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Forensic Biochemistry and Hematology

Methods Manual

Version 2.1

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I. Introduction

The goal of the Department of Forensic Biology is to develop information through the identification and individualization of physiological fluids such as blood, semen, urine and saliva. Among other benefits, this information can aid in the investigation of a crime or suspected crime, help tie a victim or suspect to a crime scene, or eliminate a suspect.

This manual contains policies and methods available for use in the Forensic Biochemistry and Hematology Laboratory. The genetic markers analyzed were chosen, in part, to yield the most information from what is often a limited sample. This results in a choice of genetic markers that have a high discrimination potential, are stable, or both.

The best use of limited evidence requires that information about the case be available. Such information as whether suspects were injured or victims transfused can help guide the analysis of evidence. Often, this may require the submittal of additional evidence such as a blood standard from the suspect or an injured, living, victim.

Depending on the case, required analysis can range from determining the species of blood present on an item to genetic marker analysis of stained items for comparison with victims and/or suspects. The decision of what analyses are to be performed is made by a supervising Forensic Scientist after evaluation of the evidence through discussions with detectives or assistant district attorneys.

Analysis follows a logical progression. Items are first examined, most often looking for human blood and staining patterns. If human blood is present, and genetic marker analysis is required, the blood standard of the victim, and any other possibly bleeding person, is typed. It is generally preferable to type the standards first for a variety of reasons:

1. By discovering the exemplar types, the most informative system or systems (i.e. discrimination potential) can be used for the evidence.
2. If during the preliminary typing, problems arise with the typing of the standards, they can be worked out before the evidence is analyzed; for example, obtaining the victim's clothing to use as a source of victim blood.
3. Sometimes the exemplars, i.e., victim and suspect, have the same genetic marker types. By knowing this in

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advance, it allows the analyst to identify a genetic marker in which a difference exists, thus preventing the wasting of valuable physical evidence specimens. If more than one person was bleeding, the preliminary typing can determine where their blood types differ and the evidence can be analyzed accordingly.

Once the blood standards are typed, decisions can be made by the supervisor and analyst regarding the potential of typing the evidence items.

Occasionally, when investigative information is desired or when the particulars of a specific case require, the genetic typing is performed before all standards are submitted and typed. An example would be when typing data is required prior to a court order being obtained.

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II. Documentation, evidence examination, and packaging

A. Case files

There is one case file per incident, which usually means one case file per victim; a double-homicide is still one incident, so there would be a file with two victims. Each incident has a unique FB (Forensic Biology) number, and all evidence associated with the victim(s) will use the same FB number.

The following are clipped to the left-hand side of each file:

1. Chain of custody forms, documenting the flow of evidence received and released.
2. Copies of evidence vouchers, the police department documentation of evidence collected.
3. Copies of request for laboratory examination forms, the NYPD request for analysis.
4. Miscellaneous correspondence, such as memos to and from outside laboratories.
5. Case contact/control forms, documenting:
 - a. basic information on the victim (and suspect, if applicable)
 - b. discussions with detectives, attorneys, or others
 - c. what items are to be analyzed and in what manner
 - d. assignment date, review dates, etc.

The following are clipped to the right-hand side of each file:

1. Autopsy case worksheet.
2. Handwritten notes documenting the evidence examinations.
3. Worksheets documenting the analyses performed.
4. Case summary sheets.

The case summary sheet, which will be the top page of the analyst's notes, has three functions:

- a. To summarize the examinations, presumptive testing, confirmatory testing, and typing results
- b. To document the total number of examinations and/or

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tests performed for laboratory statistical purposes

- c. To record the quality control numbers of reagents used in the testing.

For every piece of evidence examined there must be an entry in the summary sheet, even if no tests were performed (for example, a shoe with no stains). Whether an actual analysis is performed it takes time to examine the evidence and each examination represents, for statistical purposes, a test.

For every test performed on an item of evidence, a result must be entered in the summary sheet. The number of tests must be summed and entered as well.

The quality control (QC) numbers associated with each test must be noted in the appropriate place.

The number of standards and controls also needs to be counted. This can be most easily done by counting the total number, not worrying about what the results were. The following should be counted in this group:

- a. Positive and negative controls for presumptive tests
 - b. Positive and negative controls for confirmatory tests
 - c. Unstained/substrate controls from evidence
 - d. Standards used in electrophoresis
5. Forensic Biology laboratory reports generated at the conclusion of the analyses, as well as any reports generated by outside laboratories.

All information regarding a case, including records of phone conversations, must be kept in the case file, in a neat and organized manner. If a case file becomes very large, divide it into separate file folders, labeled "1/2", "2/2", etc. Often, this occurs when DNA testing or crime scene reconstruction is done on a case; the separate file folders can hold the different types of analyses done.

B. Notetaking - general guidelines

Notetaking is one of the most important aspects of casework. The notes are used to document the condition of the packaging and evidence, describe any stains that may be found, present the results of analytical tests, support the conclusions of the report,

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and refresh the analyst's memory when required to testify in court.

1. Each page of notes, both handwritten and worksheets, must have on it the following information:

case number
date
analyst's initials
page number (at the bottom)

2. Place page numbers at the bottom of pages. Page numbering is easiest if the bottom page in a file, usually the "Blood Processing" worksheet, is page 1. As more notes are generated, they are placed on top of existing notes and numbered. The last page, the "Case Summary" worksheet, will have the highest number and be on the top.

The numbering should also reflect the total number of pages, by using the format "1/51", where 51 is the total number of pages.

When additional analyses are done after a report has been issued, continue the page numbering. Do not start over with page one.

3. Notes should be legible and organized. If a mistake is made, draw a single line through the error and initial and date the correction. NEVER obliterate, including using "white-out", any notes or entry in a worksheet.
4. Notetaking starts with a description of the evidence, beginning with the packaging. Note the following:
 - a. Type of package - paper bag, manila envelope, zip-loc bag, etc.
 - b. Condition of package - wet, bloody, etc.
 - c. Type of seal - stapled, taped, unsealed. It is the responsibility of the person accepting the evidence to ensure that the items are properly sealed (see the Quality Control Manual 2.0, section IV.F.1.a). If an item is not properly sealed, bring it to the attention of the person who signed it in.
 - d. Identifying marks - labels, tags, handwritten notations
5. Each package must be marked by the analyst with the case number, item number, date, and analyst's initials. Finding the marks in court is easier if the analyst

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always chooses the same location to put his or her marks.

6. Next is a description of the contents, the evidence itself. Specific suggestions concerning different types of evidence will be discussed later.
7. Standardized worksheets are available with diagrams of pants, shirts, shoes, etc., to aid in documenting staining patterns. If a diagram must be hand-drawn, make sure it is large enough to allow room to document all of the stains present. It is preferable to have only one diagram per page.
8. For further analyses, such as species determination and genetic marker analyses, make use of worksheets. Make sure all worksheets are filled out completely and legibly, and that the appropriate gels or photographs are attached. If there is any deviation from the written protocol, it must be noted.

If more than one case is analyzed on a worksheet, put the original worksheet in one file and photocopies in the others. On the photocopied pages, note where the original is to be found.

9. Each stain must be given a unique identifying number, clearly shown in the notes. The evidence is marked accordingly by affixing a tag with the information or by writing directly on the item. If tags are used, color-coding can be useful: red for blood, yellow for semen, and a third color to identify the unstained control area.
10. Items may be photographed. Each photograph must have a ruler visible in the frame, either a plain straight ruler or an x,y axis ruler.

C. Preparing for the examination

Before examining evidence, certain preparations should be made:

1. Review the "Scheduled Analysis" section of the case contact/control form; this section outlines the examinations requested by the supervisor.

Review all the information provided in the case file. This includes the case contact/control form, vouchers, requests for laboratory examination, any previous laboratory reports, and police reports. If further information or clarification is needed, obtain it before beginning any analyses.

2. Plan your approach to the case. Certain items may have

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greater potential information value than others, or may need to be analyzed first as an investigative aid.

3. Prepare the work area. The bench must be clean and free of clutter. The work area should be covered with paper to prevent the loss of small particles of evidence and to prevent the cross-transfer of materials from one item to another. Change the paper when a new case is begun, between different types of evidence within a case (such as between victim's and suspect's belongings), or when necessary.
4. Make sure the necessary tools and reagents for the examination are conveniently located, that there is adequate lighting available, and that notetaking materials are at hand to record your observations. Also make sure that all reagents used have passed QC and have not expired.
5. Prepare yourself with lab coat, gloves, and any other necessary safety items.

D. Evidence examination - general guidelines

The examination of objects will be described in a general sense, covering a broad range of topics applicable to most items of evidence.

1. Examine one item at a time, being sure the bench is cleaned between items. This may involve shaking the paper covering the work area, or changing the paper entirely.
2. Be certain that the previous item has been re-packaged before opening another item on the work surface.
3. Open the packaging, avoiding the breakage of seals when possible.
4. Remove packaging with care, remembering that materials of evidential value may be adhering to the item and/or the packaging. Opening the evidence over the paper will prevent the loss of these materials.
5. If an item of evidence is found to be wet when opened, the item should be allowed to air dry. The item should not be heated or exposed to direct sunlight. If the item has become foul smelling, allow it to dry in the hood with the fan running.
6. Each item must be marked with identifying case information, either by affixing a tag with the

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information or by writing directly on the item.

7. At this point, a visual inspection should be conducted. It may be necessary to use a high intensity light source, UV light source, or alternate light source, i.e., LumaLite™ during the inspection, especially if semen or saliva is suspected. Magnification may be necessary.
8. A tactile examination is sometimes helpful for locating some biological stains, notably seminal stains. Using gloved fingertips, lightly brush over the surface of the object, feeling for changes in surface texture or stiffness.
9. Remove any easily visible surface debris such as hairs, fibers, wood fragments, etc. and package. The location on the item of all trace evidence removed should be documented by diagram and/or photography.
10. All stains must be documented by diagrams and/or photography. Note the location of the stain, size, heaviness (soaked into fabric, surface smear, etc.), and any directionality of the stain pattern. Each photograph must have a ruler visible in the frame, either a plain straight ruler or an x,y axis ruler.

If stains do not exhibit directionality, note that as well.

11. A representative unstained control should be selected for analysis. Its location should be documented in the same way as stained areas. The unstained controls are used to ascertain the effects of substrates on the test procedure and should be collected as close to the stained area as possible.
12. Cut, scrape, or swab the stain and substrate control from the evidence item and place in a labeled envelope. Small items may be kept in their entirety. It is most time-effective if all stains and controls are collected at the time of evidence examination, rather than returning at a later time.

When swabbing an area, the number of swabs collected must be recorded and each swab given a unique identifying number. Refer to the unique number when analyzing the swab.

13. When finished examining an item of evidence, it must be packaged for return to the Evidence Unit. The original packaging must be sealed and placed in a plastic bag, security envelope, or envelope which is then sealed. The

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original voucher is attached and the items signed over to the Evidence Unit. This insures adherence to NYPD evidence packaging policy.

All evidence received into the laboratory or kept by the laboratory, i.e., retained samples, must be sealed properly; only tape is an acceptable seal, staples are not.

If evidence is brought into the laboratory unsealed, have the submitting person or officer seal the package(s) with evidence tape (in the evidence desk drawer). Additionally, the tape must be initialled by the person sealing the evidence.

For items that have been analyzed but are to be kept in the laboratory as retained samples, these items must be properly sealed before being put into storage. The above guidelines apply.

Each time a retained sample is removed for analysis, the retained specimen package must be opened according to Departmental guidelines, see FBH Methods Manual v 2.0 Evidence Guidelines, # 3.

NOTE: ALL CUTTING UTENSILS MUST BE CLEANED WITH ETHANOL BETWEEN SPECIMENS. ALSO, GLOVES MUST BE CHANGED BETWEEN SPECIMENS.

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E. Evidence examination - weapons

Weapons are frequently submitted for bloodstain or tissue examinations. Be aware that latent prints may be present on the weapon. That possibility should be discussed with the detective handling the case, and a decision made whether processing for prints should be done prior to examinations by the Forensic Biology laboratory.

Weapons can consist of knives, guns, bottles, baseball bats, and numerous other items. Be aware that blood and hairs can flake off from a non-porous surface quite easily.

Weapons should be thoroughly described and examined. Follow the general guidelines for notetaking and evidence examination when examining any weapon.

1. Describe the general condition of the item, such as presence of rust or fingerprint powder.
2. Measure the physical dimensions of the item. In the case of a knife, this should include description of knife blade such as thickness, shape, cross-sectional shape, length, width, number of blades, brand names, etc. Trace and/or photograph the knife.
3. If necessary, examine under a magnifier or stereomicroscope for traces of fibers, hairs, blood, or other materials of evidentiary value. All trace evidence removed should be documented in the notes using either diagrams and/or photography.
4. Look carefully for directional spatters of blood on weapons. Discuss any directional stains with a supervisor before performing any analyses.
5. Knives, sheaths, or other weapons may be dismantled as necessary for further examination. Always photograph or diagram the intact items before dismantling.
6. All stains must be documented by diagrams and/or photography. Note the location of the stain, size, heaviness (soaked into fabric, surface smear, etc.), and any directionality of the stain pattern. Each photograph must have a ruler visible in the frame, either a straight ruler or an x,y axis ruler.

If stains do not exhibit directionality, note that as well.

7. After examining a knife or other sharp object, package it in a safe manner for return to the Evidence Unit.

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F. Evidence examination - clothing

Clothing is often submitted to the Forensic Biology laboratory for examination. Follow the general guidelines for notetaking and evidence examination when examining any item of clothing.

1. Describe the color or pattern of the item of clothing, fabric type (denim, corduroy, etc.), fabric make-up (cotton, polyester, etc., from label, if present), and size (if marked on item). If an item is submitted inside-out, record this information.
2. Spread out the item of clothing, looking carefully at the front, back, and inside for any possible evidentiary material.
3. Describe the general cleanliness of the item of clothing. Diagram any defined soiled areas on the garment, for example, knees, buttocks, or cuffs. Note whether the garment appears freshly washed or not, for example, wet or damp.
4. Describe any damage to clothing which may have evidentiary value. For example, torn or missing buttons, torn or cut areas, damaged areas, or burned areas should be described.
5. Note the presence of any suspected stab holes or bullet holes. Diagram the location, orientation, size, and shape of any holes. Do not overlook the possibility that more than one hole may be caused by a single stab or shot due to the folding of the fabric. When sampling a stain from the area of a suspected stab hole or bullet hole, DO NOT cut through or otherwise disturb the hole. Take a sample away from the existing hole.
6. Carefully examine any pockets, inside and out. CAUTION IS ADVISED WHEN PLACING THE HAND IN A POCKET. An unexpected sharp object could cause serious injury.
7. Carefully examine the waistband, lining, cuff area, and collar area. This may require turning an item inside-out.
8. Examine shoes very carefully. Shoes are less often discarded than other items of clothing. They also have many nooks and crannies which could retain material of evidentiary value. Look carefully in the groove between the sole and upper shoe. Shoes with tongues should be checked for blood which may have fallen between the shoelaces.

