1.5 Focus using the knob on the right side of the joystick controller or the "FOCUS UP"/"FOCUS DOWN" buttons on the controller screen.
- 4.1.6 Navigate the pattern scan using the "PAUSE", "CONTINUE", "BACK" and "NEXT" buttons on the controller screen.
- 4.1.7 To save a point(s) during a pattern scan, press "SAVE" while the image is in view.

Note: This step saves points on the controller screen for the current scan pattern. It does not save the points or images in the EZDoc Plus software. See Image Capture below to save images within a case file.

- 4.1.7.1 To review saved images, go to the Stored Points tab; highlight the point(s), choose "OK", highlight a point and press "GO TO".
- 4.1.7.2 To delete saved points, go to the Delete tab; choose "POINTS", highlight the point and press "DELETE". **Do not choose "DELETE ALL".**

4.2 IMAGE CAPTURE

- 4.2.1 Use the "Live Input" white screen icon on the tool bar to toggle between a still image and live input.
- 4.2.2 To save the image, click "File-Save Image" or the "Save Image" disk icon on the toolbar. When the dialog box appears, specify the image file name and description, click "OK".
- 4.2.3 Click "Check Out" to add overlays or make changes to the image.
- 4.2.4 Click "Forms-Forms" and "Tools-Overlays" or the Overlays toolbar icon to add text, graphics, scales, timestamp, etc.
- 4.2.5 Click "Check In" when finished modifying the image and note changes when prompted.
- 4.2.6 If only one to three sperm are present, the image(s) must be peer reviewed by another Scientist prior to printing a report. Highlight the image(s) to be reviewed in the Visual Directory and click "Submit For Review"; an icon with a blue mark will appear next to the image. Notify the reviewing Scientist of the images to be reviewed and their location.

4.3 REPORT

- 4.3.1 Highlight image(s) from the Visual Directory to be printed (under "DB Images" in the file menu), click "Report", select report type and click "Print".
 - 4.3.1.1 Select "Single Image Report" for an image with descriptions and notes.
 - 4.3.1.2 Select "Four Up Report" to display up to four smaller images per page.

4.4 END SESSION

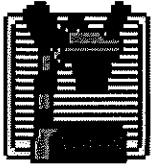
- 4.4.1 Exit out of the EZDoc Plus Software.
- 4.4.2 Choose "GO TO LOAD" on the controller screen and remove the microscope slide.
- 4.4.3 Backup the database by double clicking on the Maxtor OneTouch icon, click 'Sync', followed by 'Sync Now'. A message will appear when complete. Choose 'OK'.
- 4.4.4 Turn off the computer and monitor using "Start-Shut Down" in the bottom left corner and select "Turn Off Computer".
- 4.4.5 Switch off power to the microscope and touch screen controller.
- 4.4.6 Return the mouse to the cradle.

4.5 PEER REVIEW

- 4.5.1 Logon to EZDoc Plus as before and choose the case ID to be reviewed.
- 4.5.2 Select "DB Images" from the file menu. Select the case, evidence and user from the image location directory. Double click on the thumbnail image(s) with a blue mark, perform the review, add notes, etc.
 - 4.5.2.1 To approve an image, select "Accept"; the Peer Reviewer's ID will be added and a red mark will appear on the icon.
 - 4.5.2.2 For a questionable or rejected image, notify the creating Scientist of the objections and/or comments. The creating Scientist may then hit "Reject/Unsubmit" to return the item to its original state.

5.0 COMMENTS:

- 5.1 Original images and a record of all changes are maintained in the History and reports are available.
- 5.2 Do not press "DELETE ALL" on the controller screen or all of the patterns will be deleted.
- 5.3 While viewing a live image, the X, Y and Z coordinates are displayed on the controller screen Digital Read Out tab; to document the point coordinates add a text overlay or notes to the image.
- 5.4 To improve the image, a filter may be engaged by placement in the microscope filter slider. The brightness, contrast, and zoom enhancement features are also available via the Enhance menu.
- 5.5 Casework and crime scene photographs may also be imported, saved and stored. Images can be copied to a local directory or imported directly from a CD, disk, camera, or memory card. Go to "File-Local Images", click on the desired image(s) and select "Batch Move to DB" (files may be moved individually, if desired). A new window will appear, which allows you to rename each image or capture the current names. A report may be printed for the case file as above. Large numbers of photographs (typically associated with crime scenes) can be printed to a proof sheet; from the Visual Directory, click "Select All" and "Print Proof Sheet". This process may be lengthy for numerous photos.
- 5.6 The database should be backed-up (as above) following each user session.



IDENTIFICATION OF SEMEN BY P-30 DETECTION (ABAcard®)

1.0 BACKGROUND:

P-30 is a seminal-fluid-specific protein. 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.

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.

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.

2.0 SCOPE:

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

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), to include both positive (known semen stain extract or Seri™ semen standard [10ng; 10µl of a 1:100 dilution]) and negative (saline) controls, to ~190µl (4 drops) of saline and mix thoroughly.

- 4.3 Transfer each extract (~200 μ l) 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 saline (e.g., 1XPBS, PCR-TE, dH₂O) in 4.2.
- 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.



AMYLASE TEST (PHADEBAS)

1.0 BACKGROUND:

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.

Gaensslen, R. *Sourcebook in Forensic Serology, Immunology, and Biochemistry*. (1983) U.S. Dept. of Justice, Washington, D.C., p 184-187.

Auvdel, Michael J. "Amylase Levels in Semen and Saliva Stains" (1986) *Journal of Forensic Sciences*, 31 (2) 426-431.

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.

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).

Phadebas Amylase Test directions for use, Pharmacia AB, Uppsala, Sweden, 1994 and Magle AB, Lund, Sweden, 2007.

2.0 SCOPE:

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

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; 20µl of 1:100 and 1:500 dilutions of fresh saliva and either neat saliva, or a saliva stain (≤2mm² cutting) as positive controls] 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 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.
- 5.3 This test is not human specific, there may be reactive amylases in plants and non-human animals.

**AMYLASE TEST (STARCH IODIDE)****1.0 BACKGROUND:**

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 test takes advantage of the amylase-catalyzed starch hydrolysis that results in short polysaccharides unable to react with iodine which is detected as a 'clearing zone' around sample wells containing amylase.

Gaensslen, R. *Sourcebook in Forensic Serology, Immunology, and Biochemistry*. (1983) U.S. Dept. of Justice, Washington, D.C., p 184-187.

Auvdel, Michael J. *Amylase Levels in Semen and Saliva Stains*, (1986) *Journal of Forensic Sciences*, 31 (2) 426-431.

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.

2.0 SCOPE:

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

3.0 EQUIPMENT/REAGENTS:

Agarose (Sigma Type I or equivalent)
Soluble Starch
Amylase Diffusion Buffer
Iodine Solution
Petri Dish

4.0 PROCEDURE:

4.1 Sample and control extracts (dH₂O is used for negative control) should be prepared in dH₂O as usual (See BI-116).

- 4.2 Prepare a 0.1% agarose/0.01% starch gel by dissolving 100mg of agarose and 10mg of soluble starch in 10ml of Amylase Diffusion Buffer. Pour the gel into a (~9cm) petri dish, allow it to solidify, and punch wells ~2 mm in diameter, and at least 3 cm apart, into the gel.
- 4.3 Fill wells (do not overflow) with sample extracts and controls.
- 4.4 Mark petri dish for orientation and document sample placement.
- 4.5 Cover petri dish and allow diffusion overnight at 37°C. May be placed in a humid chamber.
- 4.6 To develop, flood the petri dish with ~10ml of 1:100 dilution of the iodine solution (100µl/10ml dH₂O), let stand a few moments to develop the purple color, then pour it off the plate's surface.
- 4.7 Record the results by measuring the diameter of the clear circles. For reporting, see 5.1.

5.0 COMMENTS:

- 5.1 Positive controls should include 1:100 and 1:500 dilutions of fresh saliva as well as neat saliva or an extract of a known saliva stain. If the clear zone of an extract \geq that of the 1:500 control, it is an indication of an elevated level of amylase in the extract and is reported as such. If an extract clears a zone smaller than the 1:500 control, there is an indication that amylase is present; however, there is no demonstration of an elevated level. An extract that demonstrates no clearing zone is reported as 'did not indicate the presence of amylase'.
- 5.2 Additional standards such as neat semen, neat urine or neat vaginal fluid may be tested as needed.
- 5.3 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.
- 5.4 This test is not human specific, there may be reactive amylases in plants, bacteria, and non-human animals.



DETECTION OF URINE (UREASE)

1.0 BACKGROUND:

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.

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

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

2.0 SCOPE:

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:

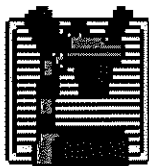
- 4.1 Cut out ~2.0cm² piece of suspected urine stain and controls, cut them into small pieces and place them into appropriately labeled 12x75mm test tubes.
- 4.2 Add 3-4 drops of dH₂O and 6-7 drops of Urease Reagent to each test tube.
- 4.3 Cut a slit into the bottom of each cork and insert a small piece 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 URINE (CREATININE)

1.0 BACKGROUND:

Creatinine, the anhydride of creatine, is a normal constituent of urine and is a waste product of normal metabolism. It is present at high levels in urine compared to other body fluids. This test is based on its reaction with picric acid and is detected by a color change from yellow to orange.

