4 SALIVA ANALYSIS

The Department does not perform tests to indicate or identify the presence of saliva. However, its presence may be inferred if a reasonable person would infer its presence, e.g., on postage stamps, envelope flaps, chewing gum, cigarette butts, mouth openings in masks, mouth openings of bottles, etc.

4.1 Examine the item for stains. In addition to locating stains visually, an alternate light source (ALS) may be useful in locating stains believed to contain saliva. Describe the item and the appearance, size, and location of stains in notes, diagrams, and/or photographs, as appropriate. In some instances, in the absence of any visible stains, random swabbing of the item or collection for DNA extraction may be appropriate.

EXAMPLE: A thighs/external genitalia sample from the victim of alleged cunnilingus may be sampled for DNA extraction regardless of the results of other examinations/testing.

4.2 If appropriate, examine stain(s) for possible mixtures of physiological fluids (blood, urine, semen and/or feces), and record results.

4.3 As appropriate, cut an appropriate sized portion of the stain/stained area and place the cutting into a labeled microcentrifuge tube for DNA extraction. If a stain is smeared over a large area of fabric, more than one microcentrifuge tube may be needed to extract the stain.

4.3.1 If more than one microcentrifuge tube is used to extract a stain, the entire sample should be condensed into one sample tube during the manual purification step or after the robotic purification step.

5 ANALYSIS OF PERSPIRATION / SKIN CELLS

The Department does not perform tests to indicate or identify the presence of perspiration or skin cells. However, the presence of these may be inferred if a reasonable person would infer their presence, e.g., on hat bands, inside gloves, on cuffs and/or collars of shirts and jackets, etc. These samples may be referred to as touch or trace DNA sample or wearer DNA samples.

5.1 Examine the item for stains. In addition to locating stains visually, an alternate light source (ALS) may be useful in locating stains believed to contain perspiration or skin cells. Describe the item and the appearance, size, and location of stains in notes, diagrams, and/or photographs, as appropriate. In some instances, in the absence of any visible stains, random swabbing of the item or collection for DNA extraction may be appropriate.

EXAMPLE: The interior sweatband/rim of a baseball cap may be sampled for DNA extraction regardless of visual examination results.

5.2 If appropriate, examine stain(s) for possible mixtures of physiological fluids (blood, urine, semen and/or feces), record and report results.

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5.3 As appropriate, cut an appropriate sized portion of the stain/stained area and place the cutting into a labeled microcentrifuge tube for DNA extraction. If a stain is smeared over a large area of fabric, more than one microcentrifuge tube may be needed to extract the stain.

5.3.1 If more than one microcentrifuge tube is used to extract a stain, the entire sample should be condensed into one sample tube during the manual purification step or after the robotic purification step.

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6 URINE ANALYSIS

The Department does not perform tests to indicate or identify the presence of urine. However, the detection of a characteristic urine odor may aid in identifying a stain in which urine may be present and may therefore be taken forward for DNA analysis.

6.1 Liquid Samples

6.1.1 When a liquid sample believed to be urine is submitted, centrifuge the sample and/or allow cellular debris to settle to the bottom of the container.

6.1.2 Preserve a portion of the sediment on swabs.

6.1.3 Return liquid to an appropriate leak proof container.

6.1.4 As appropriate, cut an appropriate sized portion of the swab(s) and place the cutting into a labeled microcentrifuge tube for DNA extraction

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6.2 Dried Stains/Swabs

6.2.1 Examine the item for stains. In addition to locating stains visually, an alternate light source (ALS) may be useful in locating stains believed to contain urine. The detection of a characteristic urine odor may also be helpful. Describe the item and the appearance, size, and location of stains in notes, diagrams, and/or photographs, as appropriate. In some instances, in the absence of any visible stains, random swabbing of the item or collection for DNA extraction may be appropriate.

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6.2.2 If appropriate, examine stain(s) for possible mixtures of physiological fluids (blood, semen or feces), and record results.

6.2.3 As appropriate, cut an appropriate sized portion of the stain/stained area and place the cutting into a labeled microcentrifuge tube for DNA extraction. If a stain is smeared over a large area of fabric, more than one microcentrifuge tube may be needed to extract the stain.

6.2.3.1 If more than one microcentrifuge tube is used to extract a stain, the entire sample should be condensed into one sample tube during the manual purification step or after the robotic purification step.

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7 FECES ANALYSIS

The Department does not perform tests to indicate or identify the presence of feces. However, the detection of a characteristic fecal odor may aid in identifying a stain in which feces may be present and may therefore be taken forward for DNA analysis.

7.1 Whole Fecal Samples

7.1.1 Preserve a portion of the fecal sample, as appropriate.

7.1.2 Place an appropriate amount of fecal sample into a microcentrifuge tube for extraction.

7.1.2.1 If the fecal material is moist and will not be extracted immediately, it should be frozen or air dried. If frozen, it will need to thaw prior to extraction.

7.1.3 Alternatively, the fecal sample may be collected onto a swab and air dried. An appropriate portion of the swab can then be cut and placed into a microcentrifuge tube for DNA extraction.

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7.2 Dried Stains/Swabs

7.2.1 Examine the item for stains. In addition to locating stains visually, the detection of a characteristic fecal odor may be helpful. Describe the item and the appearance, size, and location of stains in notes, diagrams, and/or photographs, as appropriate.

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7.2.2 If appropriate, examine stain(s) for possible mixtures of physiological fluids (blood, semen or urine), and record results.

7.2.3 As appropriate, cut an appropriate sized portion of the stain/stained area and place the cutting into a labeled microcentrifuge tube for DNA extraction. If a stain is smeared over a large area of fabric, more than one microcentrifuge tube may be needed to extract the stain.

7.2.3.1 If more than one microcentrifuge tube is used to extract a stain, the entire sample should be condensed into one sample tube during the manual purification step or after the robotic purification step.