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Shoes may be dismantled as necessary for further examination. Always photograph or diagram the intact items before dismantling.

9. All stains must be documented by diagrams and/or photography. Note the location of the stain, size, heaviness (soaked into fabric, surface smear, etc.), and any directionality of the stain pattern.

Each photograph must have a ruler visible in the frame, either a plain straight ruler or an x,y axis ruler.

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III. Bloodstain Preparation from Whole and Post-Mortem blood

1. Find the case files; the chain of custody forms should have been filled out by a member of the Evidence Unit, or lab personnel in their absence.
2. Prepare the UltraSTAIN™ cards by affixing a pre-printed FB case number sticker and writing in the following:

Deceased's or subject name
Date
Initials of person preparing the stain

3. Preparation of the bloodstain **MUST** be witnessed by another member of the laboratory staff. The witness must also initial the UltraSTAIN™ cards and the "Liquid Blood Processing" work sheet.
4. Prepare stains one at a time. Double-check that the FB number of the blood tube corresponds with the FB number on the card. Make sure a blood tube is closed before preparing the next stain.
5. Fold back the paper "flap" and make four stains on the card, placing the blood in the outlined areas. Use four drops of blood per area; apply the drops slowly, allowing them to soak in. This will prevent appreciable transfer to the paper "flap".
6. Bring down the paper "flap", turn the entire card over, and allow it to air-dry upside down.
7. Process the remainder of the blood as described in the appropriate methods section.

There is no need to witness these steps.

8. Package the air-dried stains into a 4x6" KAPAK™ bag; do not seal. Freeze the stains, cells, and sera.

Witnessing is a QC procedure used to ensure that the bloodstains are correct. This is a critical step which cannot be eliminated.

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IV. Screening Tests

A. Kastle-Meyer (KM) Presumptive Test For Blood

DO NOT MAKE YOUR OWN OR USE SUPPLIES THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

stock
solution: 10 g phenolphthalin
50 g KOH

Dissolve in 1 L deionized water.

working
solution: 200 mL stock solution
800 mL ethanol

Mix together; store in a dark tightly closed bottle over zinc dust to prevent oxidation.

OR

4 g phenolphthalein
40 g NaOH
20 g zinc dust
1 L water

Reflux this mixture until colorless. Cool and bring volume to 1200 mL with absolute ethanol. Store in a dark tightly closed bottle over zinc dust to prevent oxidation.

standards: blood and saline or water

Method:

1. A test may be performed directly on a cut out portion of a stain, an extract of a stain, or a "wipe" of the stained material. A wipe may be a piece of filter paper, thread, or swab; wet wipe with ethanol, then rub over the stained area while still wet.
2. Apply a drop of ethanol.
3. Apply a drop of KM reagent and observe any color change.

A PINK COLOR HERE IS DUE TO THE PRESENCE OF AN OXIDIZING AGENT (e.g., a chemical oxidant), NOT BLOOD

4. Add a drop of 3% hydrogen peroxide. An immediate pink color is a positive result.
5. Test the positive and negative controls at the same time.

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B. LEUCOMALACHITE GREEN PRESUMPTIVE TEST FOR BLOOD

ALL REAGENTS ARE AVAILABLE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE SUPPLIES THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

solution: 1 g leucomalachite green
 100 mL glacial acetic acid
 150 mL deionized water
 5 g zinc dust

Mix together. Simmer until the solution is almost colorless (pale yellow); this will take a few hours. Allow to cool, then filter. Add more zinc dust and store in a dark, loosely, capped bottle (to allow gases to escape) in the refrigerator.

standards: blood and saline or water

1. A test may be performed directly on a cut out portion of a stain, an extract of a stain, or a "wipe" of the stained material. A wipe may be a piece of filter paper, thread, or swab; wet wipe with ethanol, then rub over the stained area while still wet.
2. Apply a drop of ethanol.
3. Apply a drop of LMG reagent and observe any color change.

A BLUE-GREEN COLOR HERE IS DUE TO THE PRESENCE OF AN OXIDIZING AGENT (e.g., a chemical oxidant), NOT BLOOD

4. Add a drop of 3% hydrogen peroxide. An immediate blue-green color is a positive result.
5. Test the positive and negative controls at the same time.

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C. Acid Phosphatase Presumptive Test For Semen

ALL BUFFERS AND REAGENTS ARE AVAILABLE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE SUPPLIES THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Buffer: 8.21 g anhydrous sodium acetate

Dissolve in 1 L deionized water; adjust to pH 5.5 with acetic acid.

Reagents: 5 mg sodium alpha-naphthyl phosphate
5 mg Fast Blue B salt

In two separate test tubes, dissolve each in 5 mL buffer.

OR

1.58 g SERI spot test reagent

Dissolve in 5 mL deionized water.

The SERI spot test reagent contains alpha-naphthyl phosphate, Fast Blue B salt, and acetate buffer.

For either method, prepare the reagents fresh each time.

Standards: semen and water or saline

Two-step method:

1. A test may be performed directly on a portion of a stain, an extract, or a "wipe" of the stained material.
2. Apply a drop of the alpha-naphthyl phosphate reagent; wait 60 seconds.
3. Apply a drop of the Fast Blue B reagent. An immediate purple color is a positive reaction.
4. Test the positive and negative controls at the same time.

One-step method:

1. A test may be performed directly on a portion of a stain, an extract, or a "wipe" of the stained material.
2. Apply a drop of AP reagent and observe any color changes. A purple color developing within 60 seconds is a positive.

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3. Test the positive and negative controls at the same time.

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D. Presumptive Test for Saliva: Amylase Difusion

Gel buffer: 5.40 g anhydrous NaH_2PO_4
7.80 g anhydrous Na_2HPO_4
0.40 g NaCl

Dilute to 1 L with deionized water; adjust to pH 6.9.

Gel prep: 0.80 g Sigma Type I agarose
0.08 g starch (cornstarch)
80 mL gel buffer

OR

0.80 g SERI EA agarose (agarose plus starch)
80 mL gel buffer

Dissolve the agarose and starch in the buffer by heating in a flask. Pour onto a 20x20 cm glass plate and allow to solidify.

A 10x10 cm disposable petri dish can also be used; use 40 mL gel buffer and 1/2 the amount of starch and agarose.

I_2 solution: 0.05 M iodine solution (Commercially prepared)

To Prepare the solution in the laboratory
16.5 g KI
25.4 g I_2

Prepare a 0.05 M iodine solution by dissolving 16.5 g KI and 25.6 g I_2 in 1 L deionized water.

Sample prep: Extract an approximately 5 x 5 mm stain or a portion of a swab in 100 μL water for 30 minutes at room temperature using the pipet tip and test tube method.

Standards: α -amylase standard: 20, 2, 0.2, 0.02, and 0.002 units or a comparable ten-fold dilution series.

The standard can be purchased amylase or dilutions of saliva standardized against the purchased amylase.

1. Punch wells in the gel, leaving at least 1.5 cm between wells.
2. Fill wells ($\sim 8 \mu\text{L}$) with standards, blank, samples, and sample controls (unstained areas).

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3. Incubate for 3-8 hours at 37°C or overnight at room temperature; keep the plate in a humid chamber to avoid drying.
4. Pour a 1/100 dilution of the I₃ solution onto the gel; clear areas indicate regions of amylase activity.
5. Measure the diameter of the clear areas and record on the worksheet.

Entire items (blood spatter patterns, etc.) can be tested for amylase. Prepare a plate and allow to solidify; bring item (or area of item) into contact with the gel for 5 minutes. Follow steps 3-5 above to visualize any amylase pattern.

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E. Amylase₁ and Amylase₂ Differentiation by Differential Inhibition (Gel Diffusion Method)

Materials:

Kidney Bean Extract (KBE) - See Solutions Manual.

Wheat Lectin (WL) - Purchased commercially from Sigma (A-1520). Isolated from *Triticum aestivum*. Reconstitute 5 mg of solid with 1 mL deionized water.

Crude Pancreatic Extract - See Solutions Manual.

Crude Salivary Extract - See Solutions Manual.

Diffusion Gels - See Amylase Diffusion Method.

Lugol's (I₃) Iodine - See Amylase Diffusion Method.

1 mM CaCl₂

Preparation of Standardized Controls:

Determine the amylase activity of an equal amount of crude salivary extract and crude pancreatic extract using the amylase diffusion quality control test. Dilute extracts appropriately with 1 CaCl₂ to produce extracts with equal amylase activities.

Preparation of Standards:

Using standardized controls, prepare 3:1 v:v, 2:2 v:v, and 1:3 v:v mixtures of salivary and pancreatic extracts. Prepare dried stains (100 uL) on cotton swatches of the three mixtures and of the crude salivary and crude pancreatic extracts.

Assay:

Cut three 2x2 to 4x4 mm pieces of each of the samples to be tested (including the five standards) and incubate for 30 minutes at room temperature (20-25°C) in a minimal amount (approximately 50uL) of deionized water, KBE, and WL, respectively using the pipet tip and test tube method.

Place 8 uL of a deionized water blank and each of the samples into punched wells (3mm in diameter) in the diffusion gels. Allow the samples to diffuse in the gel for 16 hours at room temperature under moist conditions. Pour Lugol's Iodine over the gel after the incubation period to develop the plate, see page 13.

Immediately measure the amylase activity by determining the area ($A=\pi r^2$) of the clear circles around each well. Radius measurements should be taken under a stereoscope using a ruler with 0.5mm

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graduations if possible. The radius of each diffusion circle is calculated by averaging four readings measured at 90° from one another. Each reading is measured from the edge of the sample well to the outer border of the diffusion circle.

Calculate the percent inhibition of amylase activity for each sample or standard by using the equation:

$$1 - \frac{(\text{Area KBE or WL diluted sample})}{(\text{Area water diluted sample})} \times 100$$

Plot the inhibition ratio for each of the standards against the salivary:pancreatic ratio (4:0 v:v, 3:1 v:v, 2:2 v:v, 1:3, 0:4 v:v). Similarly, determine the inhibition ratio of the test samples and plot on the standard curve.

Interpretation of Results:

In general, samples giving an inhibition ratio which approximates the value for unmixed salivary extract can be considered Amy₁ in origin. Conversely, samples giving an inhibition ratio which approximates the value for unmixed pancreatic extract can be considered Amy₂ in origin. Values in between those for the unmixed extracts should be interpreted with caution. The origin should not be considered until after consultation with a supervisor.

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F. Presumptive Test for Urine: Urea Diffusion

Reagents

Bromothymol Blue (BTB) - Dissolve 1.5 g of BTB in 100 mL deionized water. Add one drop of phosphoric acid diluted 1:9 with deionized water. Adjust pH with 0.1 N sodium hydroxide if necessary.

Urease Solution - Dissolve enough urease in 100 mL deionized water to produce a solution containing 25-30 U/mL.

Agarose Solution - Prepare a 1% solution (w/v) by dissolving an appropriate amount of agarose (Sigma type I or equivalent) in boiling water. Cool to 56°C.

Blank Plate - Prepare a 1% (v/v) solution of BTB in agarose solution. Pour approximately 30 mL of this solution in 10x10 cm square petri dishes and allow to solidify.

Test Plates - to 1% (v/v) solution BTB in agarose solution add an appropriate amount of urease so that 30mL of test solution will contain approximately 10 units of urease activity. Aliquot 30mL fractions of test solution in 10x10 cm square petri dishes and allow to solidify.

	15 Blank Plates	15 Test Plates	20 Blank Plates	20 Test Plates
BTB	4.5mL	4.5mL	6.0mL	6.0mL
Urease	--	5.0mL	--	6.6mL
Water	445.5mL	445.5mL	594mL	594mL
Agarose (1 g/100 mL)	4.5 g	4.5 g	6 g	6 g

Sample

Preparation: For stains, extract an approximately 1x1 cm stain in 200 uL deionized water for 30 minutes at room temperature, using the pipet tip and test tube method.

Standards

Preparation: Prepare standard solutions containing 5, 0.5, 0.05, and 0.005 g Urea/100 mL deionized water.

Also prepare a 1x1 cm urine stain on either filter paper or cotton fabric. Extract the stain in 200 uL water and prepare a 1:1 dilution of the extract in deionized water.

Assay:

Punch wells in both blank plates and test plates (for 10x10 cm plates, 9 wells can easily be punched). For the deionized water blank, for the

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urea standards, both extracts of known urine stain (neat and 1:1 dilution), and for the sample extracts, place 9 uL aliquots in both blank plate and test plate wells. After a diffusion period of 20 minutes, measure the mean radius of the diffusion circle for each sample in the two corresponding plates. Record results on worksheet.

Standard Curve:

For each urea standard, plot the mean diffusion radius (determined by subtracting the mean diffusion radius of standard blank plate from mean diffusion radius of standard test plate) vs. log of urea concentration (g/10 mL). Measurement of radius should be taken in at least 3 points of the diffusion circle.

Plot the mean diffusion radius for neat and 1:1 diluted extracts of known urine stain curve.

Results:

Values obtained from the known urine stain must be positive and must have concentrations larger than the 0.005 urea standard. Concentrations from the unknown urine stain which are above the 0.005 urea standard are also considered positive. All samples whose concentrations fall below the that obtained from the 0.005 g urea standard are inconclusive. If results are in doubt consult a supervisor.

Photography:

All plates must be photographed and kept in case folders. Due to the blue and green background of the plates an orange filter is recommended when photographing plates. Recommended photographic parameters are listed below.

Test plates; *f* stop - 22
shutter speed - 1/8

(Use transmitted and oblique lighting)

Blank Plates; *f* stop - 22
shutter speed - 1/30 oblique
lighting only

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V. Confirmatory Tests

A. Takayama Hemoglobin Test

PERFORM THE TEST IN THE HOOD BECAUSE OF THE PRESENCE OF PYRIDINE.

Reagent: 5 mL 10% dextrose (glucose)
10 mL 10% NaOH
20 mL pyridine

Dilute to 100 mL with deionized water. Store in the refrigerator.

For crusts or scrapings:

1. Place a blood crust or flake on a glass slide.
2. Add a drop of reagent to the slide and cover with a cover slip.
3. Heat the slide **very gently** over a flame or on a hot plate for a few seconds.
4. Allow slide to cool slowly.
5. View under the microscope (about 400X), looking for characteristic salmon red/pink crystals.
6. Test positive and negative controls at the same time.

For clothing:

1. Extract stain with 1-3 drops water.
2. Place extract on slide and allow to evaporate. Scrape off the dried substance and gather in the center of the slide.
3. Add a drop of reagent to the slide and cover with a cover slip.
4. Heat the slide **very gently** over a flame or on a hot plate for a few seconds.
5. Allow slide to cool slowly.
6. View under the microscope (about 400X), looking for salmon red/pink crystals.
7. Test positive and negative controls at the same time.

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B. Ouchterlony Radial Diffusion

ALL BUFFERS, GELS, AND ANTISERA ARE AVAILABLE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE SUPPLIES THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Gel buffer: Cross-over electrophoresis buffer diluted 1:1 with deionized water

Mix 100 mL tank buffer with 100 mL deionized water.