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

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

2.0 SCOPE:

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

3.0 EQUIPMENT/REAGENTS:

Saturated Picric Acid Solution
5% (w/v) NaOH
Sterile/Nanopure H₂O
Concentrated Glacial Acetic Acid
12x75mm test tubes

4.0 PROCEDURE:

- 4.1 Cut out ~0.5 cm² piece of suspected urine stain and controls and place them into appropriately labeled 12x75mm test tubes.
- 4.2 Add 0.5 ml of dH₂O to each test tube and extract for 15 minutes at RT.

- 4.3 Remove the substrate. Add 1 drop (~50 μl) of Picric Acid Solution and 1 drop (~50 μl) of 5% NaOH to each tube.
- 4.4 An orange color develops fully within 15 minutes and is stable for approximately 2 hours. The orange color is a positive indication of Creatinine. The negative control stain solution should remain yellow.
- 4.5 Document results in case notes. Record positive (+) or negatives (-). Analysts may use other descriptive word(s) (e.g., strong, weak,) or numerical grading (e.g., 1+ - 4+) as well.

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 This method is not specific for Creatinine. Although other chromagens are detected by this procedure, their concentrations are negligible.
- 5.3 Among other substances, glucose is reported to produce an orange color with alkaline picrate, although the color is pale. However, if there is likely to be confusion between this and a urine stain, the addition of 2 drops of glacial acetic acid renders a creatinine-containing sample pale yellow after a few minutes. (The color can be restored by adding a few drops of 5% NaOH). Heat is necessary to achieve the color change to pale yellow if the stain is glucose.
- 5.4 The Creatinine Test is one of many presumptive tests for urine; a confirmatory test for the identification of urine in a dried stain is not available.

**DETECTION OF FECAL MATERIAL (UROBILINOGEN)****1.0 BACKGROUND:**

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.

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

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

2.0 SCOPE:

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.

EXTRACTION PROTOCOLS FOR PCR DNA TYPING TESTS**1.0 BACKGROUND:**

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.

Comey, CT et al. "DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis." J For Sci, Vol. 39, 1994, pp. 1254-1269.

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.

Hochmeister, MN et al. "PCR-based typing of DNA extracted from cigarette butts." Int J Leg Med, Vol. 104, 1991, pp. 229-233.

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:

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

3.0 EQUIPMENT:

Qiagen BioRobot® EZ1
Qiagen EZ1 Investigator Kit and card
Centricon® Concentrator Devices
Microcentrifuge
15/50ml conical tubes
56/95°C heat block/oven
Fixed Angle Centrifuge
1.5ml microcentrifuge Tubes (1.5ml tubes)
MicroAmp Tubes
Coarse Sandpaper, Blender, Hammer, Chisel, Drill or Dremel

4.0 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)
Phenol/Chloroform/Isoamyl Alcohol (PCIAA, 25:24:1)
Ethanol (EtOH)
Phosphate Buffered Saline (PBS)
Ethyl Ether
Xylene
10% SDS
Chelex Reagent

5.0 DNA EXTRACTION PROCEDURES:

NOTE: 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 samples sizes may be adjusted accordingly. 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).

Caution: See Comments 1.

5.1 WHOLE BLOOD SAMPLES (EZ1 EXTRACTION):

5.1.1 Place ~3 μ l - 10 μ l whole blood into a EZ1 sample tube provided in the EZ1 Investigator kit. Bring the volume up to 200 μ l with Stain Extraction Buffer.

5.1.2 Proceed to 6.0.

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

5.2.1 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 cotton swabs

containing sample (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), or $\sim 3\text{mm}^2$ - 5mm^2 portion of tissue.

5.2.2 Add the following to the tube:

190 μl SEB
10 μl Pro K

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

5.2.3 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.

5.2.4 Large cuttings/substrates (if applicable) may be removed by piggyback/spin basket centrifugation at low speed (3,000 - 5,000 rpm) for 3-5 minutes and discarded.

5.2.5 Proceed to **6.0**.

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

5.3.1 Place one of the following samples into a sterile 1.5ml tube: $\sim 3\text{mm}^2$ - 1cm^2 cutting/portion or swabbing of samples on cloth or porous materials (includes cigarette butts, gum, and envelope flaps/stamps), $\sim 1/8$ - $1/2$ (~equivalent of previous sample size) cutting/portion of cotton swabs containing sample (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), $\sim 3\text{mm}^2$ - 1cm^2 portion of tissue, or $\sim 10\mu\text{l}$ - $50\mu\text{l}$ whole blood.

5.3.1a Envelope Flaps/Stamps: Presoak the envelope flap/stamp cutting (steam may be used to loosen the seal prior to extraction) in 1.0ml of sterile water at 4°C for a minimum of 5 hours (may be left overnight). Remove the substrate by piggyback/spin basket centrifugation and discard. Remove all but $50\mu\text{l}$ of the supernatant and discard it. Proceed to **5.3.2** with the remaining pellet.

5.3.1b Optional (see **Comments 3**): Presoak bloodstains using 1ml of sterile PBS in a sterile 1.5ml tube. Vortex briefly, and incubate 30 minutes at RT. Vortex briefly, then spin at high speed in a microcentrifuge for ~1 minute. Using a micropipette, remove supernatant and proceed to **5.3.2**.

5.3.2 Add the following to the tube:

500µl SEB
15µl Pro K

5.3.3 Mix and incubate at 56°C for a minimum of 1 hour (may be left overnight). It is recommended that non-reference samples incubate for at least 3 hours when possible.

5.3.4 Proceed to **7.0**.

5.4 EXTRACTION OF HAIR SAMPLES

Note: For removal of hair(s) mounted on a slide, see **9.0**.

5.4.1 Examine the hair(s) under a stereomicroscope and 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.

5.4.2 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 **5.2.1** or **5.3.1**).

5.4.3 Even if the hair(s) was washed prior to proceeding to **5.4.4**, 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).

5.4.4 To an EZ1 sample tube, containing the hair sample, add:

180µl SEB
10µl 1M DTT
10µl ProK

or, for organic,

To a 1.5ml tube, containing the hair sample, add:

500µl SEB
20µl 1M DTT
15µl ProK

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

5.4.6 Proceed to **6.0** for EZ1 isolation or to **7.0** for organic isolation.

5.5 EXTRACTION OF BONE AND TEETH

5.5.1 Obtain a fragment of bone and remove any tissue present, using ethyl ether (shake vigorously with a few mls of ether in a 15ml 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 **5.5.2**.

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

5.5.3 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.

5.5.4 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.

5.5.5 To the tube, add:

500µl SEB

15µl ProK

Mix thoroughly and incubate at 56°C overnight.

5.5.6 For EZ1 isolation, spin in a centrifuge at low speed (3,000 - 5,000 rpm) for 3-5 minutes, transfer 200-500µl of the supernatant to an EZ1 sample tube and proceed to 6.0.

For organic isolation, proceed directly to 7.0.

Note: For aged bones, it may be necessary to process multiple samples and combine the extracts prior to proceeding to quantification.

5.6 EZ1 DIFFERENTIAL EXTRACTION OF SEMEN-CONTAINING SAMPLES:

Note: For removal of sample from mounted slide, see 9.0.

5.6.1 Place cutting/sample (the size of sample used will be case dependent and based upon microscopic exam and total sample amount) into an EZ1 sample tube and add ~150µl PBS or sterile deionized water. Agitate the substrate to loosen cellular material and place at 4°C for 1-4 hours (up to overnight).

5.6.2 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.

5.6.2a Optional: Resuspend the pellet and place 3-5µl on a slide for microscopic evaluation (See BI-118; BI-119). The substrate may be discarded if the pellet contains a sufficient number of spermatozoa; however, it may be desirable to add the substrate back to increase the total amount of DNA in the sample.

5.6.3 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 490 µl.

5.6.4 Mix and incubate at 56°C for 15 minutes to a maximum of 1 hour.

5.6.5 Label a new EZ1 sample tube. Remove substrate (if present) by using piggyback/spin basket centrifugation and discard. A final centrifugation on high speed for ≥1 minute should be performed to further solidify the pellet.

5.6.6 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 **6.0**.

5.6.6a 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 **5.6.3-5.6.4** may, at the scientist's discretion, be repeated 1-2 times prior to proceeding to **5.6.7**. These additional supernatants do not need to be retained.

5.6.7 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.

5.6.8 Repeat **5.6.7** 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.

5.6.8a Optional: Resuspend the pellet and place 3-5µl on a slide for microscopic evaluation (See BI-118; BI-119). If intact epithelial cells remain, the pellet should be redigested (**5.6.3 - 5.6.8**).