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8 TISSUE ANALYSIS

8.1 If appropriate, freeze the tissue until you are ready to proceed with the examination.

8.2 Describe the item appearance, size, etc., in notes, diagrams, and/or photographs, as appropriate.

8.3 If appropriate, test for blood, and record the results.

8.4 Cut a very thin slice of tissue (i.e., 1 mm thick slice of muscle) and place the sample into a labeled microcentrifuge tube for DNA extraction. If the tissue sample is degraded, a slightly larger portion of the sample may be used.

8.4.1 Alternatively, cellular material may be collected from the tissue by swabbing the sample and placing a portion of the swab into a labeled microcentrifuge tube for DNA extraction. The swab or portion of the swab placed into the microcentrifuge tube should be air dried or frozen if not proceeding directly to DNA extraction.

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9 BONE ANALYSIS

9.1 If appropriate, freeze the bone until you are ready to proceed with the examination.

9.2 Describe the item appearance, size, etc., in notes, diagrams, and/or photographs, as appropriate.

9.3 If appropriate, test for blood, and record results.

9.4 If appropriate, submit to the OCME for species, bone type, and/or sex determination.

9.5 Transfer to the Firearms Section for tool marks if required.

9.6 If appropriate, clean bone by soaking in water and washing with a strong jet of Type I water or an ultrasonic cleaner and air dry. If no analysis will be conducted on the sample, the case notes will indicate that sample has been preserved.

9.7 Using a Kimwipe and 95% ethanol vigorously clean the outer surface of the bone sample. Repeat this step 2-3 more times to remove any surface dirt or contaminants.

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9.8 Place the bone into a weigh boat or lay it on a piece of clean disposable bench paper inside of a biological safety hood to dry.

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9.9 The bone sample (i.e., small in size) may be pulverized using liquid nitrogen (9.9.1) or an electric drill and bit(s) (9.9.2) following the procedures listed below:

9.9.1 <u>Pulverizing the bone sample using liquid nitrogen</u>:

9.9.1.1 Place the bone fragment(s) into a clean mortar and pour a small volume of liquid nitrogen over the top. Ensure there is a sufficient volume of liquid nitrogen in the mortar to cover the bone fragment(s).

9.9.1.2 Allow the bone to sit in the liquid nitrogen for 30 to 60 seconds and then pour off the excess liquid nitrogen into a separate container. Using a pestle, crush the bone fragment(s). Place a barrier such as a piece of parafilm over the top of the mortar and pestle to prevent the pulverized bone fragments from flying out of the mortar.

9.9.1.3 Transfer a small portion of the pulverized bone to a 2.0 mL microcentrifuge tube for DNA extraction.

9.9.2 <u>Pulverizing the bone sample using a drill and bit(s):</u>

9.9.2.1 Clean a 1/8”, 9/64” or other appropriately sized drill bit with 10% bleach followed by 95% ethanol and allow it to dry.

9.9.2.2 Place the bone sample in a hood on a clean piece of disposable bench paper.

9.9.2.3 While holding the bone sample firmly, use an electric drill and the cleaned drill bit to drill a hole approximately 1.0 mm deep.

9.9.2.4 Tap the bone gently on the bench paper to dislodge the surface bone powder.

9.9.2.5 Replace the bench paper with a clean sheet, discarding the original sheet along with the surface bone powder.

9.9.2.6 Clean the drill bit again with 95% ethanol and a Kimwipe and allow it to dry.

9.9.2.7 Use the drill bit to drill approximately 3-5 mm further into the same hole in the bone.

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9.9.2.8 Tap the bone gently on the bench paper to dislodge the bone powder onto the paper.

9.9.2.9 Transfer a small sample of bone powder (approximately the size of a PEA or larger) to a 2.0 mL microcentrifuge tube for DNA extraction.

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10 TOOTH ANALYSIS

NOTE: Molars are the tooth of choice for DNA recovery.

10.1 If appropriate, freeze the tooth until you are ready to proceed with the examination.

10.2 Describe the tooth appearance, size, etc., in notes, diagrams, and/or photographs, as appropriate.

10.3 If appropriate, test for blood and record the results.

10.4 If appropriate, clean the outer surface of the tooth by soaking in water and washing with a strong jet of Type I water or an ultrasonic cleaner and air dry. If no analysis will be conducted on the sample, the case notes will indicate that sample has been preserved.

10.5 Clean the outer surface of the tooth with a 10% bleach, then sterile water and then 95% ethanol or isopropanol (avoiding introduction of the 10% bleach solution into any cracks in the tooth).

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10.6 Working in a hood, using a sterile diamond saw blade and a rotary shaft tool cleaned with 10% bleach and 95% ethanol or isopropanol, cut/grind away the upper crown portion of the tooth until the pulp chamber becomes visible. In addition, make small nicks in the sides of the tooth to facilitate crushing the lower portion of the tooth.

NOTE: It should take approximately 5-10 minutes to remove the crown of the tooth.

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10.7 To gain access to the pulp, place the tooth minus the crown into a small plastic zip closure bag. Insert the zip closure bag into a second zip closure bag. Ensure the bags are sealed.

10.8 Lay the zip closure bag on a hard surface. Using a hammer, pulverize the tooth sample inside the zip closure bag being careful not to puncture the zip closure bag.

10.9 Transfer the pulverized/crushed tooth sample to a microcentrifuge tube for DNA extraction.

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11 HAIR/FIBER ANALYSIS

Typically screening of hairs for nuclear DNA suitability and/or DNA analysis of hairs will not be conducted, regardless of the results of DNA analysis on any/all appropriate non-hair evidence. Screening of hairs and DNA analysis on hairs will typically be limited to specific cases in which the only evidence in the case is hair evidence or the probative value is certain (i.e., a clump of hair is recovered from a decedent's clutched hand). If, upon receipt of a Certificate of Analysis detailing that no results were obtained through other testing in a case, a specific request is made for hair examinations, a discussion between the examiner or supervisor and the investigator will occur, during which the probative nature and the information that may be gleaned from this hair examination will be considered. The determination of whether to proceed will be based on the numbers of hairs that will need to be screened and whether the information gained will reasonably be expected to provide probative information for the case when no other evidence has yielded a probative DNA profile.