Gels: Prepare tubes of 1% agarose gel in advance by dissolving 2 g of Sigma type I agarose in 200 mL gel buffer, then dispensing 7 mL aliquots into 20x150 mm test tubes. Allow to solidify, then cover with Parafilm and store in the refrigerator.

Sample prep: Prepare extracts using gel buffer, water, or saline, using the pipet tip and test tube method. Alternatively, a small, wet thread or small piece of swab can be inserted directly into the well.

Plate prep: Have a supply of 2.5 x 3.5 inch pieces of GelBond™.

Standards: Positive control, negative control, substrate controls

1. Obtain a premade test tube of gel; heat in a boiling water bath until it is liquified.
2. Pour onto the hydrophilic side of the GelBond and let solidify.
3. Punch an array of wells consisting of a central well surrounded by four wells; use the template on the worksheet.
4. Apply anti-sera to the central well.
5. Apply the positive control to one of the surrounding wells.
6. Apply the sample(s) so that a stain is always next to a positive control.
7. Apply negative and substrate controls to the remaining wells.
8. Place the plate in a moisture chamber and incubate at 37°C overnight.
9. A positive result is when the precipitin bands for the positive controls and the samples meet in a smooth curve. No spur formation should be seen.

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10. Rinse the plate in saline overnight, then do two 10 minute rinses in deionized water.
11. Press the gel between paper towels with a weight on top for 30 minutes, then dry in the oven for about 30 minutes.
12. Stain, see "Coomassie Blue Staining", Section VIII, D,1.
13. Dry either at room temperature or in the oven.

When a sample has been determined not to be human blood, it can be screened quickly against animal species.

Method:

1. Pour and prepare gels as described in steps 1-3 above.
2. Apply the sample to the central well.
3. Apply various animal anti-sera to the surrounding wells.
4. Incubate and process the gel as described in steps 3-8 above.
5. Any positive result needs to be confirmed by Ouchterlony or crossover electrophoresis using all appropriate controls and standards.

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C. Species Crossover Electrophoresis

ALL BUFFERS, GELS, AND ANTISERA ARE AVAILABLE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE SUPPLIES THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Tank buffer: 8.76 g sodium barbiturate
1.38 g diethyl barbituric acid (Barbital)
0.38 g calcium lactate

Dilute to 1 l with deionized water; adjust to pH 8.6

Gel buffer: Electrophoresis tank buffer diluted 1:1 with deionized water

Mix 100 mL tank buffer with 100 mL deionized water.

Gels: Prepare tubes of 1% agarose gel in advance by dissolving 2 g of Sigma type I agarose in 200 mL gel buffer, then dispensing 7 mL aliquots into 20x150 mm test tubes. Allow to solidify, then cover with Parafilm™ and store in the refrigerator.

Sample prep: Prepare extracts using gel buffer, water, or saline. Alternatively, a small, wet thread or small piece of swab can be inserted directly into the well.

Plate prep: Have a supply of 2.5x3.5 inch pieces of Agarose GelBond™.

Standards: Positive control, negative control, substrate controls

Method:

1. Obtain a premade test tube of gel; heat in a boiling water bath until it is liquified.
2. Pour onto the hydrophilic side of the Gelbond and let solidify.
3. Punch small wells (about 1-2 mm) in rows using the template on the worksheet. Punch enough wells for controls, samples, and substrate controls.
4. Apply anti-sera in the left-hand wells using capillary tubes.
5. Apply samples in the right-hand wells.
6. Place plate in electrophoresis tank with the antibodies toward the cathode (black).

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7. Electrophoresis is at 120V for 20 minutes.
8. A sharp, white, precipitin band (not a cloud) is a positive.
9. Rinse the plate in saline overnight, then do two 10 minute rinses in water.
10. Press the gel between paper towels with a weight on top for 30 minutes, then dry in the oven for about 30 minutes.
11. Stain (see "Coomassie Blue Staining", Section VIII D, 1).
12. Dry after staining either at room temperature or in the oven.

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D. Christmas Tree Stain For Spermatazoa

The nuclear material within the cell is stained red by the nuclear fast red stain. Sperm heads are usually well differentiated with the acrosome staining significantly less dense than the distal region of the head. Epithelial membranes and sperm tails are stained green by the PIC stain; nuclei inside epithelial cells appear purple. Yeast cells also stain red, however the stain is uniform throughout the cell and extends into polyp-like structures which are occasionally seen in yeast.

Reagents: Nuclear Fast Red (purchased commercially)
The reagent is to be kept refrigerated (0-8° C).

The reagent can be prepared in the laboratory as follows:

2.5 g aluminum sulfate
50 mg nuclear fast red (C.I. 60760)

Dissolve the aluminum sulfate in 100 mL of warm deionized water and add the nuclear fast red. Stir and allow to cool; filter. The solution is stable for many months in the refrigerator.

Picro Indigo Carmine (PIC) (purchased commercially)
The reagent is to be kept refrigerated (0-8° C).

The reagent can be made in the laboratory as follows:

1.30 g picric acid
0.33 g indigo carmine (C.I. 73015)

Dissolve the picric acid in 100 mL of warm deionized water; add the indigo carmine and stir overnight. The solution is stable for many months in the refrigerator.

Method:

1. Fix cells to the slide by heating
2. Cover cell debris with a few drops of nuclear fast red and allow to sit for at least 10 minutes (if longer, a humid chamber may be necessary)
3. Wash away the nuclear fast red with deionized water
4. Add one drop of the PIC stain to the still-wet slide; allow to sit for no more than 30 seconds
5. Wash away the PIC stain with ethanol

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6. Let slide dry; examine the slide at 100X or 400X (don't use immersion oil)

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E. P30 Crossover Electrophoresis

ALL GELS, BUFFER, AND ANTISERA ARE AVAILABLE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE SUPPLIES THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Tank buffer: 25.2 g Tris base
2.5 g EDTA, free acid
1.9 g boric acid

Dilute to 1 L with deionized water; adjust to pH 9.1

Gel buffer: same as tank buffer

Gels: Prepare tubes of 1% agarose gel in advance by dissolving 2 g of Sigma type III agarose in 200 mL tank buffer, then dispensing 7 mL aliquots into 20x150 mm test tubes. Allow to solidify, then cover with Parafilm™ and store in the refrigerator.

Sample Prep: Prepare extracts using tank buffer, water, or saline using the pipet tip and test tube method. If you don't want to make extracts, a small, wet thread or small piece of swab can be inserted directly into the well.

Plate prep: Have a supply of 2.5x3.5 inch pieces of Agarose Gelbond™.

Standards: human semen stain, saline, and substrate controls

Method:

1. Obtain a premade test tube of gel; heat in a boiling water bath until it is liquified.
2. Pour onto the hydrophilic side of the Gelbond and let solidify.
3. Punch small wells (about 1-2 mm) in rows using the template on the worksheet. Punch enough wells for controls, samples, and sample controls.
4. Apply anti-sera (approximately 5 uL) in the left-hand wells using capillary tubes or pipettes; do not fill wells to over flowing.
5. Apply samples (approximately 5 uL) in the right-hand wells using capillary tubes or pipettes; do not fill wells to over flowing.
6. Place plate in electrophoresis tank with the antisera toward

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the cathode (black).

7. Electrophoresis is at 120V for approximately 30 minutes.
8. A sharp, white, precipitin band (not a cloud) is a positive.
9. Rinse the plate in saline overnight, then do two 10 minute rinses in water.
10. Press the gel between paper towels or thick blotting paper with a weight on top for 30 minutes, then dry in the oven for about 30 minutes.
11. Stain, see "Coomassie Blue Staining", Section VIII,D,1).
12. Dry after staining either at room temperature or in the oven.

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F. P30 ELISA

ALL BUFFERS, STOCK SOLUTIONS, AND NEAT ANTISERA ARE AVAILABLE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE SUPPLIES THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Prostate specific antigen (PSA) is another name for P30 antigen.

Phosphate buffered saline (PBS)

To prepare 200 mL, dissolve 1 tablet in 200 mL of deionized water. This can be stored at 4°C for up to 2 weeks (put expiration date on label).

To prepare 1 L, dissolve 5 tablets in 1 L of deionized water. This can be stored at 4°C for up to 2 weeks (put expiration date on label).

Casein stock Solution

Thoroughly dissolve 10 g Hammerstein casein in 500 mL deionized water; adjust to pH 8.0 with 3M NaOH. Add 500 mL PBS and 0.1 g sodium azide. Freeze in 40 mL aliquots.

PBS-Casein:(PBS with 0.02% w/v Hammerstein casein)

Dilute 20 mL of casein stock to 1 L with PBS.

One plate uses approximately 800 mL.

0.1% BSA Dissolve 100 mg BSA into 100 mL PBS.

PBS- BSA:(bovine serum albumin)

Prepare a 0.1% PBS-BSA solution by mixing 100 uL BSA (0.1% BSA) with 100 mL PBS.

Alkaline substrate buffer (1M Ethanolamine)

Add 97 mL diethanolamine, 0.2 g sodium azide, and 0.1 g MgCl₂ to 800 mL of deionized water; adjust to pH 9.8 with concentrated HCl. Make up to 1 L with deionized water.

Standards P30 antigen (1 ug/mL)

When P30 antigen arrives, divide into 100 uL aliquots and freeze.

Prepare 1 ug/mL solution of P30 antigen by diluting 100 uL antigen with 100 mL PBS-BSA. Divide into 500 uL aliquots and freeze at -40°C.

Plates Immulon II microELISA plates

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Antibodies **Mouse monoclonal anti-human PSA (prostate specific antigen, P30)**

When needed, reconstitute with 100 uL of deionized water. Store at 4°C.

Rabbit polyclonal anti-human PSA

Store at 4°C.

Swine anti-rabbit IgG alkaline phosphatase conjugate

Store at 4°C.

Mouse IgG1, Kappa chain (MOPC 21, mouse myeloma protein)

When needed, reconstitute with 100 uL of deionized water. Store at 4°C.

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PLATE PREPARATION:

COATING THE PLATES

1. Prepare a 1/8000 dilution of mouse monoclonal anti-human PSA by adding 10 uL antiserum to 80 mL PBS. **Always make dilutions in glass, not in plastic.** This is enough for 16 plates.
2. Prepare a 1/8000 dilution of MOPC by adding 10 uL MOPC to 80 mL PBS. **Always make dilutions in glass, not in plastic.** This is enough for 16 plates.
3. Coat the plate as shown in the diagram. Use 100 uL of the appropriate solution per well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	M	M	M	M	M	M	M	M	M	M	M
B	PBS	M	M	M	M	M	M	M	M	M	M	M
C	PBS	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA
D	PBS	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA
E	PBS	M	M	M	M	M	M	M	M	M	M	M
F	PBS	M	M	M	M	M	M	M	M	M	M	M
G	PBS	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA
H	PBS	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA	PSA

PBS = phosphate buffered saline
PSA = mouse monoclonal anti-human PSA
M = MOPC

4. Cover plates with Parafilm and incubate overnight at 4°C.

BLOCKING THE PLATES:

1. Aspirate contents of wells. Wash each plate twice with wash buffer (PBS-casein), then fill the wells. Let the filled plate sit at room temperature for 15-20 minutes.
2. Aspirate the final wash and blot dry. Plates which are not used immediately should be wrapped in plastic and stored at 4°C; they can be stored like this for up to 2 weeks.

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SAMPLE PREPARATION:

Prepare samples and standards on the day of use.

1. **Stains/swabs** Using the pipet tip/test tube method, extract 2.5 x 2.5 mm samples in 100 uL of PBS for 30 minutes at room temperature. Centrifuge, then prepare a 1/25 dilution by adding 20 uL sample extract to 500 uL wash buffer (PBS-casein).

Freeze the remainder of the sample extract until the ELISA is complete and for amylase analysis.

2. **Standards**
 1. 10 ng/mL: 50 uL P30 (1 ug/mL) + 5 mL PBS-casein.
 2. 6 ng/mL: 3 mL 10 ng/mL P30 standard + 2 mL PBS-casein.
 3. 2 ng/mL: 1.67 mL 6 ng P30 standard + 3.33 mL PBS-casein or 0.5 uL 10 ng/uL P30 standard + 4.5 ml PBS-casein.

SAMPLE AND STANDARD APPLICATION:

1. Apply PBS, PBS-casein, standards and samples as shown in the diagram. Use 100 uL of the appropriate solution per well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	W	2ng	10ng	S2	S4	S6	S8	S10	S12	S14	S16
B	PBS	W	2ng	10ng	S2	S4	S6	S8	S10	S12	S14	S16
C	PBS	W	2ng	10ng	S2	S4	S6	S8	S10	S12	S14	S16
D	PBS	W	2ng	10ng	S2	S4	S6	S8	S10	S12	S14	S16
E	PBS	W	6ng	S1	S3	S5	S7	S9	S11	S13	S15	S17
F	PBS	W	6ng	S1	S3	S5	S7	S9	S11	S13	S15	S17
G	PBS	W	6ng	S1	S3	S5	S7	S9	S11	S13	S15	S17
H	PBS	W	6ng	S1	S3	S5	S7	S9	S11	S13	S15	S17

PBS = phosphate buffered saline

W = PBS-casein

S = samples

2. Cover the plates with Parafilm and incubate at room temperature for 1 hour.
3. Aspirate contents of wells. Wash the plate three times with PBS-casein. Make sure that there is no excess buffer

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remaining in the wells.

4. For each plate, prepare a 1/1000 dilution of rabbit polyclonal anti-PSA by adding 10 uL antiserum to 10 mL PBS.
5. Apply 100 uL of the 1/1000 dilution of rabbit anti-human polyclonal anti-PSA to each well of columns 2-12. Apply 100 uL of PBS to each well of column 1.
6. Cover the plates with Parafilm and incubate the plates at room temperature for 1 hour.
7. Aspirate contents of wells. Wash the plate three times with PBS-casein. Make sure that there is no excess buffer remaining in the wells.
8. For each plate, prepare a 1/1000 dilution of swine anti-rabbit IgG alkaline phosphatase conjugate by adding 10 uL antiserum to 10 mL of PBS.
9. Apply 100 uL of the 1/1000 dilution of swine anti-rabbit alkaline phosphatase conjugate to each well of columns 2-12. Apply 100 uL of PBS to each well of column 1.
10. Cover the plates with Parafilm and incubate at room temperature for 1 hour.
11. During the incubation, allow alkaline substrate buffer (ASB) to come to room temperature, about 30 minutes. For each two plates, dissolve one 20 mg p-nitrophenyl phosphate tablet (PNPP) in 20 mL of ASB.
12. Aspirate contents of wells. Wash the plate three times with PBS-casein. Make sure that there is no excess buffer remaining in the wells.
13. Apply 100 uL of PNNP substrate solution to each well of columns 1-12.
14. Cover the plates with Parafilm and incubate for 1 hour at 37°C. Uncover and read at 405nm.