5.6.9 To the remaining sperm pellet solution add:

180µl SEB
10µl 1M DTT
10µl ProK

Note: up to 490µl SEB may be used

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

5.6.11 Proceed to 6.0

5.7 ORGANIC DIFFERENTIAL EXTRACTION OF SEMEN-CONTAINING SAMPLES:

Note: For removal of sample from mounted slide, see 9.0.

5.7.1 Place cutting/sample (the size of sample used will be case dependent and based upon microscopic exam and total sample amount) into a sterile 1.5ml tube and add ~150µl PBS or sterile deionized water. Agitate the substrate to loosen cellular material and place at 4°C for 1-4 hours (up to overnight).

5.7.2 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 ~50µl of the supernatant and discard.

5.7.2a **Optional:** Resuspend the pellet in the remaining 50µl and place 3-5µl on a slide for microscopic evaluation (See BI-118; BI-119). The substrate may be discarded if the pellet contains a sufficient number of spermatozoa; however, it may be desirable to add the substrate back to increase the total amount of DNA in the sample.

5.7.3 To the remaining cell pellet and substrate (if present) add the following:

500µl SEB
15µl Pro K

5.7.4 Mix and incubate at 56°C for 45 minutes to a maximum of 1 hour.

5.7.5 Label a new sterile 1.5ml tube. Remove substrate (if present) by using piggyback/spin basket centrifugation and discard. A final centrifugation on high speed for ≥ 1 minute should be performed to further solidify the pellet.

5.7.6 Remove all but $\sim 50\mu\text{l}$ 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 sterile tube and store at 4°C or proceed directly to 7.0.

5.7.6a 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 5.7.3-5.7.4 may, at the scientist's discretion, be repeated 1-2 times prior to proceeding to 5.7.7. These additional supernatants do not need to be retained.

5.7.7 Wash the sperm pellet as follows: Resuspend the pellet in $1000\mu\text{l}$ PBS by vortexing briefly. Spin in a microcentrifuge for ~ 5 minutes at maximum speed ($>10,000\text{rpm}$). Remove all but $\sim 50\mu\text{l}$ of the supernatant and discard it.

5.7.8 Repeat 5.7.7 1-5 more time(s). In instances of low sperm amounts, additional washes are recommended. The final wash performed is to be done using $1000\mu\text{l}$ sterile deionized water.

5.7.8a Optional: Resuspend the pellet in the remaining $50\mu\text{l}$ and place $3-5\mu\text{l}$ on a slide for microscopic evaluation (See BI-118; BI-119). If intact epithelial cells remain, the pellet should be redigested (5.7.3 - 5.7.8).

5.7.9 To the remaining sperm pellet solution add:

500 μl SEB
20 μl 1M DTT
15 μl ProK

5.7.10 Mix and incubate at 56°C for a minimum of 2 hours (may be left overnight).

5.7.11 Proceed to 7.0.

5.8 CHELEX EXTRACTION:

Note: Chelex may also be used for clean-up of samples that have already been extracted to remove contaminants/inhibitors as needed. Start with step 5.8.4.

- 5.8.1 Place an ~3mm² cutting of a bloodstain, or 3µl whole blood into a sterile 1.5ml tube and add 1ml of sterile deionized water.
- 5.8.2 Incubate at RT for 15-30 minutes with occasional mixing or gentle vortexing.
- 5.8.3 Spin in microcentrifuge for 2-3 minutes. Remove all but 20-30µl of the supernatant and discard it. If the sample is a bloodstain, leave the substrate in the tube.
- 5.8.4 Using a wide bore pipette tip, or a tip with the end cut off, add 200µl freshly prepared 5% Chelex (0.5g Chelex resin/10ml sterile nanopure water). Make sure the Chelex solution is well mixed before adding to the sample.
- 5.8.5 Incubate at 56°C for 15-30 minutes.
- 5.8.6 Vortex at high speed for 5-10 seconds.
- 5.8.7 Incubate in boiling water for 8 minutes.
- 5.8.8 Vortex at high speed for 5-10 seconds, followed by centrifugation at high speed (≥ 10,000 rpm) for 2-3 minutes. This extract may be taken directly to realtime PCR (see BI-207) for quantification of the DNA.

Note: Care must be taken to not disturb the Chelex resin when removing sample for subsequent procedures. After storage and prior to sample removal, repeat step 5.8.8.

6.0 BIOROBOT EZ1 ISOLATION PROCEDURE

Note: The BioRobot EZ1 may also be used for clean-up of samples that have already been extracted using the organic procedure to remove contaminants/inhibitors as needed. Transfer the extract to a EZ1 sample tube, bring the volume

up to 200 μ l with Stain Extraction Buffer, and begin with step 6.1.

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.

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 "4" for the "Large Volume" protocol for 500 μ l sample volumes. **Note:** Step through the prompt regarding additional 'MTL' Buffer.

6.3 Press "2" to elute in TE.

6.4 Select either the 50 μ l or the 200 μ l elution volume from the menu (option 1 or 3, respectively). The 50 μ l elution may be preferable for FTA, dilute samples, or those suspected to be of low DNA concentration. The 200 μ l elution volume may be preferable for samples exhibiting potential inhibition.

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.

6.6 Open the workstation door.

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.

6.8 Insert the appropriate number of reagent cartridges (1-6 per extraction run) into the cartridge rack, snapping them into place. Additional samples (beyond 6) can be accommodated in subsequent instrument runs. Place the loaded cartridge rack into the instrument, followed by the tip rack.

6.9 Load 1-6 tip holders containing filter-tips into row 2 of the tip rack.

- 6.10 Load 1-6 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.
- 6.11 Load 1-6 opened sample tubes from step 5.1.1 into row 4 of the tip rack.
- 6.12 Close the workstation door.
- 6.13 Press "Start" to start the extraction protocol.
- 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.
- 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.
- 6.16 At the completion of all runs for the day, clean the piercing tool (option #3 from the tools screen), D-rings and tip adaptor, tip rack, cartridge rack, and interior of the instrument with 70% Ethanol, followed, optionally, by nanopure water.
- 6.17 Switch off the instrument, leaving the Investigator card in place.
- 6.18 Proceed to realtime PCR (see BI-207) for quantification of the purified DNA obtained in step 6.15.

7.0 ORGANIC PURIFICATION PROCEDURE:

Note: For most stains the cuttings/substrate will not interfere with organic extraction and need not be removed prior to proceeding to 7.1. Larger cuttings/samples can be removed by piggyback/spin basket centrifugation at low speed (3,000 - 5,000 rpm) for 3-5 minutes and discarded. Proceed to 7.1.

- 7.1 In a fume hood, add 500 μ l phenol/chloroform/isoamyl alcohol (PCIAA) to the extract. Mix vigorously by hand to achieve a milky emulsion. Spin in microcentrifuge for 3-5 minutes to achieve layer separation.

7.2 If the aqueous phase is clear, proceed to 8.0. If it is not clear (e.g. cloudy or large or 'dirty' interface), transfer the aqueous layer to a fresh sterile 1.5ml tube. Repeat 7.1 1-2 times until the interface is clean and aqueous phase is clear. Proceed to 8.0.

8.0 ISOLATION VIA CENTRICON CONCENTRATOR DEVICE:

Note: Centricon concentration of samples with high DNA concentrations will be performed separately from those with low DNA concentrations.

8.1 Assemble a Centricon-100 unit according to the manufacturer's directions and label the unit.

8.2 Add 1.5ml of TE to the upper Centricon-100 reservoir.

8.3 Add the entire aqueous layer (approximately 500µl) to the upper reservoir containing TE. Discard the phenol mixture (including substrate if present) into the organic waste container in the hood. Discard the tube into a biohazard waste container.

8.4 Cover the Centricon tube with the retentate cup. Spin in a fixed angle centrifuge at ~3500 rpm for 10-20 minutes. The DNA sample will be concentrated in ~20-40µl of TE in the upper Centricon reservoir while molecules with molecular weights of less than ~100,000 daltons will pass through the filter.

Note: The Centricon units are sensitive to rotor forces. Do not centrifuge above 2000 x g. Centrifugation time can be increased if the volume does not reduce to ≤40µl in the specified time.

8.5 Add 2ml of PCR TE to the concentrated DNA solution in the upper Centricon reservoir and repeat the centrifugation step as in 8.4. Discard the effluent that has collected in the lower reservoir.

8.6 Repeat 8.5 for a total of 3 washes.

8.7 Invert the upper reservoir onto the retentate cup provided with the unit. Centrifuge at ~2500 rpm for 2 minutes to transfer the DNA concentrate into the cup.

8.8 Estimate the volume of the concentrate using a pipette to transfer to a labeled sterile 1.5ml tube. Proceed to realtime PCR (see BI-207) for quantification.

9.0 REMOVING MATERIAL FROM SLIDES:

9.1 FREEZING:

9.1.1 Place slide in -20°C freezer for 3-5 minutes.

9.1.2 Wearing safety glasses, pry the cover slip off.

9.1.3 Add a drop of xylene to dissolve the mounting medium.

9.1.4 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 E21 sample tube or a 1.5ml tube. Repeat 3-4 times and proceed to 5.6.3 or 5.7.3.