If a case consists only of hairs submitted for DNA testing ("Hair Only Case"), it may, after discussion with a Trace Evidence supervisor, go to the Trace Evidence (TE) Section first, where the evidence will be examined for nuclear DNA suitable hairs and the appropriate hairs forwarded to the Forensic Biology Section (SX) examiner for DNA testing.

In the rare case that hair examinations are to be conducted, and there are a small number of hairs on an item of evidence, the SX examiner will recover the hairs from the item and subsequently screen them for suitability for nuclear DNA analysis.

In the rare case that hair examinations are to be conducted, and a large number of hairs are present on an item of evidence or a large case has many items requiring hair recovery and/or examination, the item(s) may be forwarded to the TE Section on a case by case basis for collection of the hairs and a determination of the suitability of the hairs for nuclear DNA analysis. These cases are discussed between the Forensic Biology examiner or supervisor and a Trace Evidence supervisor prior to submission. In some cases where large numbers of hairs are recovered, examination by DFS may not be possible or testing on a representative number of hairs will be attempted and the remaining hairs not examined. This case approach shall be documented in the case file.

For cases in which the evidence is examined and hairs are screened for nuclear suitability by a TE examiner, the Nuclear DNA Hair Referral form (Document 210-F206) should be used. This form can then be used by the SX examiner to supplement the notes.

Hairs previously mounted on microscope slides, typically re-submitted in cold cases, may be forwarded to the TE Section for de-mounting and assessment of the hair(s) for nuclear DNA analysis.

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11.1 Hair/Fiber Recovery and Preservation

11.1.1 When evidence is examined for biological substances, hairs/fibers may be collected from the item and/or left on the item for possible future recovery and examination.

11.1.2 With the aid of oblique lighting, if applicable, recover hairs/fibers and/or other trace evidence from items being examined for biological fluids using one or more of the following methods: forceps, post-it notes, gentle scraping and/or careful shaking over clean paper.

11.1.3 Package and appropriately label recovered hairs/fibers. When screening/examinations are complete, place the hair/fiber package in the item packaging.

11.1.4 If hairs/fibers are to remain on the item for possible future recovery, the trace evidence should be protected from loss or other deleterious effects by wrapping the item in the same clean examination paper on which it was examined and placing it in the original packaging (when possible).

11.1.5 If there is a possibility that loose hairs/fibers may be lost during the examination for biological substances (for example, while examining a broken windshield for blood, one or two hairs/fibers are noted on the glass), recover, package, and appropriately label these hairs/fibers. Place the package in the item packaging.

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11.1.6 Document findings (i.e., hairs/fibers recovered, hairs/fibers observed/not recovered, hairs/fibers recovered and more remain, etc.) along with an estimate of the numbers of hairs observed and/or recovered (small number or large number).

11.2 Procedure for Screening Hairs for Suitability for Nuclear DNA Analysis

NOTE: The SX examiner does not differentiate between hair fragments (shafts with no root) and fibers.

11.2.1 To determine suitability for nuclear DNA testing, hairs/fibers may be examined with the aid of a stereo microscope and/or by placing a cover slip over the hairs mounted in water or xylene substitute on a glass microscope slide, followed by examination with a compound microscope using bright field illumination. When using the stereo microscope, paper/post-it notes or other such material providing varying contrasts with the hairs/fibers being examined may be helpful. If there is any question about the suitability, DNA analysis should be attempted.

11.2.2 When hairs suitable for nuclear DNA analysis are found, the documentation should allow for tracking from the microscopic examination through the DNA interpretive stages. When no hairs suitable for nuclear DNA analysis are found, the documentation should reflect why the hair(s) are not suitable.

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11.2.3 While examining hairs/fibers for nuclear DNA suitability, the presence of any apparently extraneous body fluids should be noted.

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11.2.4 The documentation of the microscopic examination of hairs/fibers recovered should reflect the following information:

- Method or type of scope used, i.e., stereo and/or compound microscope/wet mount
- The approximate number of hairs/fibers recovered
- The presence or absence of a root and if the sample is suitable for nuclear DNA analysis
- The exact number of hairs from each item on which nuclear DNA analysis will be conducted
- The growth stage of the hair, if known
  - ?? may be used if growth stage is unknown - Forensic Biology examiners are only expected to be able to recognize that there is material (i.e., tissue) on a hair root that may be suitable for nuclear DNA testing and are not expected to know the exact growth stage of the hair with certainty.
  - Roots of hairs in the anagen or anagen/catagen growth stage may not appear to have tissue surrounding them, but rather appear like a crook with a natural end rather than a cut end, as a fragment or fiber would have. Although Forensic Biology examiners are not expected to be able to identify with certainty an anagen or anagen/catagen phase hair, if there is any question as to whether or not a hair examined is in the anagen or anagen/catagen phase rather than a fragment or fiber, DNA analysis should be attempted.

11.2.5 Additional specific observations such as approximate length, color, etc., of a hair may be documented.

11.3 Preparation of Hairs Deemed Suitable for Nuclear DNA Analysis

11.3.1 Wash the hair to reduce surface dirt and contaminants by immersing the hair in sterile Type I Water in a clean 50 mL beaker or other suitable container. If the hair contains a biological fluid that is important to the investigation DO NOT wash the hair.

11.3.2 Use a clean scalpel blade to cut a 0.5 to 1 cm portion from the root end of the hair and then place the hair root into a microcentrifuge tube for DNA extraction.