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CALCULATION OF P30 ELISA Results

The calculations are done automatically by transferring the data from the microtiter plate reader to an Excel™ spreadsheet set up to perform them. If necessary, the calculations can be done manually as follows:

1. Calculate the plate threshold value (PT) of the plate:

Determine the average (AVE) and standard deviation (standard deviation = $s = s^{1/2}$) (SD) of the values in column 2.

$$PT \text{ value} = 2(AVE) + 3(SD)$$

2. Subtract the PT value from the values in columns 3-12.
3. For all standards and samples, calculate the average of the duplicate samples for both the MOPC and monoclonal PSA coated wells.
4. Subtract the MOPC averages from their corresponding monoclonal PSA averages. The remaining value is the P30 ELISA result.

EXAMPLE:

The 2 ng standard gave a final ELISA value of 0.055.

Sample 1 is in wells A3-D3; sample 2 is in wells E3-H3

<u>Value</u>	<u>Value</u>	
A2 0.114	A3 0.081	} MOPC
B2 0.091	B3 0.063	} MOPC
C2 0.123	C3 0.356	} monoclonal PSA
D2 0.063	D3 0.325	} monoclonal PSA
E2 0.081	E3 0.266	} MOPC
F2 0.085	F3 0.272	} MOPC
G2 0.085	G3 0.711	} monoclonal PSA
H2 0.070	H3 0.847	} monoclonal PSA

average of column 2 values: 0.089

standard deviation of column 2 values: 0.020

$$\text{plate threshold} = 2(0.089) + 3(0.020) = 0.238$$

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Subtract the PT value from each value in column 3; if less than zero, enter zero.

A3	0.000	
B3	0.000	average of A3 and B3 = 0.000
C3	0.118	
D3	0.087	average of C3 and D3 = 0.103
E3	0.011	
F3	0.009	average of E3 and F3 = 0.010
G3	0.473	
H3	0.609	average of G3 and H3 = 0.506

P30 ELISA results:

sample 1 = (AVE C3/D3) - (AVE A3/B3)
= (0.103) - (0.000)
= 0.103

sample 2 = (AVE G3/H3) - (AVE E3/F3)
= (0.506) - (0.010)
= 0.496

Any sample with a P30 ELISA result greater than the value obtained for the 2 ng standard is positive for the presence of semen.

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VI. Red Cell Antigen Systems

A. Blood Processing Policy

See Section "Bloodstain Preparation of Whole and Post-Mortem Blood", section III.

The majority of blood samples need only to be processed for storage and possible enzyme, protein, or DNA analysis.

1. Label 12 x 75 mm test tubes and 1.5 mL microfuge tubes or cryovials (two per sample) with FB numbers.
2. Fill 12 x 75 mm test tubes about 2/3 full with whole blood and centrifuge in the Serofuge for three minutes to spin down the cells.
3. Place the serum into one of the 1.5 mL microfuge tubes or cryovials.
4. Place the packed red blood cells into the other microfuge tube or cryovial.
5. Package dried stains into their envelopes. Freeze the stains, cells, and sera.
6. Indicate on the "Liquid Blood Processing" forms that stains were made and that cells and sera were obtained (where applicable).

THE REMAINDER OF THE LIQUID BLOOD WILL BE DISCARDED IN APPROXIMATELY TWO MONTHS AS RED BAG WASTE BY MEMBERS OF THE EVIDENCE UNIT.

CELLS AND SERUM ARE RETAINED FOR TWO YEARS, AT WHICH TIME THEY ARE DISCARDED AS ABOVE. STAINS ARE STORED FROZEN FOR APPROXIMATELY ONE YEAR, THEN AT ROOM TEMPERATURE THEREAFTER.

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B. ABO TYPING

ABO typing on post-mortem blood needs to be performed only in cases where an ABO inhibition test might need to be performed. This includes cases which have vaginal, oral, or rectal swabs or evidence such as cigarette butts where saliva stains may be present. The blood samples are identified by the RED DOT on the case folder.

Reagents: anti-A serum
anti-B serum
anti-H lectin
A indicator cells
B indicator cells

Method:

See Section "Bloodstain Preparation of Whole and Post-Mortem Blood", section III.

See Previous page.

Forward (DIRECT) Typing:

- a. Add one drop of whole blood to each of three wells
- b. Add one drop of anti-A to one well
Add one drop of anti-B to another well
Add one drop of anti-H to the third well
- c. Rotate briefly (on rotator or by hand) until agglutination can be seen
- d. Interpretation of Results:

If anti-A agglutinates, the blood is type A. The anti-H may or may not agglutinate.

If anti-B agglutinates, the blood is type B. The anti-H may or may not agglutinate.

If anti-A and anti-B agglutinates, the blood is type AB. The anti-H may or may not agglutinate.

If only anti-H agglutinates, the blood is type O.

If there's no agglutination, the ABO type is inconclusive.

5. Fill a 12 x 75 mm test tube about 2/3 full with whole blood and centrifuge in the Serofuge for three minutes to spin down cells.

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Reverse Typing:

- a. Put two drops of the supernatant serum into each of two test tubes or microscope slides labeled with the case number and "A" and "B".
- b. Add one drop of the A1 indicator cells (undiluted) into the "A" tube or slide.
- c. Add one drop of the B indicator cells (undiluted) into the "B" tube or slide.
- d. Centrifuge tubes for one minute, forming a "button" of cells, or gently rock the slide.
- e. Incubate at room temperature for 10 minutes.
- f. Shake tubes gently and read macroscopically for agglutination.
- g. Interpretation of Results
 - Agglutination with B cells - type A
 - Agglutination with A cells - type B
 - Agglutination with both A and B cells - type O
 - Agglutination with neither A or B cells - type AB or inconclusive (depending on forward typing results).
7. Place the remainder of the serum into one of the 1.5 mL Eppendorf tubes or cryovials.
8. Place the packed red blood cells into the other Eppendorf tube or cryovial. If Lewis typing is to be done, take a few drops of the cells and place in a 12 x 75 mm test tube. Fill about 2/3 full with saline (see "Lewis Typing").
9. Package dried stain into its envelope. Freeze the stain, cells, and serum, see section III.
10. Indicate on the "Liquid Blood Processing" form the results of the forward and reverse typing. If it is the second typing, add the date and initials of the analyst.
11. Use the "Comments" section to document any unusual results, condition of sample, etc.

THE REMAINDER OF THE LIQUID BLOOD WILL BE DISCARDED IN RED BAG BIOHAZARED WASET IN APPROXIMATELY TWO MONTHS BY MEMBERS OF THE EVIDENCE UNIT.

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C. Lewis Typing

Lewis typing is to be performed on whole blood (autopsy blood) samples in cases where an ABO inhibition test might need to be performed. This includes cases which have vaginal, oral, or rectal swabs or evidence such as cigarette butts. The blood samples are identified by the RED DOT sticker on the lids.

Alsever's buffer: Purchased commercially

OR

4.00 g trisodium citrate*2 H₂O
0.25 g anhydrous citric acid
10.25 g dextrose
2.09 g NaCl

Dilute to 500 mL with deionized water; adjust to pH 6.0 if necessary. Store in refrigerator.

Phosphate-buffered Saline (PBS):

5.38 g NaH₂PO₄
16.35 g Na₂HPO₄
9.00 g NaCl

Dilute to 1 L with deionized water; adjust to pH 7.0 if necessary (0.9% physiological saline can be substituted). Store in refrigerator.

4% ficin:

1.00 g ficin
25 mL Alsever's solution

Mix on a magnetic stirrer until the ficin has dissolved as completely as possible. Filter through Whatman #1 filter paper using a vacuum and freeze in 0.2 mL aliquots.

Lewis antisera:

Dilute antisera 200 uL antisera with with 800 uL PBS (1:4). Store in refrigerator.

Capillary tubes:

Chown type capillary tubes
0.4 mm I.D. x 90 mm

Standards:

Le a+b- and Le a-b+

Method:

1. Wash red blood cells (RBC) three times with saline by adding saline to RBC's, mixing, and centrifuging 3 minutes in the Serofuge.

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2. Using the washed RBC, prepare a solution of 50 uL RBC, 50 uL Alsever's, and 25 uL ficin solution. Mix gently.
3. By capillary action, draw diluted Lewis antisera into the capillary tube to a distance of 2.5 cm.
4. Wipe the tip of the capillary tube with a Kimwipe.
5. By capillary action, draw the treated RBC into the capillary tube to a distance of 1.0 cm. Keep the capillary tube in a vertical position to prevent air bubbles from being trapped between the RBC and the Lewis antisera. If air gets trapped, discard the tube and repeat the process.
6. Wipe the tip of the capillary tube with a Kimwipe.
7. Seal that end of the capillary tube used to pick up the antisera and RBC by gently pushing the capillary tip into Critoseal (replace cover on the Critoseal when finished to prevent drying).
8. Invert the sealed capillary tube, sealed end up, and place in a stand set at a 60 degree angle.
9. Results may be read after 10 minutes. You may wish to use a magnified light source to aid in reading the results.

Interpretation of Results:

a positive result is indicated by a clumped, broken column of RBC along the length of the capillary tube

a negative result is indicated by a unbroken, needle-like column of RBC along the length of the capillary tube

An Le a-b- sample should have the RBC tipped through the antisera several times; clumping may eventually occur.

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D. Absorption-Elution - Thread Method

ANTISERA MUST BE QUALITY CONTROL CHECKED. DO NOT USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Reagents: polyclonal anti-A anti-sera (MUST be polyclonal)
polyclonal anti-B anti-sera (MUST be polyclonal)
anti-H anti-sera

A1, B, and O test cells
30% bovine serum albumin (BSA)

saline

Standards:

A bloodstain
B bloodstain
O bloodstain
negative control (unstained cloth)

Sample

prep: If the bloodstain is on an absorbent thread material (such as clothing), the threads may be used as is. If not (such as a knife), swab off the blood onto threads. For scrapings, dissolve in water and apply onto threads.

Always prepare an unstained area in a similar fashion as a control.

Method:

1. Label enough Petri dishes for the standards, samples, and substrate controls. Make wells on the bottom of the Petri dish with a hot test tube.
2. Using a waterproof adhesive such as nail polish, affix threads (3-10 mm) to the appropriate wells.
3. After the adhesive is dry, add one drop anti-A to the wells in the "A" columns, one drop anti-B to the wells in the "B" columns, and one drop anti-H to the wells in the "O" columns; make sure each thread is submerged.
4. Place the lids on the Petri dishes and allow to absorb for a minimum of two hours at 4 °C. Absorption can be as long as 48 hours; place Petri dishes in a humid chamber to prevent evaporation of the anti-sera.
5. Prepare 0.05 - 0.1 % suspensions of A1, B, and O test cells in the following manner:

Label three 12 x 75 test tubes "A", "B", and "O".

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Fill each tube with saline to about 2 cm from the top (about 4.5 mL); add 1 drop of 30% BSA to each tube.

To the A tube add 2 drops of 2-4% A1 test cells; to the B tube add 2 drops B cells and to the O tube add 2 drops O cells.

6. Prepare a dilute solution of BSA by mixing 100 uL 30% BSA to 10 mL saline.
7. Using cold saline (4 °C), rinse the anti-sera off the Petri dishes.
8. Rinse the Petri dishes in cold saline (4 °C) on a rotator for two hours, changing the saline every 30 minutes.
9. Gently blot dry with tissue.
10. Add a drop of dilute BSA to each well, covering each thread. Place the lids on the Petri dishes and incubate at 55 °C for approximately 25 minutes.
11. Add a drop of A1 test cell suspension to each well in the "A" columns, a drop of B test cell suspension to each well in the "B" columns, and a drop of O test cell suspension to each well in the "O" columns.
12. Place the lids on the Petri dishes and rotate at room temperature for 30 minutes.
13. Read agglutination microscopically at 100X. Score the agglutination as below:
 - cells are free; no clumps are seen
 - 1 small clumps, a few cells each, are seen; the majority of the cells are free
 - 2 more and larger clumps are seen; not many free cells
 - 3 large clumps are seen; few free cells are seen
 - 4 very large, solid, clumps are seen; essentially no free cells
14. The A, B, and O bloodstain standards should give results similar to below:

	A	B	O
A bloodstain	3-4	-	-, 1-4
B bloodstain	-	3-4	-, 1-4

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O bloodstain	-	-	3-4
negative control	-	-	-

If the agglutination is weaker than above, rotate for an additional 15 minutes.

15. Substrate controls should have no agglutination; if any agglutination is seen in the substrate control, the stain results MUST be called inconclusive.

Initials: *RU* Date: *9/6/95*

E. Absorption-Inhibition (AI)

1. Titer Determination

Reagents: polyclonal anti-A anti-sera (MUST be polyclonal)
polyclonal anti-B anti-sera (MUST be polyclonal)
anti-H anti-sera

A2, B, and O test cells

30% bovine serum albumin (BSA)

Method:

1. Prepare doubling dilutions of anti-A, anti-B, and anti-H sera in the following manner:

label column wells on a microtiter plate 1/2 - 1/512

label row wells A, B, and H

pipet 100 uL saline into each well (a total of 27 wells)

add 100 uL anti-A to the well labeled A and 1/2; this creates the 1/2 dilution of anti-A

take 100 uL of the 1/2 and add it to the saline in the well labeled A and 1/4 and mix thoroughly. Continue doing this all the way to the well labeled A and 1/512.

OR

prepare dilutions of anti-sera as described in "Absorption - Inhibition, Section F.1.

prepare the dilutions of anti-B and anti-H the same way

2. Prepare 0.05 - 0.1 % suspensions of A2, B, and O test cells in the following manner:

Label three 12 x 75 test tubes "A", "B", and "O".

Fill each tube with saline to about 2 cm from the top (about 4.5 mL); add 1 drop of 30% BSA to each tube.

To the A tube add 2 drops of 2-4% A2 test cells; to the B tube add 2 drops B cells and to the O tube add 2 drops O cells.

3. Label three 100 x 100 mm square Petri dishes, one each for anti-A, anti-B, and anti-H. Each plate will have nine wells, one for each dilution of anti-sera. Either the lids or the bottoms can be used; some dishes have convenient markings on

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the bottom.

1/2	1/4	1/8
1/16	1/32	1/64
1/128	1/256	1/512

4. Add 10 uL of saline to each well.
5. Add 10 uL of 1/2 dilution of anti-A to the 1/2 well, 10 uL of 1/4 dilution of anti-A to 1/4 well, etc.; do the same with the dilutions of anti-B and anti-H.
6. Place the lids on the Petri dishes and allow to absorb for at least 30 minutes.
7. Add 10 uL of A2 test cell suspension to each well of the "A" plate, 10 uL of B test cell suspension to each well of the "B" plate, and 10 uL of O test cell suspension to each well of the "O" plate.
8. Cover and rotate for 30 - 45 minutes.
10. Read agglutination microscopically at 100X. (It may be easier to read if the other objectives are removed). Score the agglutination as below:
 - cells are free; no clumps are seen
 - 1 small clumps, a few cells each, are seen; the majority of the cells are free
 - 2 more and larger clumps are seen; not many free cells
 - 3 large clumps are seen; few free cells are seen
 - 4 very large, solid, clumps are seen; essentially no free cells

The weakest dilutions of anti-sera which give scores of 4 are the working dilutions to be used for absorption-inhibition.