9.1.5 Rinse the hair briefly in absolute ethanol to remove the xylene and proceed to hair extraction under 5.4.

9.2 SOAKING IN XYLENE:

9.2.1 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.

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

9.2.3 Rinse the hair briefly in absolute ethanol to remove xylene and proceed to hair extraction under 5.4.

10.0 DNA EXTRACTS:

10.1 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).

10.2 Any extract remaining, following the completion of analysis will be retained in the corresponding case DNA packet (See BI-102).

11.0 Comments:

- 11.1** These methods employ the use of phenol that is a strong organic acid and may cause severe burns in addition to other effects. All operations with these chemicals should be performed in the hood with appropriate protective gear (gloves, lab coat and eyes protected by hood shield and/or goggles).
- 11.2** An appropriate reagent blank (for each type of extraction) should be carried through all extraction steps to check the purity of the reagents being used. There need only be one reagent blank per extraction run, it is not necessary to have a separate one for each case that is extracted at the same time.
- 11.3** 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).
- 11.4** 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.

DNA QUANTIFICATION: REAL-TIME PCR**1.0 BACKGROUND:**

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

Green, R.L., et al, "Developmental Validation of the Quantifiler™ Real-Time PCR Kits for the Quantification of Human Nuclear DNA Samples," Journal of Forensic Science, Vol. 50, No. 4, pp. 809-825.

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.

Quantifiler™ Kits (Quantifiler™ Human DNA Quantification Kit and Quantifiler™ Y Human Male DNA Quantification Kit) User's Manual, Applied Biosystems.

7500/7500 Fast Real-Time PCR Systems Maintenance Guide, Applied Biosystems.

2.0 SCOPE:

To provide a reliable method for the consistent quantification of small amounts of human DNA isolated from forensic samples.

3.0 EQUIPMENT/REAGENTS:

ABI 7500/Computer	96-well Reaction Plate
ABI 7500 SDS Software	96-well Reaction Plate Base
Pipettors	Optical Adhesive Covers
Pipette Tips	Centrifuge (optional)
Quantifiler™ Human Kit	Microcentrifuge Tubes
20 µg/ml Glycogen (optional)	PCR-TE

4.0 PROCEDURE:

4.1 PREPARATION OF DNA STANDARDS:

- 4.1.1 Label 8 sterile microfuge tubes A through H or 1-8.
- 4.1.2 Dispense 30 μ l (or adjusted amount according to the kit QC results: Form 419-QC) of PCR-TE into tube A (Std. 1) and 20 μ l of PCR-TE into tubes B-H (Std. 2-8).
- 4.1.3 Mix the Quantifiler Human DNA Standard thoroughly by vortexing 3-5 seconds. Transfer 10 μ l to tube A (Std. 1). Mix the dilution thoroughly.
- 4.1.4 Prepare Std. 2-8 via a serial dilution by mixing and subsequent 10 μ l transfers from tubes A through H. The dilution series consists of 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/ μ l, respectively.

4.2 REACTION PREPARATION:

- 4.2.1 Determine the number of samples to be quantified (including, at minimum, 2 sets of DNA standards).
- 4.2.2 Fill out the 7500 Load Sheet (Form 206-BI) on the 'Plate Setup' tab of the Excel spreadsheet/template. Print a copy for the case record. Choose the 'Plate Document' tab and ensure the information is correct and corresponds to the Load Sheet information entered. Perform a 'Save As' of the Plate Document Worksheet to disc (e.g. USB drive) for subsequent transfer to the ABI 7500. The document must be saved as a .txt file.
- 4.2.3 Calculate the volume of reaction components needed, based upon the number of samples to be quantified and adding 2 or 3 reactions to compensate for loss and variability due to pipetting. The following are the volumes needed per reaction.

Quantifiler PCR Reaction Mix	12.5 μ l
Quantifiler Human Primer Mix	10.5 μ l

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

- 4.2.4 Thaw the primer mix and vortex 3-5 seconds. Pulse-spin prior to opening the tube. Mix the PCR Reaction Mix by gently swirling the bottle prior to use.
- 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 Master Mix by pipetting the required volumes of primer and reaction mixes into an appropriately sized microcentrifuge tube. Mix by vortexing 3-5 seconds, followed by a pulse-spin.
- 4.2.7 Carefully pipet 23 μ l of the PCR Master Mix into the bottom of each reaction well to be used. 'Blowing-out' the pipette is not recommended to avoid splashing and/or bubbles in the well.
- 4.2.8 Add 2 μ l of sample or standard to the appropriate reaction well, being careful to avoid bubbles as much as possible.
- 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, select **File>New** and choose **Absolute Quantitation** for Assay, **96-Well Clear** for Container, and **Quantifiler Human** for Template.

4.3.5 Import the previously saved plate document by selecting **File>Import Sample Set-Up**. Browse to locate the saved .txt file and choose **OK**.

4.3.6 Review the plate document to ensure the appropriate detectors and tasks have been applied to each sample. Change the task for any unused wells to **NTC** in **View>Well Inspector** or by highlighting and double clicking on a well(s). Make any other changes, as necessary. Select the **Instrument** tab and review the thermal cycler conditions [Stage 1: 1 cycle, 95°C, 10:00 min.; Stage 2: 40 cycles, 95°C, 00:15 min, 60°C, 1:00 min.; 25µl sample volume; 9600 emulation; Data collection: Stage 2, Step 2 (60.00@1:00)]

Note: Detectors are created during the initial instrument set-up and/or kit usage. Refer to the Quantifiler Kits User's Manual (page 2-11) for instructions on creating detectors if needed.

4.3.7 Save the plate document as a .sds file with the appropriate plate name.

4.3.8 Under the **Instrument** tab, click **Start** to begin the run. When the run has completed, proceed to 4.4.

4.4 ANALYSIS AND RESULTS:

4.4.1 Open the plate document to be analyzed.

4.4.2 Select **Analysis>Analysis Settings** and verify the settings are set as follows: All for Detector, Manual Ct, 0.200000 for Threshold, Manual Baseline, 3 for Baseline Start (cycle), and 15 for Baseline End (cycle). Click **OK**.

4.4.3 Select **Analysis>Analyze**.

4.4.4 In the **Results** tab, select the **Standard Curve** tab and choose Quantifiler Human as the detector. Review the data for inconsistencies from the following:

An R^2 value of >0.99 indicates a close fit between the standard curve regression line and the individual C_T data points of quantification standard reactions.

An R^2 value of <0.98 needs further analysis of the standard curve for problems. Refer to the Quantifiler Kits User's Manual (page 5-6) for troubleshooting guidelines.

The slope should fall within the typical slope range of -2.9 to -3.3. A slope of -3.3 indicates 100% amplification efficiency.

- 4.4.5 Select the **Amplification Plot** tab (in the Results tab) and choose either the Quantifiler Human, or the IPC detector. Ensure the Threshold is set to 0.20 before proceeding (**Note:** the threshold bar will be green if the data has been analyzed and red if analysis is needed). Highlight the sample(s) of interest in the table to view the associated plot(s). Review the plots for both detectors for amplification and/or inconsistencies.
- 4.4.6 Select the **Component** tab within the Results tab. The halogen lamp may need replacement if the dye signal lines contain spikes or appear wavy/unstable and/or if the Rox value begins approaching or has fallen below 500. See Comment 2. **Note:** it is important to use the same sample well each time.
- 4.4.7 In the Results tab, select the **Report** tab and highlight the sample(s) of interest to view the results. Review the Qty column to determine the amount of DNA present in each sample. Review the Internal Positive Control (IPC) C_T value for each sample. It should fall within a range of 20-30. If the value is >30 for a particular sample, there may be an indication of inhibition.
- 4.4.8 Export the report. Within the report tab, select **Tools>Report Settings** and check the appropriate boxes to be displayed in the report and click **OK**. Print a copy of the Standard Curve for the case record. Select **File>Export** to export the report (e.g. to USB drive) as a tab-delimited text file.
- 4.4.9 Open the 7500 Results Sheet (Form 209-BI) template in Excel. Import the tab-delimited text file into the **Raw Data** tab of the worksheet. Choose the **Results** tab and

review the imported data. Delete any unused wells from the sheet. Adjust values in the Final concentration and ul Sample for Dilution columns. Print a copy of the results sheet for the case record. Perform a 'Save As' prior to exiting the template.

5.0 COMMENTS:

- 5.1 Refer to the Quantifiler Kits User's Manual for specific thermal cycler conditions, additional user information, and troubleshooting guidelines.
- 5.2 If the Component Dye signals appear unstable and/or Rox values approach 500, 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.
- 5.3 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.

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**STR AMPLIFICATION: PP16****1.0 BACKGROUND:**

DNA analyses have revolutionized forensic biology. The advent of PCR allowed scientists to analyze evidentiary material present in minute quantities and degraded states. The identification of forensically significant STR loci has allowed scientists to combine the discrimination attainable with the older RFLP technology with the speed and sampling capabilities of other PCR-based methodologies. The PowerPlex™ 16 allows the co-amplification of the core CODIS 13 loci, as well as, Amelogenin, and two pentanucleotide-repeat loci, Penta D and Penta E.