11.3.2.1 The remainder of the hair, if not consumed, should be packaged, labeled appropriately and returned to the item packaging.

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APPENDIX A – CHEMICAL TESTS FOR BLOOD

1 Combined Phenolphthalein-Tetramethylbenzidine (PTMB) Test

1.1 Safety Considerations

- Phenolphthalin - Caution! Avoid contact and inhalation!
- Potassium hydroxide - Caution! Corrosive! Poisonous!
- Tetramethylbenzidine - Caution! Harmful if swallowed, inhaled or absorbed through skin! Emits toxic fumes under fire conditions!
- Glacial acetic acid - Caution! Corrosive! Flammable!
- Ethanol - Caution! Flammable! Poisonous!
- Oxidized zinc - Caution! Danger of spontaneous combustion if allowed to dry!

1.2 Materials and Equipment

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- Dropper bottles
- Cotton swabs
- Test tubes, microtiter plates, or filter paper
- 100 ml graduated cylinder
- Weigh boats or weigh paper
- Balance
- Spatula
- Scissors
- Tweezers
- Magnetic stir plate

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1.3 Stock Solutions

1.3.1 Phenolphthalin Stock Solution

1.3.1.1 Mix the following thoroughly until dissolved:

- 1 g Phenolphthalin
- 25 g Potassium Hydroxide (KOH)
- 100 mL Type I water

1.3.1.2 Labeling

1.3.1.2.1 Label the bottle as Phenolphthalin Stock Solution with a lot number (date of preparation followed by the initials of the person preparing the stock solution) and "No Expiration" as there is no expiration date for this solution.

1.3.1.3 Storage

1.3.1.3.1 This colorless solution is stored under refrigeration over fresh granular zinc to keep it in the reduced form. The oxidized zinc in the bottle should not be allowed to dry.

1.3.1.4 Disposal

1.3.1.4.1 When the reduced Phenolphthalin Stock Solution is depleted, cover the zinc in the bottom of the bottle completely with a solution of potassium hydroxide in distilled or Type I water (25g KOH/100mL water).

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Label the bottle "For Disposal" and refrigerate. Notify the Safety Officer that the zinc is ready to be disposed of in accordance with Department procedures.

1.3.2 Tetramethylbenzidine (TMB) Stock Solution

1.3.2.1 Mix the following thoroughly until dissolved:

- 10 mg Tetramethylbenzidine (TMB)
- 30 mL Glacial Acetic Acid

1.3.2.2 Labeling

1.3.2.2.1 Label the bottle as TMB Stock Solution with a lot number (date of preparation followed by initials of person preparing the stock solution) and "No expiration", as there is no expiration date for this solution.

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1.3.2.3 Storage

1.3.2.3.1 The TMB Stock Solution should be stored at room temperature.

1.3.2.4 Disposal

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1.3.2.4.1 Dispose of the TMB Stock Solution and other materials contaminated with this solution as hazardous waste in accordance with Department procedures.

1.4 Working Solutions

- Type I water
- Ethanol
- 3% Hydrogen Peroxide
- 1:5 dilution of Phenolphthalin Stock Solution in Type I water (1 part Stock Solution plus 4 parts Type I water)
- TMB Stock Solution

1.4.1 Labeling

1.4.1.1 Each bottle containing a working solution will be labeled with the identity of the contents

1.4.1.2 Each bottle containing a working solution with a lot number will be also be labeled with that lot number (all but the Type I water).

1.4.1.2.1 The bottle containing the 1:5 dilution of phenolphthalin Stock Solution must be labeled appropriately. If the lot number of the diluted stock solution is recorded in the reagent log book, then the bottle must be labeled with this lot number. If only the neat stock solution is recorded in the reagent log book, then the bottle must be labeled with the lot number of the neat stock solution, the date of the dilution, and the initials of the person making the dilution.

1.4.1.3 Each bottle containing a working solution with an expiration date will also be labeled with that expiration date. If no expiration date applies, the bottles will be labeled to indicate this.

1.4.2 Storage

1.4.2.1 All working solutions may be stored at room temperature.

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1.5 Minimum Standards and Controls

1.5.1 On the day of use, a positive reagent control (known bloodstain) and a negative reagent control (Type I water or same substrate type as used for positive control with no stain) must be tested to ensure the reagents are working properly. The results of this testing must be documented in the case file.

1.5.2 If either control does not give the expected result, testing of evidence samples will not proceed until the problem has been resolved and a new set of positive and negative controls gives the expected result.

NOTE: Substrates such as leather or suede may cause the results to be more difficult to interpret.

1.6 Combined Phenolphthalein-Tetramethylbenzidine (PTMB) Test Procedure

NOTE: If it is possible that a stain will be consumed, do not perform the PTMB testing and proceed directly to collection of the stain for DNA analysis.

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1.6.1 Gently rub a suspected stain with a cotton swab which has been moistened with Type I water or place a small cutting of the stain in a small test tube or microtiter plate, or on filter paper and moisten with Type I water if desired.

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1.6.2 Add a drop of ethanol.

1.6.3 Add a drop of the working solution of phenolphthalein.

1.6.4 Add a drop of 3% hydrogen peroxide.

1.6.5 Note any color change. An immediate pink color is expected if blood is present.

1.6.6 Add a drop of tetramethylbenzidine stock solution.

1.6.7 Note any color change. An immediate blue-green color is expected if blood is present.

1.6.8 Interpretation

1.6.8.1 Positive reaction = immediate pink color at 1.6.5 followed by immediate blue-green color at 1.6.7

1.6.8.2 Negative reaction = no color change at 1.6.5 followed by no color change at 1.6.7

1.6.8.3 Inconclusive reaction = development of color combinations other than those specified for a positive reaction, including one test positive and the other test negative

2 Luminol Test

2.1 Safety Considerations

- Sodium perborate - Caution! Harmful if swallowed, inhaled or absorbed through skin!
- Aminophthalhydrazide (luminol) - Caution! Irritant! Emits toxic fumes under fire conditions!

