Initials: RCJ Date: 5/5/89

Forensic Biochemistry

Methods Manual

Version 4.0

Initials: RCJ **Date:** 5/5/25

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General guidelines

1. 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.

2. In general, screening tests and/or confirmatory tests are used to identify physiological fluids such as blood, semen, urine and saliva prior to further analysis.

3. While this laboratory does not analyze for biochemical or immunological genetic markers routinely, those methods remain in the method manual for those instances when their analysis is necessary.

4. Required analysis can range from determining only 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.

5. Analysis typically follows a logical progression. Items are first examined, most often looking for human blood and staining patterns. If blood is present, the samples are prepared as if DNA testing is required.

6. 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 or when a scene is suspected but the body was found at another location.

Initials: RG **Date:** 2/13/03

Processing of postmortem specimens

This task should be performed as soon as a batch of samples arrives in the laboratory. The task is separate from the review of the autopsy sheets and will generally be performed by the criminalists assigned to the rape kit rotation.

1. Retrieve the postmortem specimens. Specimens from all five boroughs are delivered to the laboratory in sealed red plastic containers.

2. Inventory the contents of the red plastic containers by cutting the plastic ties, removing the contents, and comparing the contents with the enclosed transport sheet. Inventory each container separately. Check each item off on the transport sheet, check for completeness, and make a note of any discrepancies. Any discrepancies must be reported to the evidence exam supervisor.

3. For discrepancies, the evidence exam supervisor must send an email to the pathologist. These discrepancies may include:

   unlabeled specimens
   specimen collected but not listed on transport sheet
   broken blood vials
   blood vial with a rubber stopper off
   container not sealed with black ties
   missing paperwork

4. Place the transport sheets into the appropriate files by borough at the post mortem desk.

5. Place a blank transport sheet and two plastic ties into each container after they have been emptied. Place the red containers alongside the hood.

6. For each borough fill out a PM sample batch chain of custody sheet and, if necessary, a PM sexual assault evidence batch chain of custody sheet. Record the ME numbers, indicate where blood was received, and list any other non-sexual assault items in the additional items column. Please use the abbreviations.

   Use the separate PM sexual assault evidence sheet for all sexual assault evidence such as kits, dried secretions, orifice swabs or underwear. All of these cases will receive an FB number.

   Use the comments section to document damaged packaging or other unusual observations. IMPORTANT: If a sample was not received in a purple top tube, list the type of blood tube that had been used. .

7. Place all non blood items in refrigerated storage. Proceed to prepare staincards for the PM blood samples by following the steps below.

8. Non blood items in FB cases will be signed over later and will be stored in permanent retained

storage. Additional items in non FB cases will be discarded on a regular basis, such as every six months.

9. Postmortem bloods that are not transferred to an FB file will be discarded every three years.

Bloodstain preparation from whole blood

Staincards are prepared from all vouchered blood samples and all post-mortem blood samples.

Initial steps for PM blood samples:

1. Date and initial the blood processing section of the PM sample batch chain of custody sheet on the day the bloods are being processed.

2. For PM blood samples label the staincard with:

   ME case number
   date
   initials of person preparing the stain

   Wear latex gloves when handling these cards.

3. The witness must initial the PM blood batch processing worksheet as well.

Initial steps for vouchered blood samples:

4. Find the case files and obtain the blood vials; sign the chain of custody forms. Fill out a Evidence packaging blood processing worksheet.

5. Label the staincard with:

   FB case number
   subject's name
   date
   initials of person preparing the stain

   Wear latex gloves when handling these cards.

Continued procedure:

6. The setup of the staincards must be witnessed by another laboratory staff member. The stain cards and tubes of blood should be setup in the hood so the witness may examine this setup. The witness will then date and initial each staincard as well the worksheets..

7. Prepare stains one at a time. Staining of the cards and the opening of liquid blood samples MUST be performed under a biological safety cabinet with the exhaust fan operating. A new KimWipe™ be used to open each vial stopper. Make sure a blood tube is closed before preparing the next stain.

8. Fold back the paper "flap" and make four stains on the card, placing the blood in the outlined areas. Using a transfer pipet, make 4 stains for each sample, filling in the 4 circles on each staincard.

9. Allow the staincards to dry overnight.

10. Package the air-dried stains into a 4x6" KAPAK™ bag. Seal the bag with evidence tape, date and initial the seal. Continue the chain of custody to reflect their final location, which should be retained storage.

11. **CLEAN THE BIOLOGICAL SAFETY CABINET (refer to QC125 in Appendix B.2 of the Quality Manual).**

12. Place all case files that contain **any** sexual assault evidence or NYPD evidence on the evidence sign-in desk so that they may be processed. Place all remaining case folders in the file rack designated PM files.

13. Disposal of blood and blood vials:

    The remainder of the PM blood in each vial is emptied into a beaker half full of 10% bleach. Empty blood vials are then discarded into the plastic BIOHAZARD "sharps" container.

    For vouchered blood, empty any unused blood into the beaker. Rinse the empty vial with 10% bleach. The empty vial is placed back into its original package and returned to the Evidence Unit.