Butler, J. *Forensic DNA Typing: Biology and Technology Behind STR Markers*. (2001) Academic Press.

GenePrint® PowerPlex™ 16 System Technical Manual

2.0 SCOPE:

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:

BioHood
10% Bleach or Dispatch®
UV light
Thermocycler
Microcentrifuge
MicroAmp tubes
PowerPlex™ 16 Kit Contents
AmpliQa Gold® DNA Polymerase

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-0.4ng/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. For those samples that were deemed to be <1ng (or not detected at all), the maximum amplification volume (19.2μl for PowerPlex 16) should be used. For larger volume samples, it may be necessary to concentrate the sample prior to amplification. The analyst may also choose to extract, quantify, and combine additional sample prior to amplification as an alternative.
- 4.1.2 The amount of DNA template added to an amplification reaction should be targeted at 0.5-1.0ng.

4.2 AMPLIFICATION SET-UP:

- 4.2.1 Determine the number of samples to be amplified and label microAmp tubes (200μl) for identification. Label a microfuge tube(s) for the Master Mix. Place the labeled sample tubes in a rack or microAmp tray. The scientist may choose to irradiate the tubes with UV light at this point (≥ 15 minutes) while performing other preparations.
- 4.2.2 Thaw the Gold ST★R 10X Buffer and the PowerPlex™ 16 10X Primer Pair Mix.
- 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 1 or 2 reactions to compensate for loss and variability due to pipetting. Use Form 210-BI for recording this information. The following is a list of the 'fixed' amounts to be added for a 25μl reaction.

Gold ST★R 10X Buffer	2.5μl
PowerPlex™ 16 Primer Mix	2.5μl

*AmpliTag Gold® 0.8µl
DNA Template + dH₂O 19.2µl

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

* AmpliTag Gold® volume is based upon its typical concentration of 5U/µl. Check tube to verify concentration and adjust volume as necessary to add 4U of enzyme per reaction.

- 4.2.4 Pipet PCR Master Mix into each reaction tube. The negative amplification control should be the last sample processed.
- 4.2.5 If DNA concentrations were not the same, add appropriate volume of dH₂O as necessary.
- 4.2.6 Pipet each DNA sample into the appropriate tube. 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 (with the exception of the negative control which remains open throughout the process). Use 9947A control DNA for the positive amplification control and dH₂O for the negative amplification control. Again, making additions to the negative control last.
- 4.2.7 Ensure all of the sample tubes are closed tightly. Mix by finger or standard vortex and spin in microfuge, if necessary, to bring the reaction components to the bottom of the tube and remove any bubbles. Return samples to the rack or MicroAmp tray, placing them in position for the thermal cycler (record position on Form 210-BI).
- 4.2.8 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 the rack down in this room. Remove gloves and return the rack to the biology lab. The rack may be placed in the hood under UV light for ~30 minutes at this time.

4.3 THERMAL CYCLING PARAMETERS:

After the samples have been placed in the thermal cycler, turn on the power and select the pre-programmed 'pp16stdrun' cycling profile with the following conditions:

95°C for 11 minutes, then:

96°C for 1 minute, then:

ramp 100% to:

94°C for 30 seconds,

ramp 29% to:

60°C for 30 seconds

ramp 23% to:

70°C for 45 seconds

for **10 cycles**, then:

ramp 100% to:

90°C for 30 seconds

ramp 29% to:

60°C for 30 seconds.

ramp 23% to:

70°C for 45 seconds

for **20 cycles**, then

60°C for 45 minutes, then:

4°C soak

5.0 AMPLIFIED DNA PRODUCT:

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 4°C. For longer storage periods, samples should be frozen at -20°C. Amplified product is ONLY stored in the Amp/PostAmp room.

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 to the dishwashing room. The container

will be placed into a second biohazard bag, sealed and disposed of with other biohazardous material.

6.0 COMMENTS:

- 6.1 Clean surfaces with freshly made 10% bleach solution or Dispatch® prior to set-up.
- 6.2 Wear gloves at all times during amplification set-up.
- 6.3 Mix all reagents thoroughly (e.g., vortex) and pulse-spin them in microfuge prior to dispensing.
- 6.4 A precipitate may form in the Gold STAR 10X Buffer, this may be eliminated by briefly heating the solution at 37°C prior to mixing.

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STR TYPING: CAPILLARY ELECTROPHORESIS AND DATA ANALYSIS**1.0 BACKGROUND:**

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 2-6bp 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 addition to the 13 core CODIS loci, the PowerPlex™ 16 multiplex includes Amelogenin, a gender identification locus, and two pentanucleotide repeat STR loci, Penta D and Penta E. STR typing, with amplified products generated from this kit, separated by capillary electrophoresis on the 310 and/or 3130 Genetic Analyzer with data collection and analysis software employed in developing the genetic profiles, will be used to produce STR profiles from evidentiary material for entry into CODIS.

Butler, J. *Forensic DNA Typing: Biology and Technology Behind STR Markers*. (2001) Academic Press. GenePrint® PowerPlex™ 16 System Technical Manual

ABI PRISM™ 310 Genetic Analyzer User's Manual

ABI 3130/3130xl Genetic Analyzer Getting Started Guide

ABI 3130/3130xl Genetic Analyzer Maintenance Troubleshooting and Reference Guide

GeneMapper™ ID Software User Guide

2.0 SCOPE:

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

3.0 EQUIPMENT/REAGENTS:

310 and 3130 Genetic Analyzers with Data Collection Software
GeneMapper™ ID Software
Computers
Heating Block (or 9700 Thermal Cycler)
Benchtop Cooler
Capillaries
Capillary Arrays
Syringe
Sample Tubes and Septa
96 Well Reaction Plates and Septa
Buffer Jars and Septa
Buffer Reservoirs and Septa
POP4 Polymer
Genetic Analyzer Buffer
PowerPlex® 16 Kit Contents
PowerPlex® 16 Matrix Standards
Deionized Formamide
Nanopure Water

4.0 PROCEDURE:

4.1 AMPLIFIED FRAGMENT DETECTION USING THE 310

Note: Prior to using the ABI PRISM™ 310 Genetic Analyzer for samples, 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, four standards are run under the same capillary electrophoresis conditions that will be used for samples and allelic ladders. Use the 310 Matrix Standard set, which includes the Fluorescein Matrix, JOE Matrix, TMR Matrix and CXR Matrix for the blue, green, yellow and red 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.

4.1.1 Turn on instrument, turn on computer and refer to ABI PRISM® 310 Genetic Analyzer's User's Manual for detailed instructions on instrument set-up. Shut down is performed in opposite order (computer, then 310). The computer may be shutdown after each run; the 310 should only be shut down if it will not be in use for extended periods. Fill-in appropriate information in the 310 Injection Log (Form 422A-OC).

4.1.2 Open the ABI PRISM® 310 Collection Software. In the manual control window, the scientist may use 'temperature set' to set the heat plate to 60°C so that it will be ready to run. Using File/New/Sample Sheet, create a 48-well Genescan® sample sheet as described in the ABI PRISM® 310 Genetic Analyzer's User's Manual. If there is room on the sample sheet, 'CCD' and 'SEQFILL' may be added (generally, as the last two samples). There is a 'Setup Check' sample sheet already on the instrument so that these samples may be placed in a pre-run by themselves, rather than adding them to the new sample sheet. Enter appropriate identifying information for other samples into the sample column as follows:

Matrix samples: FLUOR, JOE, TMR or CXR

Allelic Ladder: LADDER (or PP16 LADDER)

Controls: POS [or (+), etc.], NEG [or (-), etc.], BRB (sperm reagent blank), SPRB (male reagent blank), ECRB (e-cell reagent blank), etc.

Case Samples: **XY99999999-(or /)ZZ...**,
(e.g., VM20010112-1AEC or VM20010112/1AEC) where:

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.)

Y = Letter for Lab (M, C or P)

999999999= Lab Case Number

ZZ...= numbers and letters that designate case
Item (including 'SP' for sperm cell and 'EC' for
epithelial cell at end of number to delineate
fraction).

4.1.3 Using File/New/Injection List, create a new
Genescan® Injection List, selecting the
appropriate sample sheet from the pull-down menu.
Using pull-down selections, order samples,
placing allelic ladders in the 1st and last
injection positions as well as, at least every
20-25 samples in a long run. Move the 'CCD
DUMMY' and 'SEQFILL DUMMY' to the 1st and 2nd
injection positions, respectively if they were
not run separately. Matrix samples are often
analyzed in a separate run. However, they may be
run with other samples, in which case they are
run as contiguous samples either at the beginning
or the end of a run.