2.2 Materials and Equipment

- Spray bottle (must contain no metal parts as the luminol will react with some metals)
- 50 mL graduated cylinder
- Balance
- Weigh boats or weigh paper

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- Spatula
- Zip-closure bags, conical tubes or other appropriate containers (optional)
- Magnetic stir plate

2.3 Stock Solutions

2.3.1 Solution A

2.3.1.1 Mix the following ingredients (proportionally) until thoroughly dissolved.

- 0.7 g sodium perborate
- 50.0 mL Type I water

2.3.1.2 Solution A should be stored in the dark at room temperature for up to 6 months.

2.3.2 Solution B

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2.3.2.1 Mix the following ingredients (proportionally) until thoroughly dissolved.

- 0.1 g Aminophthalhydrazide (luminol)
- 5.0 g sodium carbonate
- 50 mL Type I water

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2.3.2.2 Solution B should be stored in the dark at room temperature for up to 6 months.

NOTE: The dry chemicals in Solutions A and B may be weighed out and placed in appropriately labeled containers and stored in the dark at room temperature. Each container must be labeled with the date prepared, the initials of the preparer and the amount of Type I water to be added. The Type I water can be added when needed.

2.4 Minimum Standards and Controls

2.4.1 Immediately prior to use as outlined in 2.5.2, a positive reagent control (known dilute bloodstain) and a negative reagent control (Type I water or same substrate type as used for positive control with no stain) must be tested to ensure the reagents are working properly. The results of this testing must be documented in the case file.

2.4.2 If either control does not give the expected result, testing of evidence samples will not proceed until the problem has been resolved and a new set of positive and negative controls gives the expected result.

2.5 Luminol Procedure

2.5.1 Immediately prior to conducting the tests, mix equal parts of solutions A and B and place in a spray bottle.

2.5.2 Under darkened conditions (total darkness is best), spray the positive and negative controls and document results in the case file.

2.5.3 Spray any areas of interest on the evidence being tested.

2.5.3.1 Areas containing blood will luminesce immediately.

2.5.4 Mark areas of luminescence for subsequent testing with the PTMB test.

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2.5.5 Interpretation

2.5.5.1 Positive reaction = immediate luminescence

2.5.5.2 Negative reaction = no luminescence

2.5.5.3 Inconclusive reaction = Slow and/or weak luminescence

3 BLUESTAR® Forensic Test for blood

NOTE: BLUESTAR® Forensic Test Kit information is available at http://www.bluestar-forensic.com

3.1 Safety Considerations

- Beige Tablet - Ca Beige Tablet - Caution! Avoid contact with skin and eyes, gloves must be worn.
- White Tablet - Caution! Avoid contact with skin and eyes, gloves must be worn.

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3.2 Materials and Equipment

- Spray bottle (must contain no metal parts as chemicals may react with some metals)
- 100 mL graduated cylinder

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3.3 BLUESTAR ® Working Solution

3.3.1 Dissolve the white and beige tablets in 125 mL Type I water.

USE IMMEDIATELY! DO NOT STORE!

3.4 Minimum Standards and Controls

3.4.1 Immediately prior to use as outlined in 3.5.2, a positive reagent control (known dilute bloodstain) and a negative reagent control (Type I water or same substrate type as used for positive control with no stain) must be tested to ensure the reagents are working properly. The results of this testing must be documented in the case file.

3.4.2 If either control does not give the expected result, testing of evidence samples will not proceed until the problem has been resolved and a new set of positive and negative controls gives the expected result.

3.5 BLUESTAR® Forensic Test Kit for blood Procedure

3.5.1 Immediately prior to conducting the tests, dissolve the tablets in Type I water, as described in 3.3.1 in a spray bottle.

3.5.2 Under darkened conditions (total darkness is best), spray the positive and negative controls and document results in the case file.

3.5.3 Spray any areas of interest on the evidence being tested.

NOTE: Areas containing blood will luminesce immediately.

3.5.4 Mark areas of luminescence for subsequent testing with the PTMB test.

3.5.5 Interpretation

3.5.5.1 Positive reaction = immediate luminescence

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3.5.5.2 Negative reaction = no luminescence

3.5.5.3 Inconclusive reaction = Slow and/or weak luminescence

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APPENDIX B – SCREENING TESTS FOR SEMINAL FLUID / SEMEN

1 Acid Phosphatase Test

1.1 Safety Considerations

- Glacial acetic acid - Caution! Corrosive! Flammable!
- Sodium acetate - Caution! Irritant!
- Sodium α-naphthyl acid phosphate - Caution! Irritant! Emits toxic fumes under fire conditions!
- o-Dianisidine (Naphthanil diazo blue B) - Caution! Highly toxic! Emits toxic fumes under fire conditions!
- Naphthanil diazo red - Caution! Avoid contact and inhalation! Emits toxic fumes under fire conditions!

1.2 Materials and Equipment

- 5 ml and 500 ml Graduated cylinders
- Balance
- Spatula
- Scissors
- Tweezers
- Filter paper or microtiter plate (optional)
- Weigh boats or weigh paper
- Cotton swabs
- Test tubes or bottles
- Disposable transfer pipets or droppers

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1.3 Stock Solutions

1.3.1 Acid Phosphatase (AP) buffer

1.3.1.1 Mix the following thoroughly until dissolved:

- 2.5 ml Glacial acetic acid
- 10.0 g Sodium acetate (anhydrous)
- 450.0 ml Distilled water

1.3.1.2 Labeling

1.3.1.2.1 Label the bottle as AP buffer with a lot number (date of preparation followed by the initials of the person preparing the stock solution) and “No Expiration” as there is no expiration date for this solution.