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2. Absorption-Inhibition (AI)

ANTISERA MUST BE QUALITY CONTROL CHECKED AND TITERED. DO NOT USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Reagents: polyclonal anti-A anti-sera (MUST be polyclonal)
polyclonal anti-B anti-sera (MUST be polyclonal)
anti-H anti-sera

A2, B, and O test cells

30% bovine serum albumin (BSA)

Standards:

A secretor saliva
B secretor saliva
O secretor saliva
non-secretor saliva

Sample

prep: Extract an approximately 1 x 1 cm stain or half of a swab in 200 uL saline for 30 minutes at room temperature, using the pipet tip and test tube method. Centrifuge at high speed to separate extract from sample.

Any cell pellet formed should be retained and stored frozen for possible DNA analysis.

Method:

1. Check the QC files for the current titer of anti-sera being used. Prepare the appropriate dilutions of anti-sera using the amounts below:

dilution	anti-sera	saline
1/8	200 uL	1.4 mL
1/16	100 uL	1.5 mL
1/32	100 uL	3.1 mL
1/64	100 uL	6.3 mL
1/128	50 uL	6.3 mL
1/256	25 uL	6.3 mL

Any other necessary dilutions would be prepared similarly.

2. Prepare 0.05 - 0.1 % suspensions of A2, B, and O test cells in the following manner:

Label three 12 x 75 test tubes "A", "B", and "O".

Fill each tube with saline to about 2 cm from the top (about 4.5 mL); add 1 drop of 30% BSA to each tube.

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To the A tube add 2 drops of 2-4% A2 test cells; to the B tube add 2 drops B cells and to the O tube add 2 drops O cells.

3. Label enough 100 x 100 mm square Petri dishes for the standards, samples, and substrate controls. Either the lid or the bottom can be used; some dishes have convenient markings on the bottom.
4. Transfer the extracts to a microtiter plate. Prepare 1/10 dilutions by diluting 20 uL of the extracts in 180 uL saline.
5. To the appropriate spaces across the labeled Petri dishes, add three 10 uL aliquots of the neat samples and three 10 uL aliquots of the 1/10 diluted samples in the rows labeled "N" and "1/10", see below.
6. Add 10 uL of anti-A to each space in the "A" columns, 10 uL of anti-B to each space in the "B" columns, and 10 uL of anti-H to each space in the "O" columns, see below.
7. Cover and allow to absorb for at least 30 minutes at room temperature.
8. Add 10 uL of A2 test cell suspension to the "A" columns, 10 uL of B test cell suspension to the "B" columns, and 10 uL of O test cell suspension to the "O" columns.
9. Cover and rotate for 30 - 45 minutes.
10. Read agglutination microscopically at 100X. (It may be easier to read if the other objectives are removed). Score the agglutination as below:
 - cells are free; no clumps are seen
 - 1 small clumps, a few cells each, are seen; the majority of the cells are free
 - 2 more and larger clumps are seen; not many free cells
 - 3 large clumps are seen; few free cells are seen
 - 4 very large, solid, clumps are seen; essentially no free cells
11. The A, B, and O secretor standards and the non-secretor standard should give results similar to below:

	A	B	O
A secretor, N	-	4	-
, 1/10	-	4	-, 1-4

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B secretor, N	4	-	-
, 1/10	4	-	-, 1-4
O secretor, N	4	4	-
, 1/10	4	4	-, 1-4
non-secretor, N	4	4	3, 4
, 1/10	4	4	3, 4

If the agglutination of the standards is too weak, cover and rotate for an additional 15 minutes. Read as before.

12. Substrate controls should give results similar to the non-secretor standard. If any inhibition or weak agglutination (1 or 2) is seen in the substrate control, the stain results MUST be called inconclusive.

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VII. SEMEN ANALYSIS - POST MORTEM SPECIMENS

A. Semen Analysis Policy

Once a week an assigned analyst processes the accumulated swabs, sexual assault evidence collection kits, and any other applicable autopsy items.

1. Remove the swabs and kits from the freezer and/or coldroom.
2. Obtain the case files associated with the swabs and kits; they may be in the file cabinet, already assigned to an analyst, or in the "waiting to be assigned" box.
3. Fill out the "Swab Processing" worksheet or the "Kit Inventory" worksheet, indicating which envelopes were submitted, whether the sample was taken, and whether the envelope was sealed. For swabs and slides, note the number submitted.
4. Perform blood presumptive tests on any swabs that appear to be bloody (see section IV, "Kastle-Meyer or Leucomalachite Green Presumptive Tests for Blood").
5. Cut an approximately 2.5 x 2.5 mm piece out of each set of swabs (e.g., only test one vaginal swab if more than one is submitted). Place the samples in clearly labeled blue pipet tip/test tubes and place in the freezer in the area designated for P30 ELISA.

Each extract must be tested for the presence of P30 see section V, E or F.

Each vaginal and penile swab extract must also be tested for the presence of amylase. Use the diffusion method, see IV, D.

6. Put the P30 ELISA worksheet, a P30 ELISA plate worksheet, and the raw P30 data in each casefile. For the P30 ELISA plate worksheet and raw data, note where the original can be found.
7. For swabs that are borderline positive, that is, swabs whose P30 level is just under 2 ng, examine their corresponding slides for sperm (see "Christmas Tree Stain for Spermatozoa".) If there are two slides, stain only one.
8. If the swab is not to be processed for DNA, check the Blood Processing worksheet for the ABO and Lewis typing results of the deceased and proceed as follows.

If no ABO or Lewis type exists, save the swab according to laboratory policy.

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If there is an ABO type, the Lewis type of the deceased is a-b-, AND the oral swab is negative for semen, an absorption inhibition and an amylase test (see sections VI and V respectively), may be done on the oral swab. **NOTE: A proficiency test must be passed before performing ABO A/I tests.**

If the oral swab is KM positive, follow the procedure in "Processing of KM Positive Oral Swabs."

If the case has physical evidence associated with it, the A/I and amylase are the responsibility of the assigned analyst.

If there is no physical evidence, the A/I and amylase are the responsibility of the analyst processing the swabs.

9. Repackage all swabs and/or slides in small envelopes, seal according to Departmental guidelines, see FBH Methods "Evidence examination general guidelines" pg 9, and label with:
 1. case number
 2. date
 3. analyst's initials
 4. type of swab

If the swabs and/or slides came from a kit, they stay in their original envelopes.

10. If an envelope of cuttings from a case already exists (ask assigned analyst), place the swabs and slides in that envelope. If not, prepare an envelope for them and place in the freezer along with other retained samples.
11. Once all the items in a kit have been processed, the kit box itself can be discarded. Any unused items should be discarded.
12. If a case doesn't have an analyst assigned, give the file to the clerical staff for typing of a report.

NOTE: If P30 ELISA is not available, analyze the swabs using the acid phosphatase presumptive test, followed by the Christmas Tree stain and/or P30 crossover electrophoresis.

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B. Processing of KM Positive Oral Swabs

When determining the secretor status of a decedent from an oral swab (contingent on obtaining an ABO type), care must be taken to remove any possible interference from a KM-positive sample. To remove blood ABO antigens from KM-positive oral swabs, the following method is suggested.

Method:

1. Extract a swab as per the absorption-inhibition method (Section: VI,E, "Absorption-Inhibition").
2. Centrifuge sample test tubes (maximum high speed) for 5 minutes.
3. Decant supernatant into another test tube and discard debris.
4. Take sample test tubes, place in a water bath, and boil for 5 minutes.
5. Centrifuge the sample (at high speed) test tubes.
6. Proceed with the amylase and absorption-inhibition methods using the supernatant.

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VIII. Electrophoresis

A. Guidelines

1. Agarose Gel Electrophoresis

Reagents: Miscellaneous reagents, i.e., gel, tank, and reaction buffers, are quality control checked before use. Do not make your own or use reagents that have not been quality control checked.

Equipment: The following equipment is needed:

1. glass plates, various sizes
2. leveling stand
3. cooling bath and platens
4. power supplies capable of 500V
5. electrophoresis tanks
6. incubator
7. photography equipment
8. miscellaneous glassware

**Tank
prep:**

The buffer reservoirs must be filled with tank buffer; the amount depends on the type of tanks being used.

Discard tank buffer after each use.

**Gel
prep:**

The amounts of gel buffer and agarose depend on the size of the gel:

gel size	g agarose	mL gel buffer
10 x 20 cm	0.40	40
15 x 20 cm	0.60	60
20 x 20 cm	0.80	80

The type of agarose (amount of electroendosmosis, EEO) is specified by the method.

The agarose is dissolved in the gel buffer by heating. Make sure the agarose is completely dissolved by checking the solution for visible agarose granules.

Pour gel solution onto a leveled glass plate, making sure the gel is evenly distributed. Allow gel to solidify at room temperature or on a cooling platen.

**Sample
prep:**

Stains on thread (clothes or stains swabbed off items) can be used directly; alternatively, an extract can be

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made and applied to cotton threads.

Soak threads in a minimal amount (just enough to wet the sample) of 0.05 M DTT. Do not allow threads to dry out.

Scrapings need to be dissolved in a minimal amount of 0.05 M DTT and applied to cotton threads.

Lysates need to be treated with 0.05 M DTT and applied to cotton threads.

Sample

slots: Using a metal comb, make slots in the gel at the origin location specified by the method. Place sample threads in slots.

Unless otherwise specified, it is generally preferable to use 1 cm wide sample slots.

Wicks: Moisten filter paper wicks, 12 cm wide and as long as the gel, with tank buffer. Use 2-6 wicks, depending on the thickness of the wicks.

Gel Assembly:

Place gel on platen at 4 °C. All enzyme genetic marker electrophoresis methods used in the laboratory run at this temperature.

Make sure there is a THIN layer of water between the plate and the platen; avoid air bubbles and do not use so much water that the plate is "skating" around. The layer of water ensures even cooling of the gel. Any bubbles will result in a "hot spot" on the gel which can affect migration and may cause unreadable results.

Place wicks on either side of the gel to give a wick distance of 12-17 cm. To make sure the wicks stay in contact with the gel, place a border stick on each wick and cover with a glass plate.

Plug in electrodes (if applicable) and close the tank lid. Connect the power supply, making sure the positive on the tank is connected to the positive on the power supply.

Parameters:

Conduct electrophoresis at the voltage and for the time specified by the method.

Agarose electrophoresis is run at constant voltage; the amperage and watts can be set at the maximum for the power supply being used.

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Overlays: If water has condensed on a gel, remove excess moisture with filter paper or Kimwipes.

Filter paper overlays should be wet, but not dripping, with the reaction mixture. Remove filter paper before viewing or photographing.

Cellulose acetate membrane (CAM) overlays should be wet, but not dripping, with the reaction mixture.

To pour an agarose overlay, make a "corral" of black border sticks around the area where the overlay is to be poured. Use molten 1% agarose to plug the corners where the sticks meet. Pour on the overlay, making sure the overlay is evenly distributed.

An agarose overlay can be removed upon solidifying and a new one poured if necessary.

Incubation:

Incubate the gel with overlays at 37 or 55 °C. Use a humid chamber or a glass plate lid to keep the gel from drying out.

Results: For a phenotype to be called, the banding pattern and band separation must fulfill the requirements specified by the method.

Results are recorded by noting the phenotype on the worksheet. If the sample can't be called, indicate the reason why using the following symbols:

1. -,NR: no visible activity
2. inc: activity, but no clear bands
3. (): possible type, not clear enough to call

If the required standards don't work, the entire plate must be called inconclusive, **UNLESS** there are secondary standards that did work on the plate. For example, a secondary standard could be a victim blood sample that was previously typed.

Double-reading:

All electrophoresis results must be called **INDEPENDENTLY** by a second reader. The date and the initials of the second reader must be on the bottom of the electrophoresis worksheet.

If there is a disagreement on a call, a supervisor should be consulted. If there is no agreement, the sample must be called inconclusive.

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Photography:

All electrophoresis results should be documented by photographs. The quality of a photo is determined by film speed, *f*-stop, and shutter speed.

The *f*-stop controls the size of the aperture (lens opening); a small *f*-stop means a large aperture that allows more light.

The shutter speed controls how long the film is exposed. The "faster" the shutter speed, the less light that reaches the film. On the camera, the speeds are in sec^{-1} ; therefore, 1=1 sec, 2=1/2 sec, all the way to 60=1/60 sec. The B setting holds the aperture open until the lever is released.

The lab generally uses two types of film - fast (3000 ASA, Polaroid type 667) and slow (80 ASA, Polaroid type 665). A slow film requires more light; therefore, a larger aperture and/or a longer exposure time.

Suggested settings are:

1. Type 667

ASA 3000	UV photo	non-UV photo
<i>f</i> -stop	11	22 or 32
shutter	1/8	1/60

2. Type 665

ASA 80	UV photo	non-UV photo
<i>f</i> -stop	5.6	8
shutter	B (10 sec)	1/30

A filter must be used with U.V. light. The exposure settings given are for use with the orange filter.

If a photo is too dark (under-exposed), increase the amount of light reaching the film by either increasing the aperture (moving the *f*-stop to a smaller number) or decreasing the shutter speed (moving it to a smaller number). The reverse is true for a photo that is too light (over-exposed).

For UV photos, it may be helpful to turn the plate over and photograph the underside of the gel.

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2. Isoelectric Focusing (IEF)

Reagents: Reagents, buffers, and IEF gels are prepared by lab personnel and quality control checked before use. Do not make your own or use reagents that have not been quality control checked.

Equipment:

1. glass plates, various sizes
2. cooling baths and platens
3. power supplies capable of 2000 V
4. isoelectric focusing tanks
5. isoelectric focusing electrodes
6. incubator
7. photography equipment
8. miscellaneous glassware

Plate prep:

Described in detail in "Preparation of ultrathin polyacrylamide gels".

Gel prep:

Described in detail in "Preparation of ultrathin polyacrylamide gels".

The amount of gel solution prepared depends on the size and numbers of the gels being prepared:

Gel Size	mL Solution
10 x 20 cm	3.5
15 x 20 cm	6-7
12 x 20 cm	4-5
12 x 25 cm	6-7

The acrylamide and other reagents are dissolved in deionized water. Make sure the acrylamide and sucrose is completely dissolved by checking the solution for visible granules.

Sample prep:

Use the pipet tip and test tube method for the strongest extracts.

For stains, extract an approximately 3x3 mm stain in 50 uL of the appropriate extractant for 30 minutes at room temperature.

If a stain is small or light, use less; if it is heavy, it may need to be diluted. Experience will tell you if sample size or volume of extractant need to be adjusted.

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For lysates, prepare a solution of 5 uL of red blood cells with 100 uL of the appropriate extractant.