4.1.4 Select a run module with the following settings:

GS STR POP4 (1ml) A
Inj. kV: 15.0
Run kV: 15.0
Run °C: 60
Run Time (minutes): 30
Matrix File: none
Autoanalyze: No

Inj.Secs: 3-10 secs

3 secs - Matrix Standards, Allelic Ladders and
1ng POS control DNA (injection times may
be adjusted [3-10 seconds per analyst's
discretion] but a 3 sec. inj. time for

single-source samples estimated at $\geq 1\text{ng}$ generally produce good results).

5 sec.- Samples $< 1\text{ng}$ generally produce good results.

4.1.5 To prepare samples for capillary electrophoresis:

Label sample tubes. For amplified products (including controls), typically $1\mu\text{l}$ - $1.5\mu\text{l}$ rxn is added to $25\mu\text{l}$ of ILS Master Mix (made by adding $0.5\mu\text{l}$ ILS600 size standard/sample; $24.5\mu\text{l}$ deionized formamide/sample and adding quantities for N+2 in Master) that has been dispensed into sample tubes. For Allelic Ladders add $1\mu\text{l}$ Ladder to $25\mu\text{l}$ Master Mix. **Note:** The master mix may be altered by adding $1\mu\text{l}$ ILS600 size to $24\mu\text{l}$ deionized formamide if ILS peaks are too low.

Matrix samples ($2\mu\text{l}$) are added to $25\mu\text{l}$ of deionized formamide (without size standard).

4.1.6 Following sample addition, place septa on sample tubes, mix (spin as necessary) and heat denature for ~ 3 minutes at 95°C . Immediately chill in benchtop cooler (or on ice) for ≥ 3 minutes (perform on all sample types - ladders, matrix, controls and samples).

4.1.7 Assemble tubes for run into appropriate order (based on the sample sheet) in a 48-tube autosampler sample tray removing any moisture with a Kimwipe if necessary.

4.1.8 Place the autosampler tray in the instrument and close the doors.

4.1.9 Prior to hitting the 'Run' button to start the capillary electrophoresis, make sure that the autosampler has been calibrated if necessary, the syringe has sufficient polymer for the run and its current position is correct, and there are no bubbles that may interfere with the run. Click 'Run' and monitor electrophoresis in the 'Raw Data' and 'Status' windows. Each sample will take ~ 40 minutes.

4.1.10 If, at any point in the run, prior to the last injection, 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]) the scientist may insert a new row (Ctrl -I) and select that sample from the pull-down menu, changing the injection time if necessary.

4.1.11 After completion of the run finish filling out the 310 Injection Log (Form 422A-QC).

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, four standards are run under the same capillary electrophoresis conditions that will be used for samples and allelic ladders. Use the 3130 Matrix Standard set, which includes the Fluorescein Matrix, JOE Matrix, TMR Matrix and CXR Matrix for the blue, green, yellow and red 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). Fill-in appropriate information in the 3130 Injection Log (Form 422B-QC).

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 'ga3130xl'. 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 <100 for injection priority.

Instrument Protocol 1: Choose the Spectral instrument protocol from the drop down menu

PowerPlex 16 specific run module and protocol settings for Spectral Calibrations are as follows:

Module Type:	Spectral
Template:	Spect36_POP4
Inj. kV:	3
Data Delay Time:	100
Run Time (seconds):	800
Protocol Type:	Spectral
DyeSet:	F
Array Length:	36
Chemistry:	Matrix Standard
Lower condition bound:	4.0

Upper condition bound: 12.0

Inj.Secs: 5

4.2.2.2 For a sample plate fill out the 3130 Load Sheet (Form 216-BI) on the '3130 Load Sheet' tab of the Excel spreadsheet/template. Print a copy for the case record. Choose the '3130 Plate Template' 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 (or PP16_LADDER)

Controls: POS [or (+), etc.], NEG [or (-), etc.], BRB (blood reagent blank), SPRB (sperm reagent blank), ECRB (e-cell reagent blank), etc.

Case Samples: **XY99999999_ZZ...**, where:
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.)
Y = Letter for Lab (M, C or P)
999999999= Lab Case Number
ZZ...= numbers and letters that designate case Item (including 'SP' for sperm cell and 'EC' for

epithelial cell at end of number to delineate fraction).

(e.g., VM20010112_1AEC)

Priority: May optionally be changed to a number <100 for injection priority.

Sample Type: Sample Categories of 'Sample', 'Allelic Ladder', 'Positive Control', or 'Negative Controls' 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., PP16_5 sec, PP16_3 sec, PP16_10 sec).

PowerPlex 16 specific run module and protocol settings for sample plates are as follows:

Module Type:	Regular
Template:	HIDFragmentAnalysis36_POP4
Inj. kV:	3
Run Time (seconds):	2000
Protocol Type:	Regular
DyeSet:	F

Inj.Secs: 3-10

3 secs - Allelic Ladders and 1ng POS control DNA (injection times may be adjusted [3-10 seconds per analyst's discretion] but a 3 sec. inj. time for single-source samples estimated at \geq 1ng generally produce good results).

5 secs. - Samples < 1ng generally produce good results.

- 4.2.2.3 Delete any unused wells. Perform a 'Save As' of the Plate Template Worksheet 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 record 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 For amplified products (including controls), typically 1µl-1.5µl rxn is added to 10µl of ILS Master Mix (made by adding 0.5µl ILS600 size standard/sample; 9.5µl deionized formamide/sample and adding quantities for N+2 in Master) 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 ILS600 size to 9.75µl deionized formamide if ILS peaks are too high.

4.2.4.2 Matrix samples are diluted 1:10 in Nanopure H₂O. 5µl of each matrix dye fragment is then added to 480µl of deionized formamide (without size standard). Load 25 µl of the fragment mix into each of four wells on the pre-labeled plate, which will include each of the four capillaries (e.g. wells A1 through D1).

4.2.5 Following sample addition, place a plate septa on the plate and heat denature for ~3 minutes at 95°C. Immediately chill in benchtop cooler (or on ice) for ≥3 minutes (perform on all sample types - ladders, matrix, controls and samples). **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 F is selected and the correct active calibration for dye set F 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 F is selected. Click on individual wells in the plate diagram to see results for each of the four capillaries. For each capillary, verify that four peaks are present in the spectral profile (upper pane), that the order of the peaks are, from left to right, blue-green-yellow-red, and that the peaks are regular in appearance. Next verify that four peaks are present in the raw data profile (lower pane), that the order of the peaks are, from left to right, red-yellow-green-blue, and that the peak heights are above 750RFU (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.

4.2.12 After completion of the run finish filling out the 3130 Injection Log (Form 422B-QC).

4.3 DATA ANALYSIS: GENEMAPPER® ID (GMID)

4.3.1 Data analysis is NOT performed on the instrument computers. Transfer the run folder (including the sample sheet for 310 runs and plate record for 3130 runs) to an analysis computer using a portable USB drive. After analysis and review are complete, a copy of the run folder and GMID project(s) will be stored on an analysis computer until CD/DVD archiving has been completed. 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® ID. Additionally, previously run 310 Macintosh data must first be converted to PC files using the 'Mac to Win' conversion program.

4.3.2 Set up the analysis methods for GMID 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. 310PP16-150RFU).

'Allele' Tab: Choose the appropriate bin set. Choose 'Use marker-specific stutter ratio if available', and ensure 'minus stutter distances' are from 3.25 to 4.75 for tetra and from 4.25 to 5.75 for penta. All others should be 0.

'Peak Detector' Tab: Advanced Peak Detection Algorithm, partial sizing (80-550 or 600), 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 80 to ≥ 500 bases.

Peak Amplitude Thresholds will depend on sample quality. Generally 150 rfu threshold in all colors. Rfu threshold may be raised in Blue, Green and Yellow for Allelic ladders. Rfu threshold may be lowered to 50 rfu at the analyst's discretion (see 4.4.2 RFU Threshold). Lowering of rfu threshold below 70 rfu (to ≥ 50) should be done with caution and only if the data generally appears to be good, and without excessive baseline background or artifacts. Peaks below 50 rfu are deemed inconclusive.

'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. The signal level and 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.

- 4.3.3 Create and store a size standard for GMID analysis, under the 'Size Standards' tab in 'GeneMapper Manager'. Name the size standard so that it reflects what the standard is (e.g. ILS600 80-600).

Data analysis will be performed using the 'Basic or Advanced' size standard. The size standard consists of the following peaks: 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 550, and 600 (the 60 and 600 peaks may be optionally defined by the analyst).

4.3.4 Create a matrix for GMID analysis of 310 data (3130 data do not require a matrix) under the 'Matrices' tab in 'GeneMapper Manager'. Matrix name is the date "MMDDYY" followed by "Matrix".

4.3.4.1 Review the Raw Data of the Fluor, JOE, TMR and CXR standards in the GMID project (see 4.3.5 and 4.3.6) and record an 'x' value that is after the primer peak, in an area of relatively flat baseline signal for each matrix standard. **Note:** the 'Analysis Range' must include ≥ 5 peaks for each matrix standard.

4.3.4.2 In the 'Matrix Editor' window, Click on a 'dye color' and select the corresponding Matrix Standard/.fsa file(e.g., Blue dye=FLUOR standard), and then enter the 'x' start value that you recorded from the Raw Data for that sample. Repeat for each of the Matrix Standards and click 'Create' to generate a new Matrix file.