1.3.1.3 Storage

1.3.1.3.1 AP buffer may be stored at room temperature.

1.4 Working Solutions

- Type I water
- Sodium α-Naphthyl Acid Phosphate Solution (see 1.4.1)
- Dye Solution (see 1.4.2)

1.4.1 Sodium α-Naphthyl Acid Phosphate Solution

1.4.1.1 Add a small amount (approximately 4 mg) of sodium α-naphthyl acid phosphate to

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approximately 3 mL of AP buffer (proportionally) in an appropriately labeled container and mix well.

1.4.1.1.1 Discard by the end of the day.

1.4.2 Dye Solution

1.4.2.1 Add a small amount (approximately 4 mg) of o-dianisidine or naphthanil diazo red to approximately 3 mL of AP buffer (proportionally) in an appropriately labeled container and mix well.

1.4.2.2 The solution may be protected from light by covering the container with foil or using a brown bottle.

1.4.2.2.1 Discard by the end of the day.

1.5 Minimum Standards and Controls

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1.5.1 On the day of use, a positive reagent control (known seminal fluid stain) and a negative reagent control (Type I water or same substrate type as used for positive control with no stain) must be tested to ensure the reagents are working properly. The results of this testing must be documented in the case file.

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1.5.2 If either control does not give the expected result, testing of evidence samples will not proceed until the problem has been resolved and a new set of positive and negative controls gives the expected result.

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1.6 Acid Phosphatase (AP) Test procedure

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NOTE: If it is possible that a stain will be consumed, do not perform the AP testing and proceed directly to collection of the stain for DNA analysis.

1.6.1 Moisten filter paper/swab with distilled water. (Do not use buffer solution, as this will contaminate the stained area.) Press the filter paper against the suspected stain or gently rub the stained area with the moistened swab. Alternatively, a small piece of the stain/swab can be placed on filter paper, in a small test tube, or in a microtiter plate.

1.6.1.1 If filter paper is used for AP mapping, the appropriate sized piece of filter paper should be moistened with Type I water, taking care not to soak the paper as this can dilute the stain and/or transfer the stain to another layer or portion of the item.

1.6.1.2 Apply the filter paper to the questionable stain or stained area by pressing the filter paper down onto the item carefully with moderate pressure long enough to transfer just a small amount of stain for testing (typically ~15 to 30 seconds).

1.6.1.3 Remember the orientation of the filter paper or mark a corner.

1.6.1.4 Apply the solutions in the order described below (see 1.6.2); however, in order to ensure each solution covers the entire piece of filter paper, disposable pipettes can be used to apply each solution over the paper while holding at an angle; place the paper down on bench paper or paper towel and read the reaction after 10-15 seconds as described below.

1.6.1.5 Mark the item or document in a diagram using the filter paper as reference where any positive areas were detected and note strength of reaction.

1.6.1.6 Be sure to change gloves between each application of filter paper to additional stains/stained areas for testing.

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1.6.1.7 If necessary air dry the evidence item before repackaging.

1.6.2 Add 1-2 drops of sodium α-naphthyl acid phosphate solution.

1.6.3 Add 1-2 drops of dye solution.

1.6.4 Note any color change. The development of a blue/purple color with o-dianisidine or an orange/red color with naphthanil diazo red within 10 to 15 seconds is indicative of acid phosphatase levels in seminal fluid/semen.

NOTE: Slow color development or development of a color not defined above may be attributed to increased acid phosphatase levels from non-seminal fluid sources (e.g., increased acid phospatase levels in vaginal fluid due to the victim's age, bacteria and/or white blood cells).

1.6.5 The presence of semen in all samples exhibiting an inconclusive result (not attributable to an increase in phosphatase levels from non-seminal fluid sources) or a positive result must be either confirmed by identifying spermatozoa or, in the absence of spermatozoa indicated with p30.

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1.6.6 Interpretation

1.6.6.1 Positive reaction = Blue/purple color with o-dianisidine within 10 to 15 seconds OR Orange/red color with naphthanil diazo red within 10 to 15 seconds

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1.6.6.2 Negative reaction = No color development, slight/slow color development

1.6.6.3 Inconclusive reaction = Slow moderate to strong color development

2 Extraction of Spermatozoa from a Substrate

2.1 Materials and Equipment and Reagents

- Rotator, vortex, sonicator, or centrifuge (depending on extraction method used)
- Scissors
- Tweezers
- Dissecting needle
- Microscope slides
- Test tubes
- Type I water

2.2 Extraction Methods

NOTE: Any of the following methods are acceptable. Once complete, a 3.0 µL spotting of the sediment created should be placed on a microscope slide (if the method does not already involve placement of the extract on a microscope slide). If the extraction is performed in a microcentrifuge tube, the tube can be centrifuged to collect the sediment at the bottom, the supernatant drawn off and discarded and the sediment at the bottom used for the spotting.

- If the slide will be stained for examination, it should be allowed to air dry.
- If the slide will be unstained for examination, it should be examined while wet.

2.2.1 Cut a small portion of a stain and soak in a test tube in Type I water.

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2.2.2 Soak a small portion of a stain in Type I water and rotate.

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OR

2.2.3 Soak a small portion of a stain in Type I water and sonicate on low for 10 seconds, followed by a second 30 second sonication on low.

OR

2.2.4 Tease fibers apart and soak in a small amount of Type I water.

OR

2.2.5 Soak a small portion of a stain in Type I water and vortex.

OR

2.2.6 Soak a small portion of a stain in Type I water on a microscope slide, stain side down (may be followed by maceration).

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OR

2.2.7 In some instances, a small portion of the stain can be cut for the p30 procedure and a sperm search can be conducted on a 3.0 µL spot from the p30 extract.

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OR

2.2.8 If the stain is small enough that consuming any portion of it for a sperm search may negatively impact the ability to obtain DNA analysis results, cut an appropriate amount of the stain (consuming it if necessary) for DNA extraction.