If a sample needs to be analyzed in both PGM and ACP or ESD, there are two approaches:

1. extract in DTT and use that extract for all analyses; there will be a blue discoloration at the PGM origin

OR

2. extract in water and run the PGM first. Take the remaining ~40 uL extract and add 10 uL of a 0.25 M DTT (5X) solution

Plate

loading: Use a silicone applicator strip to apply samples at the origin specified by the method. Comparison of the strips with applicator tabs showed a clear improvement in results with the applicator strip.

It is generally preferable to use the 1x7 mm sample slots.

Generally, 10 uL of extract is used. If a sample is very light, up to 15 uL can be used in the 1x7 mm slots. For very weak samples, use up to 15 uL in the 2x3.5 mm slots.

If a sample has been run previously and was too intense, use less extract or dilute it the next time.

If a sample has been run previously and gave distorted results, it may be due to salts in the sample (especially in semen samples or samples contaminated with sweat or dirt). Try running a volume reduction series of the sample (10, 5, and 2.5 uL) or a dilution series (neat, 1/2, 1/4, etc).

Post-mortem samples are especially prone to intense and/or distorted PGM subtype results. The sample extract may need to be diluted as much as 1:100 in gel buffer to obtain callable results.

It may be helpful to place standards right next to samples whose type was ambiguous on a prior run.

It may be helpful to leave blank spaces around samples that have caused distortion during a prior run.

Wicks: Moisten filter paper wicks, 6 mm wide and as long as the gel (not the plate), with the appropriate electrolyte.

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The wicks should be uniformly wet, but not dripping or too dry. The wicks must be parallel.

Place wicks on gel to give the wick distance specified by the method.

Gel

assembly: Place gel on platen at 4 °C. All enzyme genetic marker electrophoresis methods used in the laboratory run at this temperature.

Make sure there is a THIN layer of water between the plate and the platen; no air bubbles and not so much water that the plate is "skating" around. The layer of water ensures even cooling of the gel. Any bubbles will result in a "hot spot" on the gel which may affect migration and may cause unreadable samples.

Adjust electrodes to give the electrode gap specified by the method. Make sure the electrodes are parallel by measuring at each end. Lower electrodes onto wicks making sure there is contact all the way along the wicks.

Plug in electrodes and close the tank lid. Connect the power supply, making sure the positive on the tank is connected to the positive on the power supply.

Parameters:

Conduct electrophoresis at the voltage and for the time or volt-hours specified by the method.

Isoelectric focusing is run with a power (10 watts) maximum to limit heat build-up; the voltage and amperage are set at 2000V and 15mA.

Two gels can be run simultaneously from one power supply. Change the Watt setting to 20W.

Overlays: See Section VIII Electrophoresis

Incubation:

See Section VIII Electrophoresis

Results: See Section VIII Electrophoresis

Double-reading:

See Section VIII Electrophoresis

Photography:

See Section VIII Electrophoresis

Initials: RL Date: 7/6/95

3. Preparation of Ultrathin Polyacrylamide Gels

Method:

1. Prepare the ultra-thin mold by sticking strips of smooth PVC adhesive tape, approximately 1 cm wide, 0.15 mm thick, to the edges of clean 15 x 20 cm or 12 x 25 cm glass plates. Avoid over-stretching the tape and avoid gaps and overlaps at the corners since this can hinder polymerization. Use different colored tape for each system to allow clear and rapid identification of the plates:

PGM	blue
ACP	green
ESD	red
Hb	red/yellow

2. Prepare enough plates and plate "lids" (plain glass) by cleaning their surfaces thoroughly with alcohol; this removes any oils left on the plate and helps prevent bubbles and tearing. Place the mold up on a petri dish or other support. **DO NOT TOUCH THE SURFACE OF THE PLATES WITH YOUR FINGERS ONCE THEY ARE CLEAN;** handle by the edges.
3. Prepare the acrylamide solution using the amount of reagents as shown in Table I. Ensure that the solids are completely dissolved (let sit for about 10 minutes) and avoid mixing too vigorously.

TABLE I

REAGENTS	NUMBER OF PLATES REQUIRED				
	5-6	10-12	15-18	20-24	Units
Deionized Water	20	40	60	80	mL
Sucrose	2.5	5.0	7.5	10	g
3% Acrylamide Premix	1	2	3	4	g
Riboflavin or APS*	150	300	450	600	uL
Total Volume (Approx)	24	48	72	96	mL

*: Ammonium Persulfate

Polymerization

To initiate polymerization, two different methods are available. These are chemical polymerization using ammonium persulfate and photopolymerization using riboflavin (RIBOFLAVIN GIVES BETTER Results). Use one or the other:

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Solutions: Riboflavin 1.0 mg/mL water
APS: 0.228 g/5 mL water

ALTERNATIVELY

Add the appropriate volume of riboflavin solution (1.0 mg/10mL stored in the refrigerator) to the gel solution; mix well.

ALTERNATIVELY

Add the appropriate volume of freshly prepared 0.2M APS (0.228 g/10mL water); mix well.

If necessary, TEMED may be added to aid in polymerization. use approximately 10 uL for each 10 mL of acrylamide solution.

1. Pour 6-7 mL of the solution onto the upper edge of a glass mold. Carefully and slowly lower the plain glass "lid" down, allowing the solution to spread aover the mold. Take care not to trap any air bubbles; if this happens, raise the top plate and lower again. - **DO NOT PRESS THE PLATES TOGETHER OR USE A WEIGHT** - once the force is removed, the solution will pull away from the edges and ruin the plate. Any excess solution will flow out and can be removed using tissues.

With practice, 6 mL will cover one large plate. At first, use 7-8 mL.

2. Allow the plates to polymerize:

For riboflavin plates, expose them to UV light overnight. They may be stacked, but no more than three in a pile and each plate must be separated by strips placed along the edges to prevent them from sticking together.

For APS plates, leave the plates at room temperature for 12 hours.

3. The plates are then placed in the refrigerator, even if they are to be used that day; cooling aids in separating the plates. The plates can be stored in the refrigerator up to 2 months, but do not use them if they have dried, become discolored, or have obvious damage.
4. To use the plates, separate them immediately before sample application by placing the whole plate on a firm, horizontal surface and inserting a spatula between the plates. Twist gently and slowly and carefully lift the top plate; once you start, don't stop.
5. If the gel has torn or has ragged edges, trim them so they are straight. If there is a bubble or gap in the gel, do not

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apply a sample in that position.
 apply a sample in that position.

6. Add the appropriate amount of the correct ampholyte as shown in Table II. Note that each system has a different ampholyte.

(1.0 mg per 10 mL water, stored in the refrigerator) to the gel solution; mix well

SYSTEM	AMPHOLYTE	NUMBER OF PLATES REQUIRED*				UNITS
		5-6	10-12	15-18	20-24	
ACP	pH 4-8	1.0	2.0	3.0	4.0	mL
	or pH 4-6	0.5	1.0	1.5	2.0	mL
	pH 6-8	0.5	1.0	1.5	2.0	mL
ESD	pH 4.5 -5.4	1.0	2.0	3.0	4.0	mL
	plus HEPES	0.17	0.34	0.51	0.68	g
	MOPS	0.50	1.00	1.50	2.00	g
PGM	pH 5-7	1.0	2.0	3.0	4.0	mL
	plus EPPS/HEPPS	0.25	0.50	0.75	1.00	g
GC	pH 4.5-5.4	1.0	2.0	3.0	4.0	mL
	plus MOPS	0.50	1.00	1.50	2.00	g
	+ HEPES	0.17	0.35	0.52	0.69	g
Hb	pH 6-8	0.50	1.0	1.5	2.0	mL
	pH 7-9	0.50	1.0	1.5	2.0	mL
	pH 3-10	0.20	0.40	1.5	2.0	mL

*: The number of plates is approximate and is based on the medium sized plates. With the given amounts of reagents, there is plenty to make the lesser number of plates; with careful pouring, more plates can be prepared. If different sized plates are being poured, calculate how many can be made using the following:

SIZE	DIMENSION	mL REQUIRED
S	10 x 20 cm	3 - 3.5
M	13 x 20 cm	4 - 4.5
L	15 x 20 cm	6 - 6.5
L	13 x 27 cm	6 - 6.5

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B. Red Cell Isoenzyme Determination

1. Esterase D and Phosphoglucomutase

ALL BUFFERS AND PGM REACTION MIXTURE ARE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Tank

buffer: 12.11 g Tris base
11.62 g maleic acid
2.92 g EDTA free acid
2.03 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
5.00 g NaOH

Dissolve in 1 L deionized water; adjust to pH 7.4 with NaOH.

Gel

buffer: Dilute 60 mL tank buffer with 840 mL deionized water; adjust to pH 7.4 if necessary.

ESD reaction buffer:

4.1 g sodium acetate, anhydrous

Dissolve in 1 L deionized water; adjust to pH 6.5 with 1% acetic acid.

PGM reaction buffer:

12.0 g Tris base
4.0 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

Dissolve in 1 L deionized water and adjust to pH 8.0 with HCl.

Gel prep: 0.8 g Sigma type I agarose
80 mL gel buffer

Dissolve agarose in gel buffer by heating until clear. Pour gel onto a leveled 20x20 cm plate making sure that the gel is evenly distributed; allow gel to solidify.

If a different plate size is used, adjust reagents accordingly.

Standards: ESD 1 and 2-1, PGM 1 and 2-1

Sample

prep: Stains are soaked in a minimal amount (just enough to wet the sample) of 0.05 M DTT (Cleland's reagent) in a spot

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plate.

Stains can also be extracted in a minimal amount (just enough to wet the sample) of 0.05 M DTT using the pipet tip/test tube method; apply the extract to cotton threads.

For most stains, 2-4 threads should be sufficient. Up to 6 threads can be used for weak stains.

Origin: 3 cm from cathode

Parameters:

Place gel on platen at 4°C.

Conduct electrophoresis at 400 V for approximately 3 hours.

Development for ESD isoenzymes:

Reagents: 6 mg methylumbelliferyl acetate (MUA)
15 mL reaction buffer
Acetone

Method:

1. Dissolve the MUA in a small amount of acetone and mix in the reaction buffer. Soak onto a strip of 20x8 cm filter paper, allowing excess reaction mixture to drip off, and apply the paper to the gel from the origin forward.
2. Incubate in a humid chamber for 15 minutes at 37 °C and read under UV light (it may be helpful to remove the filter paper).
3. Photograph the plate as bands develop.
4. Mark the position of the ESD 2 band.
5. After ESD development, rinse plate in water for about 30 seconds to remove any remaining ESD reaction mixture.

Development for PGM Isoenzymes:

Reagents: 47 mg glucose-1-phosphate with 1% glu-1,6-diphosphate
3 mg NADP sodium salt
4 mg MTT
15 mL reaction buffer
75 uL glucose-6-phosphate dehydrogenase
200 uL meldola blue (1 mg per mL)

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15 mL overlay agarose, 1% Sigma type I in deionized water

Method:

1. The volume of enzyme used is determined by the concentration of enzyme in the solution. Adjust the volume if necessary to use approximately 10 units.
2. Dissolve dry ingredients (available pre-measured in freezer) in reaction buffer; add meldola blue and enzyme. Mix in agarose and pour onto plate between the origin and the ESD 2 band.
3. Incubate in a humid chamber at 37°C or 55°C until bands are clear.
4. Photograph the plate as bands develop.

Interpretation of Results:

Compare the banding pattern to the ESD and PGM controls. The photograph is used to interpret the final results, not the gel.

The allowable separation between bands is:

ESD 1-2	> 4	mm
ESD 2-3	> 4	mm
PGM a-b	> 3	mm
PGM a-c	> 7	mm
PGM b-d	> 7	mm
PGM a-d	> 14	mm

The separations are based on experimental data. If the actual band separation is less than that allowed, all of the results must be called inconclusive.

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2. Acid Phosphatase by Isoelectric Focusing

ALL BUFFERS, GELS, AND REAGENTS ARE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Gel

preparation: Prepare the gels as described, using pH 4-6 and 6-8 ampholytes from LKB; SERVA ampholytes are not acceptable.

ACP typing gels can be identified by the green tape.

Standards: B, BA, and A

If standards are placed in lanes 2, 5, 8, 11, etc., a sample will always be next to a standard. THIS IS NOT MANDATORY, but can assist with typing problem samples.

Sample

preparation: For lysates, mix 5 uL of washed packed red blood cells with 100 uL DTT; store frozen if the sample may be typed again.

For stains, extract an approximately 3x3 mm stain in 50 uL DTT for 30 minutes at room temperature. Experience will tell you if the samples need to be further diluted.

For the strongest extracts, use the pipet tip/test tube method; let the tubes lie horizontally during the 30 minutes so the water stays in contact with the sample. Centrifuge at high speed to separate extract from sample.

Plate loading: See above for instructions.

Electrolytes: Prepare electrode wicks as long as your gel; they should not be touching the plastic tape. Saturate the anode wick with acid (1% acetic acid) and the cathode wick with base (1% ethanolamine). The wicks should be uniformly wet, but not dripping.

Apply the wicks to give an electrode gap of 10.5 cm.

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Run conditions: Place gel on platen at 4 °C. Apply the electrodes and cover. Conduct electrophoresis at 2000V for a total of approximately 3000 volt-hours (1.5 hrs); the Hb should be focused.

Power Supply Settings

Volts	Amps	Watts
2000V	15mA	10W

This method is **POWER LIMITED**; the wattage will never be allowed to be greater than 10 W, even if the voltage is forced to be lower. This means that the voltage may not be 2000V at the beginning.

Reaction buffer: 1.92 g citric acid
0.8 g NaOH

Dissolve the citric acid in 200 mL deionized water and adjust to pH 5.0 with the NaOH.

Reaction mixture:

Have the reaction mixture already dissolved and the cellulose acetate membrane (CAM) cut before your plate is finished.

ACP reaction mixture:

3 mg Methylumbelliferyl Phosphate

Dissolve in 3 mL reaction buffer.

1. Absorb reaction mixture onto a single piece of 5 cm wide CAM as long as your gel.
2. Apply the CAM to the gel surface beginning 4 cm from the origin and towards the anode.
3. Place a glass plate over the gel or place in a humid chamber.
4. Do not have the CAM extending from under the glass plate lid. This can cause distortion of the samples on the edges.
5. Incubate for 10 minutes at 37°C or 55°C and view the gel (with CAM) under UV light; leave up to 30 minutes if bands are weak.

Initials: *RC* Date: *9/6/95*

Interpretation of Results:

Compare the banding pattern to the ACP controls and record the actual ACP type on the "ACP by IEF Worksheet". If the specimen is from post-mortem blood, determine whether the ACP type identified could have occurred because of degradation. If it could, report ACP type as an ACP "class". If it could not, report the ACP type as read from the plate. If the ACP type appears to be a variant, rerun using rare variant controls to confirm they type.

All results are read from the photographs and not the original gels.

In order for a sample to be called, at least one secondary band (a_3 , a_4 , b_3 , b_4 , c_3 , c_4) must be visible.

The allowable separation between bands is:

B1/B2 bands	>	8 mm
B/A bands	>	10 mm
A/Hb bands	>	1 mm

The separations are based on experimental data. If the actual band separation is less than that allowed, all of the results must be called inconclusive.