4.3.4.3 Check to see that the numerical value trends indicate a good matrix (numbers on diagonal are '1.0000' and decrease from that value in each column.

4.3.4.4 Prior to clicking 'OK' to save the newly created matrix, it must be printed. This is done by pressing "shift" and "printscreen" simultaneously to take a snapshot of the matrix screen. Next, open Microsoft Word and paste the screen-shot into the blank document. The screen-shot may then be printed as a Word document. Once the matrix has been printed, click 'OK' on the matrix editor window.

- 4.3.4.5** Check Matrix quality by applying it to previously run samples, such as ladder, positive, and negative controls. Print each of the four, color plots and file in the QC log for the instrument.

The matrix may also be evaluated by applying it to the individual matrix samples in the GMID project. When applying it to itself, the Analysis Method chosen for analysis is as described in 4.3.2, except the peak detection algorithm must be Classic instead of Advanced. Examine the data generated. The samples should have peaks in the standard color but profiles should be relatively flat in the other 3 colors. With the exception of TMR (yellow) into CXR (red), bleed-through should not exceed 10%. If satisfied, print out a 4-color electropherogram plot, for each Matrix Standard. File in the QC log for the instrument.

- 4.3.5** Create a GeneMapper® ID Project:

4.3.5.1 From the GMID 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.

4.3.5.2 In the Samples table, for each sample, select the sample type, analysis method, panel, size standard, and matrix (310 data only) from the pull-down lists. 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'.

4.3.5.3 Save the project as the date MMDDYY, followed by Matrix, or case # (and any other descriptors that may be necessary). A separate project should be created for individual cases. To do this, highlight the samples not associated with the particular case and choose Edit/Delete from the project main menu. Optionally, samples may be added to the project individually, rather than the entire run folder. **Note:** the analyzed project will be exported to the run folder at the completion of analysis/review.

4.3.5.4 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.

4.3.6 Evaluate GeneMapper® ID Data:

4.3.6.1 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.

4.3.6.2 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. 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.

Note: Data may still be deemed acceptable without the ILS 60 and/or 600 bp peaks present. If additional peaks are assigned because of bleed-through of TMR peaks (typically Amelogenin peaks), the scientist may choose an Analysis Method, with an increased rfu threshold for the red channel to prevent these peaks from being detected, if desired.

4.3.6.3 Examine the blue, green, and yellow allelic ladders. Check that correct allelic assignments were made.

Note: GMID automatically averages all valid ladders in a run for genotyping. Genotypes are assigned by comparing the sizings of unknown alleles from samples with the sizings 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.

4.3.6.4 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).

4.3.6.5 GeneMapper® ID 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.

4.3.6.6 All negative controls (including reagent blanks) should be examined to verify that each displays a relatively flat baseline in blue, green and yellow.

4.3.6.7 Review all samples (including positive controls) for the above listed 'artifacts' and evaluate: peak height and shape, matrix 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.

- 4.3.6.8 Reanalyze individual samples with different Analysis Methods, as necessary if the rfu cut-off will need to be changed.
- 4.3.6.9 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.
- 4.3.6.10 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 nonsuspect case). Microvariants will be labeled and reported as "X.Y" (where X is the number of complete repeats and Y is the number of basepairs 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.
- 4.3.6.11 GMID 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 (see 4.4.2.4).
- 4.3.6.12 Export an allele/genotypes table to Excel and save it in the run folder. The

table will be printed for the case file. 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.

4.3.6.13 Print the 'Samples Plots' for case files. Only one of the allelic ladders need be printed for documentation purposes.

4.4 STR INTERPRETATION GUIDELINES AND STATISTICAL ANALYSES

4.4.1 CONTROLS

4.4.1.1 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®, ID peaks above threshold should only appear in the CXR (red dye) lane, corresponding to the ILS600 size standard. Electropherograms for the blue, green and yellow dyes should show a relatively flat baseline throughout the range (discounting primer signal, fluorescent 'spikes' or CXR 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 50 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 in terms of volume and amount amplified, injection time/amount, and

analysis threshold. Additionally, the reagent blank will be reamplified with samples from the set if any of the amplifications conditions are more sensitive than the original.

4.4.1.2 The purpose of the **POSITIVE AMPLIFICATION CONTROL** (9947A DNA supplied with the PP16 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 are below 150-rfu threshold) **AND** the other positive control (Extraction Control) appears as expected (i.e. the problem is confined to the 9947A sample).

LOCUS	GENOTYPE	LOCUS	GENOTYPE
D3S1358	14, 15	TH01	8, 9.3
D21S11	30, 30	D18S51	15, 19
Penta E	12, 13	D5S818	11, 11
D13S317	11, 11	D7S820	10, 11
D16S539	11, 12	CSF1PO	10, 12
Penta D	12, 12	AMELOGENIN	X, X
vWA	17, 18	D8S1179	13, 13
TPOX	8, 8	FGA	23, 24

4.4.1.3 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® ID electropherograms, peaks above threshold should only appear in the CXR

(red dye) lane, corresponding to the ILS600 size standard.

Electropherograms for the blue, green and yellow dyes should show a relatively flat baseline throughout the range (discounting primer signal, fluorescent 'spikes' or CXR 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). It is possible, since this control is processed last and its tube deliberately left open during the amplification set-up (to demonstrate maximum contamination potential), that it would be the only sample affected.

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.

4.4.1.4 The purpose of an **EXTRACTION CONTROL** sample is primarily to assess correct genotyping, however, it does take measure of all of the steps in the analytical process from extraction through allele designation. The extraction controls consist of ~3mm² cuttings of previously typed bloodstains prepared in batches. An extraction control must be run with every batch of forensic cases (will generally be extracted with reference samples or non-semen evidence). The reviewing scientist will complete an Extraction Control Check Form (Form 212-BI) for verifying correct genotype(s). A copy of this form will

be included in each associated case file. Failure of the extraction control, if isolated to that sample, will not deem other samples inconclusive.

4.4.2 RFU THRESHOLD:

4.4.2.1 For reference blood or oral standards (excluding autopsy or other samples that may be degraded or of limited quantity), a minimum of 100 rfu should be achieved for data acceptance. If necessary, go back in the process as follows: repeat injection (changing injection time; 3-10 seconds allowable range), or perform re-analysis (i.e., changing amount of amplified product added for fragment analysis), or re-amplification (increase DNA template), or re-extraction.

4.4.2.2 For minor mixture components (or low-copy single-source forensic samples), a threshold of 50 rfus may be used (see **4.3.2** Peak Detection). However, depending on signal/baseline may be deemed inconclusive.

4.4.2.3 Peaks below the analysis threshold (based on data obtained and signal/baseline) will not be interpreted but should be noted as being present in the case notes (eg. on the table of results).

4.4.2.4 Peaks marked as off-scale in GeneMapper® ID (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 (see **4.3.6.11**). If the overall quality of the data is not acceptable, the sample must be diluted, re-injected (3-10 seconds), reanalyzed (decrease the

amount of amplified product added) or re-amplified (decrease DNA template) as deemed appropriate by the scientist.

4.4.2.5 Multiplex amplification kits are designed so that heterozygous loci in single-source samples generally demonstrate relatively balanced peak heights [typically $\geq 70\%$ 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® ID.

4.4.3 EXTRA PEAKS (NON-MIXTURES)

4.4.3.1 PCR amplification of STR loci typically produces a minor product peak one core repeat unit shorter than the main allele peak ($n-4$ 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 $\sim 150-4500$ RFU). The measurement of percent stutter may be unnaturally high for main peaks that are off-scale or due to problems with matrix performance and can be corrected by diluting (or reamplifying less DNA) the sample and/or applying a new matrix. Loci stutter values are listed in Appendix A to assess potential contribution to peaks in stutter positions.

4.4.3.2 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® ID software so that it appears on the printed electropherograms.

4.4.3.3 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 GMID) if it falls within a diagnostic region and is of significant size to potentially interfere with analysis.

4.4.3.4 **Bleed-through** or pull-up peaks are a result of the matrix not correcting for all of the spectral overlap (most common with the PowerPlex 16 kit from yellow into red) 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 GMID) 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 red, and is not due to off-scale data, a new matrix may be used at the analyst's discretion to correct for the problem.

4.4.3.5 *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 45 minutes, followed by dilution prior to reinjection. It may be necessary to re-amplify the sample with less template DNA.

4.4.4 MIXTURES

4.4.4.1 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.

4.4.4.2 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.

4.4.4.3 Mixture assessment, in terms of determining the presence of a mixture

(# of potential contributors) and probable locus genotypes is performed prior to examining the reference profiles.

4.4.4.4 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.

4.4.4.5 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.

4.4.4.6 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 (see Appendix B for calculation examples).

4.4.4.7 For samples where distinct genotypes are discernable, 'single-source' statistics are calculated for the individual profile(s) in the event of a profile match. It is more common; however, to only report a distinct major profile, due to the possibility of shared and/or dropped-out alleles in a minor component. Caution should be

exercised when reporting a distinct minor profile for a sample.