2.2.8.1 3.0 µL of the sperm fraction produced during the differential extraction procedure should be spotted on a microscope slide for a sperm search if no sperm search is conducted prior to extraction.

3 Staining Slides/Smears to Aid in the Visualization of Spermatozoa (Optional)

3.1 Kernechtrot-Picroindigocarmine Stain

3.1.1 Safety Considerations

- Aluminum sulfate - Caution! Harmful if inhaled, in contact with skin, and if swallowed! Emits toxic fumes under fire conditions!
- Nuclear fast red - Caution! Irritant! Emits toxic fumes under fire conditions!
- Saturated picric acid solution - Caution! Toxic! Explosive when dry! Emits toxic fumes under fire conditions!
- Indigocarmine dye - Caution! Harmful if swallowed! Emits toxic fumes under fire conditions

3.1.2 Materials and Equipment

- Filtration apparatus
- 500 ml glass beakers
- Balance
- Spatula
- Plastic bottles
- Glass rod
- Filter paper
- Weigh boats or weigh paper

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3.1.3 Reagents

- Aluminum sulfate
- Nuclear Fast Red
- Type I water
- Picroindigocarmine dye
- Saturated picric acid solution - **Purchase saturated solution. DO NOT PURCHASE DRY PRODUCT!**

3.1.4 Kernechtrot Solution (KS)

3.1.4.1 Dissolve 5 g aluminum sulfate in 100 mL of hot Type I water in a beaker.

3.1.4.2 Immediately add 0.1 g Nuclear Fast Red and stir with a glass rod.

3.1.4.3 Allow to cool and filter through filter paper.

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3.1.4.4 Labeling

3.1.4.4.1 Label the bottle as KS with the lot number (date of preparation followed by initials of preparer) and the expiration date.

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3.1.4.5 Storage

3.1.4.5.1 The KS can be stored at room temperature for up to 6 months, but may need to be re-filtered after standing.

3.1.5 Picroindigocarmine Solution (PICS)

3.1.5.1 Dissolve 1 g Indigocarmine dye in 300 mL of a commercially purchased saturated solution of picric acid.

3.1.5.2 Filter through filter paper.

3.1.5.3 Labeling

3.1.5.3.1 Label the bottle as PICS with the lot number (date of preparation followed by initials of preparer) and the expiration date.

3.1.5.4 Storage

3.1.5.4.1 The PICS can be stored at room temperature for up to 6 months, but may need to be re-filtered after standing.

3.2 SERI Christmas Tree Stain (R540) Kit

3.2.1 Contents

- Solution A (Kernechtrot Solution – KS) – 30 mL
- Solution B (Picroindigocarmine Solution – PICS) – 30 mL
- Directions for use

3.2.2 Store and discard according to manufacturer’s recommendation and expiration date.

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3.3 KPICS/Christmas Tree Staining procedure

3.3.1 If not already prepared and dried, prepare a thin smear of an extract of a suspected semen stain on a microscope slide and allow to air dry or use a smear provided in a Physical Evidence Recovery Kit (PERK).

3.3.2 Fix the sample to the slide/smear with a quick flame heating, with a spray fixative or by placing it on a heat block overnight.

3.3.3 Add a sufficient volume of KS (red stain) to cover the stained portion of the slide/smear.

3.3.4 Allow the KS stand on the slide/smear for at least 15 minutes.

3.3.5 Wash the KS off the slide/smear with a gentle stream of water.

3.3.6 Add a sufficient amount of PICS (green stain) to cover the stained portion of the slide/smear.

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3.3.7 Allow the PICS to stand on the slide/smear for ~5-15 seconds.

3.3.8 Wash the PICS off the slide/smear with methanol, 95% Ethanol or a gentle stream of water.

3.3.9 Dry the slide/smear at room temperature.

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4 Microscopic Examination of Stained Slides/Smears for Spermatozoa

4.1 Materials, Reagents and Equipment

- Type I water, xylene substitute or another appropriate mounting medium
- Coverslips
- Microscope (with approximately 200X-400X total magnification, with or without phase contrast capability)

4.2 Procedure

4.2.1 If necessary, follow the procedure for Köehler Illumination (section 7 of this appendix) on the microscope.

4.2.1.1 This should not often be necessary. The microscopes should maintain Köehler Illumination through several to many uses.

4.2.2 Scan the slide/smear at either ~200X or ~400X total magnification. Confirm at ~400X total magnification.

4.2.2.1 With phase microscopy: Spermatozoa heads are neon-like pink/red with darker pink/purple acrosomal caps and green tails. Epithelial cells and most bacteria stain green with some of the nuclei pink/red; however, these are shaped differently than spermatozoa. Yeast cells take on the same color as spermatozoa, but are shaped differently.

4.2.2.2 Without phase microscopy (bright field): Spermatozoa heads are neon-like pink/red with pale pink (almost colorless) acrosomal caps, blue-green necks/midpieces, and green tails. Epithelial cells appear bright blue with red to purple nuclei. Yeast cells take on the same color as spermatozoa, but are shaped differently.

4.2.3 Document the approximate number of spermatozoa/spermatozoa heads on the slide/smear per hpf (~400X), per lpf (~200X), per length of slide/smear (low or high power sweep), or per slide/smear as appropriate. Documentation of intact vs. heads only can be helpful in some cases.

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4.2.4 If only one spermatozoon or spermatozoon head is observed, a second qualified examiner ***must*** confirm its presence and document that confirmation in the notes.

5 Microscopic Examination of Unstained Slides/Smears for Spermatozoa

5.1 Materials, Reagents and Equipment

- Type I water
- Applicator sticks
- Coverslips
- Microscope (with approximately 200X-400X total magnification, with or without phase contrast capability)

5.2 Procedure

5.2.1 If necessary, follow the procedure for Köehler Illumination (section 7 of this appendix) on the microscope.

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5.2.1.1 This should not often be necessary. The microscopes should maintain Köehler Illumination through several to many uses.