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3. Esterase D by Isoelectric Focusing

ALL BUFFERS, GELS, AND REAGENTS ARE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Gel

preparation: Prepare the gels as described, using pH 4.5 -5.4 ampholytes, plus HEPES and MOPS.

ESD typing gels can be identified by the red tape.

Standards: ESD: 2-1 and 5-1

If standards are placed in lanes 2, 5, 8, 11, etc., a sample will always be next to a standard. THIS IS NOT MANDATORY, but can assist with typing problem samples.

Sample preparation:

For lysates, mix 5 uL of washed packed red blood cells with 100 uL DTT.

For stains, extract an approximately 3x3 mm stain in 50 uL DTT for 30 minutes at room temperature. Experience will tell you if the extract needs to be further diluted.

For the strongest extracts, use the pipet tip/test tube method; let the tubes lie horizontally during the 30 minutes so the DTT stays in contact with the sample. Centrifuge at high speed to separate extract from sample.

Plate loading: See above for instructions.

Electrolytes: Prepare electrode wicks as long as your gel; they should not be touching the plastic tape. Saturate the anode wick with acid (1% acetic acid) and the cathode wick with base (1% ethanolamine). The wicks should be uniformly wet, but not dripping.

Electrode wicks should have a gap of 10 cm.

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Run conditions: Place gel on platen at 4 °C. Apply the electrodes and cover. Conduct electrophoresis at 2000V for a total of approximately 4500 volt-hours (2 1/4 hrs).

Power Supply Settings

Volts	Amperage	Watts
2000V	15mA	10W

This method is **POWER LIMITED**; the wattage will never be allowed to be greater than 10 W, even if the voltage is forced to be lower. This means that the voltage may not be 2000V at the beginning.

Reaction buffer: 4.1 g anhydrous sodium acetate

Dissolve the sodium acetate in 1 L deionized water; adjust to pH 6.5 with 1% acetic acid.

Reaction mixture:

Have the reagents measured and the cellulose acetate membrane (CAM) cut before your plate is done.

ESD reaction mixture:

3 mg methumbilliferyl acetate (MUA)

1. Dissolve the MUA in MINIMAL (a few drops) acetone; add 3 mL reaction buffer.
2. Absorb reaction mixture onto a single piece of 5 cm wide CAM as long as your gel.
3. Apply the CAM to the gel surface beginning 4 cm from the origin and towards the anode.
4. Place a glass plate over the gel or place in a humid chamber.
5. Do not have the CAM extending from under the glass plate lid. This can cause distortion of the samples on the edges.
6. Incubate for 10 minutes at 37 or 55 °C and view the gel (with CAM) under UV light; leave up to 30 minutes if bands are weak.

Photograph the plate.

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Interpretation of Results:

Compare the banding pattern to known ESD IEF type. All results are read from the photographs and not the original gels.

The allowable separation between bands is:

type 1	> 3	mm
type 2-1	> 1	mm
	> 1	mm
type 5-1	> 3	mm

The separations are based on experimental data. If the actual band separation is less than that allowed, all of the results must be called inconclusive.

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4. Phosphoglucosyltransferase by Isoelectric Focusing

BUFFERS, GELS, AND REAGENTS ARE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT PREPARE YOUR OWN OR USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Gel

preparation: Prepare the gels as described, using pH 5-7 ampholytes and N-2-hydroxyethylpiperazine-N-2-propanesulfonic acid (abbreviated EPPS or HEPPS) as a separator.

PGM subtyping gels can be identified by the blue tape.

Standards: 2+2-1+1- in any combination (i.e., 2-1+ and 2+1-) or use a 4-band standard (available from SERI or prepared in the laboratory).

If standards are placed in lanes 2, 5, 8, 11, etc., a sample will always be next to a standard. THIS IS NOT MANDATORY, but can assist with typing problem samples.

For samples whose PGM type is known, an appropriate PGM sub standard can be placed next to it: e.g., a PGM sub 1+1- next to samples that are PGM 1.

Sample

preparation: For lysates, mix 5 uL of washed packed red blood cells with 100 uL deionized water.

For stains, extract an approximately 3x3 mm stain in 50 uL deionized water for 30 minutes at room temperature.

For the strongest extracts, use the pipet tip/test tube method; let the tubes lie horizontally during the 30 minutes so the water stays in contact with the sample. Centrifuge at high speed to separate extract from sample.

A reducing agent (DTT) is not necessary for PGM typing and will cause a blue discoloration near the origin. If DTT is used (for example, if the same extract is to be used for ESD), trim away the blue discoloration on the agarose overlay. Alternatively, use a water extract and apply part of the extract to the PGM plate. Treat the remainder of the sample (approximately 40 uL) with 10 uL 0.25 M DTT, bringing the concentration of DTT in the sample to 0.05 M.

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Plate loading: See above for instructions.

Electrolytes: Prepare electrode wicks as long as your gel; they should not be touching the plastic tape. Saturate the anode wick with acid (1% acetic acid) and the cathode wick with base (1% ethanolamine). The wicks should be uniformly wet, but not dripping.

Apply the wicks to give an electrode gap of 10.5 cm. Use the LKB template for the proper placement of the electrode wicks.

Run conditions: Place gel on platen at 4 °C. Apply the electrodes and cover. Conduct electrophoresis at 2000V for approximately 2 hours, for a total of 4000 volt-hours.

The settings on the power supply are:

2000V 15mA 10W

This method is POWER LIMITED; the wattage will never be allowed to be greater than 10 W, even if the voltage is forced to be lower. This means that the voltage may not be 2000V at the beginning.

Reaction buffer: 12.0 g Tris base
4.0 g MgCl₂*6H₂O

Dissolve in 1 L deionized water and adjust to pH 8.0 with HCl.

Reaction mixture:

Have the reaction mixture already dissolved and the agarose melted before your plate is done.

PGM reaction mixture:

47 mg glucose-1-phosphate with
1% glucose-1,6-diphosphate
3 mg NADP sodium salt
4 mg MTT
15 mL reaction buffer
75 uL glucose-6-phosphate dehydrogenase
200 uL meldola blue (1 mg/mL)
15 mL overlay agarose, 1% Sigma type I in
deionized water, melted

Dissolve dry ingredients (available pre-measured in the freezer) in reaction buffer; add meldola blue and enzyme. Mix in agarose and pour onto plate between the origin and the hemoglobin. Incubate at

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37°C or 55°C or until bands are clear.

Post-mortem samples can have enhanced PGM activity and may develop quickly; check the plate often.

Interpretation of Results:

Compare the banding pattern to a known PGM subtype. All results are read from the photographs and not from the original gels.

The allowable separation between bands is:

type 2+2-	> 4	mm
type 2-1+	> 6	mm
type 1+1-	> 2	mm

The separations are based on experimental data. If the actual band separation is less than that allowed, all of the results must be called inconclusive.

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C. Protein Polymorphism Determination

1. Group Specific Component (GC) - Conventional

ALL BUFFERS AND ANTISERA ARE PRE-MADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Tank

buffer: 41.2 g anhydrous sodium phosphate, dibasic
19.2 g anhydrous citric acid

Dissolve in 1 L deionized water; adjust to pH 5.5

Gel

buffer: 0.81 g anhydrous sodium phosphate, dibasic
0.48 g anhydrous citric acid

Dissolve in 1 L deionized water; adjust to pH 5.5

Plate

prep: 0.8 g Sigma Type I agarose
80 mL gel buffer

Dissolve agarose in gel buffer by heating until clear. Pour gel onto a leveled 20x20 cm plate, making sure that the gel is evenly distributed; allow gel to solidify.

If a different plate size is used, adjust reagents accordingly.

Standards: Gc 2-1

Sample

prep: Stains are soaked in a minimal amount (just enough to wet the sample) of deionized water.

For most stains, 5-6 1 cm long threads should be sufficient. Up to 8 threads can be used for weak stains.

Origin: Midpoint of gel

Parameters:

Place gel on cooling platen at 4 °C.

Conduct electrophoresis at 400 V for 2.5 hours.

Development of GC:

5 x 20 cm cellulose acetate membrane (CAM)

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anti-Gc, diluted with deionized water to the concentration specified by the QC file. For a 20 cm long CAM, a total volume of 600-750 uL is sufficient.

Soak the CAM in diluted anti-sera. Apply to the anodic end of the gel from the edge of the wick towards the origin.

Incubate the plate in a moisture chamber at room temperature for 2 hours.

Processing:

Following incubation, remove the overlays and submerge the gel in saline and wash overnight in the refrigerator (2-5 °C).

Rinse by submerging the gel in deionized water for 30 minutes.

Remove the gel from the glass plate and place it on the hydrophilic side of a piece of GelBond. Put a sheet of filter paper on top of the gel, then several layers of paper towels; press the gel for 30-60 minutes.

Dry the gel in the oven at 55-60 °C for approximately 60 minutes. Do not overdry; the gel may separate from the Gel Bond.

Once the gel is dry (the gel should be clear, flat, and tightly adhered to the GelBond), stain in Coomassie Blue for approximately 10 minutes then destain (see "Coomassie Blue Staining").

Results: Compare the banding pattern to a known Gc pattern. The original dehydrated gel is kept as the official record of the results.

The allowable separation between bands is:

all bands > 2 mm

The separations are based on experimental data. If the actual band separation is less than that allowed, all of the results must be called inconclusive.

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2. Hemoglobin (Hb) by Isoelectric Focusing

GELS ARE PREMADE AND QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROL CHECKED.

Gel

preparation: Prepare the gels as described, using pH 6-8, 7-9, and 3-10 ampholytes (use the ammonium persulfate procedure).

Hb typing gels can be identified by the yellow/red tape.

Standards: AFSC (Helena Labs' 4-band standard)

If standards are placed in lanes 2, 5, 8, 11, etc., a sample will always be next to a standard. THIS IS NOT MANDATORY, but can assist with typing problem samples.

Sample

preparation: For whole blood, lysates, or AFSC standard, mix 10 uL with 100 uL 0.05% KCN.

For stains, extract an approximately 1x3 mm stain in 50 uL 0.05% KCN for 30 minutes at room temperature.

All samples should be slightly lighter than the diluted AFSC standard; dilute if necessary.

For the strongest extracts, use the pipet tip/test tube method; let the tubes lie horizontally during the 30 minutes so the water stays in contact with the sample. Centrifuge at high speed to separate extract from sample.

Plate loading: See above for instructions.

The application mask with 2x3.5 mm slots can be used; use 5 uL of extract.

Electrolytes: Prepare electrode wicks as long as your gel; they should not be touching the plastic tape. Saturate the anode wick with acid (1% acetic acid) and the cathode wick with base (1% ethanolamine). The wicks should be uniformly wet, but not dripping.

Apply the wicks to give an electrode gap of 10.5 cm.

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Run conditions: Place gel on platen at 4 °C. Apply the electrodes and cover. Conduct electrophoresis at 2000V for approximately 3000 volt-hours, or until the bands are focused and the separation meets the allowed separation.

Power Supply Settings

Voltage	Amperage	Watts
2000V	15mA	10W

This method is **POWER LIMITED**; the wattage will never be allowed to be greater than 10 W, even if the voltage is forced to be lower. This means that the voltage may not be 2000V at the beginning.

Photograph the bands.

Interpretation of Results:

Compare the banding pattern to a known Hb pattern. Results are read from the photograph and not the original gel.

The allowable separation between bands is:

A - F	> 2	mm
F - S	> 3	mm
S - C	> 6	mm

The separations are based on experimental data. If the actual band separation is less than that allowed, all of the results must be called inconclusive.

NOTE: VERY WEAK BANDS CAN BE ENHANCED BY DEVELOPING THE PLATE WITH O-TOLIDINE. REMEMBER THAT THIS STAIN IS EXTREMELY SENSITIVE AND IT MAY BE NECESSARY TO DILUTE THE STANDARDS TO THE SAME COLOR AS THE WEAK SAMPLE BEING ANALYZED AND REPEAT THE ANALYSIS, SEE HAPTOGLOBIN (SECTION VIII,C,3).

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3. Haptoglobin by Horizontal Discontinuous Electrophoresis

ALL PREMADE GEL COMPONENTS AND DEVELOPMENT REAGENTS ARE QUALITY CONTROL CHECKED. DO NOT MAKE YOUR OWN OR USE REAGENTS THAT HAVE NOT BEEN QUALITY CONTROLLED.

Tank Buffer

tris
glycine
HCl
deionized water

Dissolve 90.0 g tris and 432.0 g glycine in 4000 mL deionized water.

Place solution in an appropriate size carboy and add an additional 11 L of deionized water.

Adjust solution to pH 8.3 with HCl.

Dilute 1/2 with deionized water when used.

Resolving Gel

acrylamide
bisacrylamide
deionized water

Dissolve 28.8 g acrylamide and 1.2 g bisacrylamide in 50ml deionized water by gently shaking.

Filter the solution and bring to a final volume of 100ml with deionized water.

Stacking Gel:

tris
HCl
deionized water

Dissolve 60.6 g tris in 750 mL deionized water.

Adjust to pH 6.8 with HCL.

Bring to a final volume of 1000ml with distilled water.

Preparation of Sample Wells:

Sample wells are created by attaching approximately 6x1x0.5mm strips of labeling tape 2.5cm from the top of the

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unframed edge of a 125x260x0.5mm silianized U-frame glass plate.

Another unframed glass plate is placed over the U-frame glass plate. The two are held together using flexiclamps. The gel casting apparatus is placed vertically with the open edge of the U-frame facing up.

Pouring the Gel:

The resolving gel is poured into the top end of the gel casting apparatus to a approximately 1cm below the labeling tape (the gel solution must have an even front).

After 5 minutes, a thin layer of water is poured over the top of the resolving gel surface and the gel is allowed to polymerize.

After polymerization occurs (a visible interface can be seen at the gel, water interface) the water is decanted by tilting the gel casting apparatus.

The stacking gel is poured onto the surface of the resolving gel until it reaches the top of the gel casting apparatus and is allowed to polymerize.

After polymerization occurs, the two plates are separated. The gel should adhere to the unbordered plate.

Standards: Hp 1, Hp 2, and Hp 2-1

Sample preparation: (Operation should be done in a fume hood).

40 uL of stacking gel buffer is added to a 3x3mm bloodstain and extracted using the pipet tip test tube method.

200 uL of methylene chloride is added to the extract, vortexed, and centrifuged at approximately 500 g for 1 minute.

10 uL of the aqueous top layer is placed in a sample well on the gel just prior to electrophoresis.

**Electrophoresis
and Development:**

Dilute tank buffer 1:1 using deionized water.

Maintain a current of 40mA at 4°C until the hemoglobin reaches the gel interface.

At this time, a thin strip of filter paper moistened in tank buffer is placed over the region of the gel encompassing the sample wells and gel interface to prevent burning.

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The current is increased to 90mA for the duration of the run.

Electrophoresis is terminated after 90 minutes.

Interpretation

of Results:

Compare the banding pattern to a known haptoglobin pattern.