4.4.4.8 Minor contributors in which a distinct minor genotype cannot clearly be determined will be reported as an inclusion/cannot be excluded (all minor alleles in the sample accounted for) or an exclusion (all or majority of the minor alleles in the sample not accounted for) and statistics will not be calculated for that minor contributor. It is possible that an individual may not be excluded as a possible contributor of the minor component, even if some of the reference alleles may not be present. This would occur with low level DNA and when there is an indication of possible allele drop-out.

4.4.4.9 Possible contributors to a mixture which distinct genotypes cannot be determined and/or mixtures of more than two individuals will be reported as inclusions (all reference sample alleles present in the mixture), cannot be excluded (majority of reference alleles present in the mixture but may be low level and have some indication of allele drop-out), or exclusions (majority or all of reference alleles not present in the mixture). Statistical interpretation will demonstrate the significance (or lack thereof) of the data.

4.4.4.10A sample with interpretable peaks at one or more loci may be reported even if no peaks are detected at additional loci (i.e. partial profiles); statistical interpretation will demonstrate the significance (or lack thereof) of the data.

4.4.5 STRs: STATISTICAL GUIDELINES

To present the significance of a match between STR profiles, the scientist uses the population distribution (frequency) of alleles at the various loci examined to assess how likely it is that this match might occur by chance. This general concept forms the basis of all calculations used in the reporting of forensic "matches".

4.4.5.1 The frequency of occurrence of a STR profile obtained from an evidentiary sample will be determined by examination of the frequency in the FBI's Caucasian, African American and Hispanic databases. Calculations will be performed using the Popstats and/or DNAView programs. Additional population data may also be used when available and relevant to a particular case (See **Biology QA Manual, section 11.2.6** for reporting of statistical frequencies).

4.4.5.2 The frequency for a heterozygous profile is determined by the equation $f_{(pq)} = 2pq$.

4.4.5.3 The frequency for a homozygous profile is determined by the equation $f_{(pp)} = p^2 + p(1-p)\theta$, where $\theta = 0.01$ except where small isolated populations (e.g., Native Americans) may be relevant, in which case, $\theta=0.03$.

4.4.5.4 For single-source evidentiary samples (or mixtures for which a distinct genotype(s) is discernible) the statistical consideration will be in the form of a **RANDOM MATCH PROBABILITY** (RMP; or inverse probability of inclusion). The RMP is the inverse of the calculated profile frequency (e.g., for $f_{(STR\ profile)} = 2 \times 10^{-14}$, $RMP = 1$ in 5×10^{13} ; See **Biology QA Manual, section 11.2.6** for reporting of statistical frequencies).

4.4.5.5 For mixtures for which distinct genotypes are not discernible, and one or more of the associated reference samples are included in the mixture, the scientist may elect to use either the **LIKELIHOOD RATIO (LR)**, **PROBABILITY OF EXCLUSION (CPE)**, or **PROBABILITY OF INCLUSION (CPI)**.

The **LR** compares the probability of the occurrence of the evidentiary profile under two hypotheses regarding the composition of the profile and is in the form:

$$LR = \frac{P(\text{evidentiary STR profile}|H_1)}{P(\text{evidentiary STR profile}|H_2)}$$

The larger the **LR**, the more likely H_1 was the true hypothesis (See **Biology QA Manual, section 11.2.6** for reporting of statistical frequencies). For a paternity calculation, this corresponds to the **PI** (Paternity Index).

The **PE** (P_E) represents the probability that a randomly selected individual would possess one or more alleles inconsistent with the crime scene stain (or paternity). It is the complement of the **RANDOM MAN NOT EXCLUDED** (or "inclusion probability"; P_I).

The **PE** and **PI** do not take into account the number of contributors, the principals' genotypes (i.e., the fact that they could account for the profile) or the evidence (e.g., peak height differences allowing probable donor assignment). They are calculated as follows:

$$P_E = 1 - P_I$$

Where $P_I = (p_1 + p_2 + p_3 \dots p_x)^2$ (the square of the sum of the frequencies of all alleles present in the evidentiary sample). The $P_{I\text{combined}}$ (**CPI**) may be

calculated by multiplying the P_i for each locus.

The $P_{E\text{combined}}$ (for all of the loci combined/CPE) is as follows:

$$P_{E\text{combined}} = 1 - P_{I\text{combined}}, \text{ or } 1 - [(1 - P_{E1})(1 - P_{E2})(1 - P_{E3}) \dots (1 - P_{E15})]$$

(See **Biology QA Manual, section 11.2.6** for reporting of statistical frequencies).

4.4.5.6 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 11.2.6** for reporting of statistical frequencies).

4.4.5.7 In many forensic cases, the denominator of the RMP obtained for an evidentiary item, from the analysis of several polymorphic STR loci, exceeds the population of the world several-fold. However, no reasonable individual would make the assertion that every individual in the world need be considered a potential DNA source in the context of a given case. '**SOURCE ATTRIBUTION**' (see Budowle, B. et al, Source Attribution of a Forensic DNA profile. *Forensic Science Communications*. 2(3) July 2000) is the result of a statistical approach to 'operationally' define uniqueness (assess whether a given multi-locus DNA profile could be considered unique for a given case).

The equation $p_x \leq 1 - (1 - \alpha)^{1/N} \approx \alpha/N$, is used to determine maximum RMP (p_x) that would support 'source attribution' for a relevant population sample size (N) and

selected confidence limit (i.e., $\alpha=0.01$; $1-\alpha=99\%$ confidence).

The FBI has selected an upper confidence limit (UCL) of 99% ($\alpha=0.01$) and an "N" equivalent to the U.S. population (2.6×10^8 pre-2000 census). This is reasonable as the FBI performs casework for jurisdictions all over the country and this calculation would provide a uniform approach to be used regardless of jurisdiction. For these figures, an RMP of $<3.9 \times 10^{-11}$ (or less than 1 in 2.6×10^{10}) would confer 99% confidence that the evidentiary profile is unique in the population. However, an additional 10-fold conservation factor, as recommended in NRC II, is added to this figure resulting in a frequency of less than 1 in 2.6×10^{11} for the reporting of source attribution.

In Idaho, using 2000 consensus figures, an operational population (N) of 1.6×10^7 has been selected (representing the sum of the populations of Idaho and the six surrounding states: ID= 1.3×10^6 ; MT= 0.9×10^6 ; NV= 2.0×10^6 ; OR= 3.4×10^6 ; UT= 2.2×10^6 ; WA= 5.9×10^6 and WY= 4.9×10^5). Therefore, an RMP of less than 1 in 1.6×10^{10} (including 10-fold conservation) will define source attribution (at 99% UCL) for analyses performed in this laboratory (See **Biology QA Manual, section 11.2.6** for reporting of statistical frequencies).

5.0 Comments:

- 5.1 The 310 POP4 Polymer and the 3130 POP4 Polymer are different and are not to be used interchangeably.
- 5.2 The 3130 Data Collection Software does not allow the entry of spaces or dashes in titles, sample names, etc. An underscore must be used in place of spaces when entering information.

DRIFTCON FFC: TEMPERATURE VERIFICATION**1.0 BACKGROUND:**

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 cyclers 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 cyclers 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.

Driftcon Operations Manual, version 1.1

Driftcon Quick Start Guide, version 1.4

2.0 SCOPE:

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 The analyst need not be present once the test has started; however, additional views/information are available during the run by pressing the F6 key for a surface view (heat dispersment), F7 for measurement values (shown in real time), and F8 for measurement locations (probe wells).

4.1.9 Once the run has completed, print the report and place it in the QC binder. Connect to the printer as follows:

- a. Right click 'My Computer'
- b. Choose 'Manage' > 'Device Manager'
- c. Select 'Network Adaptors'
- d. Double click 'Intel(R) PRO/Wireless LAN 2100 3A Mini PCI Adaptor'
- e. Open the 'Wireless Network' tab
- f. Click the 'Security' button and change the Data Encryption to 64 bit from the dropdown menu
- g. Ensure that the ssid is Linksys
- h. Click 'OK' and close all windows

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.

4.2.3 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 The analyst need not be present once the test has started; however, additional views/information are available during the run by pressing the F6 key for a surface view (heat dispersment), F7 for measurement values (shown in real time), and F8 for measurement locations (probe wells).
- 4.2.12 Once the run has completed, print the report and place it in the QC binder. Connect to the printer as follows:
- Right click 'My Computer'
 - Choose 'Manage' > 'Device Manager'
 - Select 'Network Adaptors'
 - Double click 'Intel(R) PRO/Wireless LAN 2100 3A Mini PCI Adaptor'
 - Open the 'Wireless Network' tab
 - Click the 'Security' button and change the Data Encryption to 64 bit from the dropdown menu
 - Ensure that the ssid is Linksys
 - Click 'OK' and close all windows

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 may be found beginning on page four, for each temperature. The measured value, along with the target specifications are shown in this section.

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