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5.2.2 If not already prepared, place a small amount of an extract of a suspected semen stain on a microscope slide and cover with a coverslip, or add a drop of Type I water to a smear provided in a Physical Evidence Recovery Kit (PERK), agitate with an applicator stick and cover with a coverslip.

5.2.3 Scan the slide/smear at either ~200X or ~400X total magnification. Confirm at ~400X total magnification.

5.2.3.1 When the coverslip is touched gently, the spermatozoa/heads will roll, exhibiting their characteristic 3-dimensional shape. Use the distinctive size and morphology to identify the spermatozoa/heads.

5.2.4 Document the approximate number of spermatozoa and spermatozoa heads on the slide/smear per hpf (~400X), per lpf (~200X), per length of slide/smear (low or high power sweep), or per slide/smear as appropriate. Documentation of intact vs. heads only can be helpful in some cases.

5.2.5 If only one spermatozoon or spermatozoon head is observed, a second qualified examiner ***must*** confirm its presence and document that confirmation in the notes.

6 p30 ABACARD® Procedure

NOTE: The "High Dose Hook Effect" is a false negative result that is obtained in the presence of high concentrations of p30 (usually undiluted semen). This effect results from large amounts of human p30 binding to the antibody to form an antigen-antibody complex and free p30 migrating toward the test area "T". The antibody in the test area "T" is blocked by this free p30. Therefore, the mobile antigen-antibody complex cannot bind to the antibody. As a result no pink line will form in the test area "T". To confirm the presence of "High Dose Hook Effect", repeat the test using a 10-10,000 fold dilution of the sample.

6.1 Materials, Reagents and Equipment

- ABAcard® p30 Test cards (test devices) – one per sample tested, including positive and negative controls
- Dropper (included in each test device pouch)
- Buffer (included in kit)
- Microcentrifuge
- Microcentrifuge tubes with lids and/or lids
- Spin-Ease baskets

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- Timer
- Scissors
- Tweezers
- Microcentrifuge tube rack
- Pipettes (1000 µl and/or 200 µl)
- Pipette tips
- Dissecting needle
- Known semen or seminal fluid sample

6.2 Minimum Standards and Controls

6.2.1 On the day of use a positive reagent control (known seminal fluid/semen) and a negative reagent control (buffer) must be tested to ensure that the reagents and test device are working properly. The results of this testing must be documented in the case file.

6.2.2 If either control does not give the expected result, do not proceed with testing evidence samples until the problem has been resolved as demonstrated by testing another set of positive and negative reagent controls and achieving the expected results with both controls

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6.3 Procedure

6.3.1 Cut a portion of the dried stain into small piece(s) (size based upon the substrate and the intensity of the acid phosphatase test) and place into a labeled microcentrifuge tube.

6.3.2 Add 350 µl of the buffer provided within the kit and cap the tube.

6.3.3 Allow the sample to extract at room temperature for a minimum of 2 hours to overnight.

6.3.4 Briefly spin the tube to force liquid to the bottom.

6.3.5 Remove the cutting(s) and place them into a Spin-Ease basket.

6.3.6 Centrifuge for 5 minutes at ~10,000 rpm to recover the liquid and pellet the sediment.

6.3.7 Remove approximately 320 µl of the extract and place into a new labeled microcentrifuge tube (if storing for later use) or remove approximately 200 µl of the extract and add directly into the sample well "S" on a labeled test device.

6.3.7.1 If conducting a microscopic sperm search, spot 3 µl of the remaining liquid with sediment onto a microscope slide. Refer to 3.3.1 or 5.2.1 for further direction. Refer to 2.2.3.3-2.2.3.5 for direction regarding the disposition of the laboratory created slide.

6.3.7.2 The aliquot in the new microcentrifuge tube may be stored between 2-8°C or frozen if not used immediately. The sample must then be allowed to warm to room temperature prior to conducting the test.

6.3.7.2.1 Once warmed to room temperature, add approximately 200 µl of the aliquot into the sample well "S" on a labeled device.

6.3.8 Record the result for each test device at 10 minutes.

6.3.8.1 A positive result can be seen as early as 1 minute.

6.3.8.2 For negative results, one must wait for the full 10 minutes.

6.3.8.3 Do not record the results after 10 minutes.

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6.3.8.4 All control samples must give the expected results before the result on an unknown sample can be used.

6.4 Interpretation

6.4.1 Positive Result = 2 pink lines, one in the test area “T” and one in the control area “C”. Regardless of how faint a line may be in the T area, the result is positive if a line can be seen.

6.4.2 Negative Result = 1 pink line in the control area “C”.

6.4.2.1 Consider the High Dose Hook Effect described above and dilute the remaining sample and perform the test again, if indicated.

6.4.3 Invalid Result = No pink line in the control area “C” or incomplete line in the test “T” area. The test is inconclusive. Repeat if sufficient sample remains.

7 Procedure for Köehler Illumination

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7.1 Determine that the lamp is centered according to the instructions for the microscope in use.

7.2 Using a medium to low power objective (approximately 10X), place a specimen in position and focus.

7.3 Close the field diaphragm.

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7.4 Focus the image of the field diaphragm by adjusting the sub-stage condenser.

7.5 Center the field diaphragm using the centering screws on the condenser.

7.6 Open the field diaphragm so that the rim just disappears beyond the field of view.

7.7 Adjust the condenser diaphragm (aperture diaphragm) to about ½ of the full aperture.

NOTE: Resolution, contrast, and depth of field can be regulated with the condenser diaphragm. It should not be used to regulate the brightness. For this purpose, either the regulating transformer or neutral density filters should be used.

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