For Hp 2-1, four cathodic and one anodic band must be present.

For Hp 2, four cathodic must be present and, for Hp 1, one anodic band must be present.

Hp 2-1M should typed as a 2-1

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D. Related Techniques

1. Coomassie Blue Staining of Gels

Destain

solution: 500 mL water
500 mL methanol
100 mL glacial acetic acid

Stain solution: 0.2 g coomassie blue

Dissolve in 200 mL destain solution; filter if necessary.

Have three jars labeled STAIN, DESTAIN 1, and DESTAIN 2. Put the stain solution into the STAIN jar, and about 200 mL of destain into the other two jars.

Two or three gels can be stained and destained simultaneously; more than that can make destaining difficult or result in scratches to the gel surface.

Method:

1. Place pressed, dried GelBond plate into the STAIN jar for 3-5 minutes.
2. Place into the DESTAIN 1 jar for 5 minutes, then remove.
3. Place into the DESTAIN 2 jar for 5 minutes.
4. Remove and dry at room temperature or in the oven.

As DESTAIN 1 gets too dark, discard into the appropriate waste container and replace with DESTAIN 2. Put the new destain into DESTAIN 2.

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IX. Report Writing

A. General guidelines

A report is the last step in a case. It brings together all of the analytical results and conclusions found in the case notes, in an easily readable style. Overly technical terminology or misleading statements must be avoided.

1. Each homicide report will have specific identifying information at the top. Some of the information required for homicide reports may be either missing or altered depending on the case type and/or whether the case is an NYPD submitted case.
 - a. date report was written
 - b. name of deceased or victim
 - c. FB number
 - d. ME number (if applicable)
 - e. physician that conducted the autopsy (if applicable)
 - f. precinct submitting evidence (if applicable)
 - g. NYPD complaint number (if applicable)

With this information, both the ME and the detective who receive the reports will know where to file them.

2. The bottom of a handwritten report should have your name or initials and the date you wrote the report.
3. If an additional report is generated, this will be noted immediately prior to the SUMMARY section using the following standard statement:

ADDITIONAL REPORT

This is an additional report. For previous examinations, evidence submitted, and disposition, see report dated (insert date or dates of prior reports).

4. In those instances when additional reports are generated for a particular case, the most recent report will be signed by the analyst who works on that portion of the case. If the new data includes genetic testing, the report will include the new genetic results plus the genetic testing results from past reports. This will be noted using the following standard statement:

With the exception of (insert description of new testing results), all genetic marker analysis was previously performed. See report dated (insert date or dates of prior reports).

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5. The body of a report will have three or four sections, depending on the complexity of the case. For examples of reports, see completed case files beginning in 1994 and later.

B. SUMMARY OF RESULTS

The summary should be a brief synopsis of the analytical results; it should **answer the questions** that were posed by the submission of the physical evidence, such as: Is there blood? Could it be the victim's? Are there blood types foreign to the victim? Is there semen? Could the suspect be the semen donor?

Before you write your summary, ask yourself **"WHAT DOES READER OF THE REPORT NEED TO KNOW?"** Then write a short, clear summary answering those questions! The summary should give all of the answers in a simple manner; save all technical explanations for the EXAMINATIONS section.

1. For the majority of cases, the following type of summary is sufficient:
 - a. Human blood was found on the knife, but the amount was insufficient for further enzyme analysis.
 - b. Human blood was found on the shirt "from suspect." Typing results show that it could have come from the victim and could not have come from the suspect (see EXAMINATIONS).
 - c. Amylase, a component of saliva, was found on all three cigarette butts found in the "living room." Analysis showed types foreign to the victim (see EXAMINATIONS).
 - d. No blood was found on the pants or shoes.
2. For cases where there are similar items, but they can be differentiated by color or other descriptions:
 - a. Human blood was found on the blue shirt. No blood was found on the green shirt.
 - b. Human blood was found on the samples from the "doorway" and "hall."
3. Avoid the exclusive use of voucher and item numbers, since that forces the reader to look elsewhere to find out what is being referred to. However, voucher and item numbers may be used in conjunction with the item description if necessary to avoid confusion.

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For example, if the items need to be identified by item and/or voucher numbers:

- a. Human blood was found on the shirt (item 1). No blood was found on the shirt (item 2).
 - b. Human blood was found on the shirt (item 1, voucher E111111). No blood was found on the other shirt (item 1, voucher E111112).
4. If items were removed from an object, location or person, it is useful to put that information in the summary. Since you don't have personal knowledge of this, use quotation marks. Remember that quotation marks means you are copying EXACTLY information written elsewhere.
- a. Human blood was found on the sample taken from the "bedroom door."
 - b. Human blood was found on the shirt taken from "the defendant."
5. If when examining evidence, you collect trace evidence (hairs, fibers, etc.), they should be mentioned in the summary:
- a. Hairs and/or fibers were collected from the shirt.
 - b. Glass fragments were found on the sneakers.
6. All items submitted must be mentioned in the report. If nothing of evidentiary interest was found on an item:
- a. No blood was found on the shirt or pants.
 - b. No semen was found on the vaginal, oral, or rectal swabs from (V).
7. If items were not examined, the item should be mentioned and the reason for not examining the evidence should be given.
- a. The clothes from (V), (V) head hair, and (V) fingernails because, for example, exemplars were not available for comparison purposes.
 - b. The hairs taken from "deceased's hand" were not examined due to lack of serological value.
 - c. The shirt was submitted wet, making it unsuitable for analysis.

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8. After you write a summary, review it carefully. Does it answer all of the questions? Is it clear? Are all submitted items accounted for?

C. EXAMINATIONS

This section is used when there are analytical results, such as enzyme or antigen typing, that need a more detailed presentation than is used in the summary. In most cases, this will take the form of a table comparing typing results with the blood of the victim and/or suspect.

The table consists of four sections: a standard explanatory statement, the table of results, a key to explain any symbols in the table, and an interpretation following the table.

1. A standard explanatory statement regarding conventional genetic markers, i.e., isoenzymes etc, and DNA, i.e., HLA-DQA1, has been prepared and should be used for all reports where these results are to be reported. The standard statement consists of several paragraphs; choose those that apply to the table.

The standard statement can be further modified to reflect the analyses performed in a specific case.

The standard statement follows:

Blood and other physiological fluids and tissues contain polymorphic ("many forms") genetic markers which can differ from person to person. These genetic markers are inherited, that is, passed from generation to generation and can be used to compare biological samples from different sources.

Genetic markers occur because of changes (mutations) that occur in a person's hereditary material, DNA (Deoxyribonucleic Acid). These genetic markers can be identified through the analysis of enzymes, such as esterase D (ESD), phosphoglucomutase (PGM), and erythrocyte acid phosphatase (ACP) and red blood cell antigens such as ABO (blood group) and Lewis, or by direct analysis of the DNA itself, i.e., HLA-DQA1 (previously, HLA-DQ α).

Alternative forms of DNA are called alleles; they are found at the same location of the DNA (locus) on homologous (matching) chromosomes. An individual can have a maximum of two different alleles at a particular locus, one on each homologous chromosome.

With HLA-DQA1, several alleles can be identified. The alleles are combined into six allelic groups which divide the population into 21 types. The HLA-DQA1 alleles are typed using a technique known as the polymerase chain reaction (PCR).

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In a restriction fragment length polymorphism (RFLP) test, several small polymorphic regions of an individual's DNA is examined. Each region contains alleles of multiple sizes which are visualized on an autoradiograph. Samples are compared by comparing the sizes of their alleles. Each allele sizing has a measurement imprecision associated with it. Two alleles are considered the same size if their sizes are within the measurement imprecision of +/- 2.5% of each other. Because of the measurement imprecision, population frequencies are determined from bins which are larger than the imprecision and contain statistically significant numbers of alleles.

To analyze sample which potentially contain a mixture of semen and other body fluids, a technique known as "differential extraction" is used. This technique is designed to physically separate DNA in epithelial cells from the DNA in sperm cells. This results in the sample being divided into two "fractions":

"Epithelial Cell Fraction" - is enriched for DNA from the source of physical evidence; this is typically DNA from the victim.

"Sperm Cell Fraction" - is enriched for DNA from the semen donor(s), if sperm were present.

Genetic marker analysis was done with the following results:

2. Then comes the table itself. The table should list all genetic marker analyses.

a. For a simple case.

ITEM	ABO	ESD	PGM
(S) Smith blood	B	1	2-1
(V) Jones blood	O	2-1	2-1
shirt from "suspect"	*	2-1	-

b. For many different items and/or multiple stains on one item it may be necessary to identify different stains on clothing by their location on the item. This is important information that can be used to help interpret results.

ITEM	ABO	ESD	PGM
(S) Smith blood	B	1	2-1
(V) Jones blood	O	2-1	2-1
shirt from "suspect"			

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stain 1A, right sleeve	*	2-1	2-1
stain 1B, left chest	*	1	2-1
pants from "suspect"			
stain 2A, right leg	*	1	2-1

c. In a case such as above where there are stains of different types, the table could also be arranged so that items of the same type are together:

ITEM	ABO	ESD	PGM
(V) Jones blood	O	2-1	2-1
shirt from "suspect"			
stain 1A, right sleeve	*	2-1	2-1
(S) Smith blood	B	1	2-1
shirt from "suspect"			
stain 1B, left chest	*	1	2-1
pants from "suspect"			
stain 1A, right leg	*	1	2-1

d. For typing with DNA such as HLA-DQ α , the table may appear as:

ITEM	ESD	PGM	HLA-DQ α Alleles
(S) Smith	1	2-1	1.1, 4
(V) Jones	1	2-1	2, 3
Pants from "Deft"			
Stain A, L Knee	1	2-1	2, 3
Control From Stain A	*	*	**
Stain B, L hem	1	*	2, 3
Control From Stain B	*	*	NEG
Stain C, L Knee	*	*	no DNA

e. Cases involving semen might require additional columns:

ITEM	ABO	Lewis
(S) Smith	B	a-b+
(V) Jones	O	a+b-
(V) vaginal swab	B,H	-

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3. The Forensic Biology Department has prepared a standard set of symbols to be used in genetic marker tables. The standard symbols are below; choose those that apply to the table.

* = Typing not attempted
- = Typing attempted with inconclusive results
** = Alleles were detected which do not meet laboratory criteria for allele identification; therefore, these additional alleles were not used to make any comparisons and are not reported.
(1.2) = Indicates that this sample may contain a 1.2 allele; however, due to the number of alleles detected in the sample it is not possible to make a definitive determination.
NEG = No alleles detected
no DNA = No human DNA detected therefore, this sample was neither amplified nor hybridized.

4. Interpretation

After the table, an explanatory section is needed to explain conclusions, especially in cases with lots of stains or semen evidence.

a. For simple cases:

The stain on the sleeve had the same PGM subtype as the (V). Therefore, the stain could have come from the (V). The PGM subtype was different than the PGM subtype of the (S). Therefore, it could not have come from the (S).

b. For cases involving secretor status:

As a non-secretor, ABO antigens would not be expected to be found in the body fluids of (V) Jones (such as vaginal secretions). As an ABO type B secretor, both B and H antigens would be expected to be found in the body fluids of (S) Smith.

Both B and H antigens were found on the vaginal swab from (V); these antigens are foreign to her and could not have come from her. They could have come from either an ABO type B secretor or a combination of ABO type B and O secretors. As a B secretor, (S) Smith could be the donor.

c. For cases involving potential mixtures.

PGM subtypes 2+ and 1+ were found on the vaginal swab; the 2+ activity is foreign to the (V) and could not have come from her. The 2+ activity

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could have come from a PGM subtype 2+ or 2+1+ individual. As a PGM subtype 2+, (S) Smith could be the donor.

d. For limited typing results:

The stain on the chest of the (S)'s shirt was ESD type 2-1. Since both the (V) and (S) are the same ESD type, either one could have been the source of the blood.

e. For inconclusive results:

The stain on the chest of the shirt gave inconclusive typing results. No conclusion can be drawn concerning its source.

D. EVIDENCE RECEIVED:

This section will list all evidence received, whether from the agency or from the autopsy. The items from autopsy are given PM numbers to differentiate them from other evidence.

Make sure that all items submitted, whether or not you examined them, are listed in the EVIDENCE RECEIVED section.

- Using the paperwork and your notes, list the items numbers, voucher numbers, and a description of the item. If items were removed from an object, location or person, it is useful to put that information in the description. Since you don't have personal knowledge of this, use quotation marks. Remember that quotation marks means you are copying EXACTLY information written elsewhere.

ITEM	VOUCHER	DESCRIPTION
1	E111111	sample from "bedroom door"
2	"	knife
1	E222222	shirt from "suspect"
2	"	pants from "suspect"
3A, 3B	"	socks
PM1	-	(V) blood sample
PM2-4	-	(V) vaginal and rectal swabs
PM5	-	hairs from (V) hands

- If there are several items submitted as one, give them all individual identifiers, both in your notes and in the report:

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ITEM	VOUCHER	DESCRIPTION
1A-C	E111111	three cigarette butts

On the voucher, the cigarette butts were identified as "item 1". Upon opening the package, there were three; they were then given the identifiers 1A-C.

3. If there are items submitted that weren't included on the voucher, they still need to be listed in the evidence section:

ITEM	VOUCHER	DESCRIPTION
1A-B	E111111	shoes
2A-B	"	socks (not listed on voucher)

4. If upon opening items it was discovered that the description on the voucher was wrong (for example, a tank top was submitted, but the voucher says "T-shirt"), put the correct description in the EVIDENCE RECEIVED section.

E. DISPOSITION:

This section tells what has happened to the vouchered evidence, post-mortem evidence, and samples removed from the evidence.

1. Always keep a dried stain of (V) blood. If there are hair standards, fingernails, etc., retain them as well.

The head hair from (V) and a dried stain prepared from (V) blood will be retained in the laboratory.

2. If semen is found on vaginal, oral, or rectal swabs, they are retained. If they are negative, they are discarded:

The vaginal, oral, and rectal swabs were discarded after analysis.

3. Any remaining stains from clothing, stains taken off of knives, etc., will be retained for possible further analysis.

Stains and unstained controls from the pants and shirt will be retained in the laboratory.

The blood swabbed off the knife will be retained in the laboratory.

4. If an entire item is retained:

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Item 1, sample from "bedroom door", will be retained in the laboratory.

5. If numerous items are being kept, it is easier to write it in this way:

The following items are being retained in the laboratory:

dried stain prepared from (V) blood
(V) head and pubic hairs
stains and unstained controls from shirt and pants
item 1, sample from "bedroom door"

6. If an item has left the lab, NOT through our evidence unit:

The gun was returned to Det. Smith on 5-7-90.

The vaginal swabs from (V) have been submitted to the FBI for further analysis.

7. If a sample was consumed during the analysis, that must be mentioned in the disposition.
8. For DNA cases, all DNA extracts are discarded after analysis. The only exception is if a stain was consumed during analysis.
9. For items that have been transferred to the Evidence Unit:

The remainder of the evidence has been released to the Evidence Unit.

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