14. Staincards that have an FB case number should be stored in the coldroom at 520 in the proper box.

15. Staincards without an FB case number should be stored by borough and numerical order. These staincards may be discarded after three years.

16. If a staincard that has not been previously labeled with an FB case number is to be tested, this staincard should be retrieved, labeled with the appropriate FB case number and tested. This staincard should then be stored in the coldroom at 520 in the proper box.

Processing of autopsy sheets

1. Retrieve the autopsy case worksheets and daily case census sheets for all five boroughs. This paperwork is delivered to the 6th floor of 520.

2. Arrange the autopsy case worksheets and daily case census sheets in numerical order. Screen all the paperwork for potential Forensic Biology cases. The following types of cases should be signed into the laboratory and given an FB case number:

   homicides
   any case in which sexual assault evidence has been collected
   any case in which a Forensic Biology test is requested such as hemoglobin
   any unknown bodies with bones requiring DNA identification
   any case in which evidence from the NYPD or DA's office has been submitted

3. All other remaining cases are not assigned an FB case number. These would include:

   motor vehicle accidents (MVA's) in which there is an investigation (hit and run)
   any case which involves child abuse or suspected child abuse
   possible homicides and cases that are pending
   unidentified decedents with blood samples

   If case circumstances are unclear, immediately consult with a supervisor.

4. Compare each autopsy case worksheet with the transport sheet and the specimens received to ensure that all of the specimens designated for Forensic Biology have been received.

5. **For cases that will NOT be assigned an FB case number**: all autopsy case worksheets should be attached to the relevant PM sample batch chain of custody sheet. The batch sheet and the daily case census sheets must be placed into their designated folders (by borough) for retention. Place all paperwork into the appropriate folders in order of date received.

6. **For cases that will be assigned an FB case number**: separate the autopsy worksheets from the others. Check the Paradox database to determine if evidence from the NYPD or DA's office has already been submitted on any case. If so, any specimens will be signed into the preexisting case numbers. Retrieve any existing case folders.

7. Assign the remaining specimens new FB case numbers and enter the appropriate information into the FB log book.

   Create a new case folder by obtaining a manila folder with a new label (FB case number), a chain of custody form, and an evidence packaging worksheet.

8. Fill in ALL the appropriate information on each form. Numerically list each specimen in the evidence received section. Give each specimen a unique PM number (*i.e.*, blood is usually

designated "PM 1" and sexual assault kits are usually designated "PM 2"). In the chain of custody section enter the date the specimens were received from Forensic Biology storage.

9. On the corresponding PM sample or PM sexual assault evidence batch chain of custody sheet indicate which ME number was signed over to an FB file. Make a copy of the PM batch chain of custody sheets and place it in the casefile.

10. Autopsy case worksheets and evidence packaging worksheets are affixed to the right side of the folder. Chain of custody forms, scheduled analysis and case contact sheets are affixed to the left side of the folder.

11. Remove the bloodstaincards from Forensic Biology storage and label them with the Forensic Biology number and the victim's name. Reseal the staincard pouch and place with the other FB bloods. Label the additional items with the corresponding FB case number and PM number.

12. Sexual assault kits and sexual assault evidence that is not part of a kit must be placed in the designated storage area in order that they may be processed. All other specimens must be placed in retained storage. Continue the chain of custody for these items to reflect their final location.

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Kastle-Meyer (KM) presumptive test for blood

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 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 control and negative control at the same time.

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Leucomalachite green (LMG) presumptive test for blood

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 control and negative control at the same time.

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Acid phosphatase presumptive test for semen

Standards: semen and water or saline

Prepare the reagents fresh each time.

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 control and negative control at the same time.

One-step method (commercial AP spot test reagent):

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 AP reagent and observe any color changes. A purple color developing within 60 seconds is a positive.

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

Initials: RG **Date:** 7/30/01

Amylase diffusion presumptive test for saliva:

1. Prepare starch-containing agarose gel by adding the ingredients listed below:

   100 ml batch size (enough for 2 plates)
   1.0 g Sigma Type I agarose
   0.1 g potato starch
   100 ml amylase gel buffer

   To dissolve, mix and boil this solution. Allow to cool and pour 40 ml each into 10 x 10 cm disposable petri dishes. Scale up batch size when necessary.

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

3. Prepare α-amylase standards containing 0.02, and 0.002 units each per 10 uL of deionized water (dH_2O) from purchased amylase.

   Do this by first preparing 1 mL of the 20 unit/10 uL standard (see example calculation below). Continue by performing four serial ten-fold dilutions; the last two dilutions will be the 0.02 and 0.002 unit standards. This is easily accomplished by first adding 900 uL of dH_2O to each of four microcentrifuge tubes. Then transfer 100 uL of your 20 unit standard into the first tube containing 900 uL of dH_2O. The concentration of amylase in this tube is a ten-fold of 20 units/10 uL or 2 units/10 uL. Continue making three more ten-fold serial dilutions in the same manner.

   When doing the serial dilutions, make sure to mix each tube before the subsequent transfer. Use a fresh (unplugged) pipette tip for each transfer.

   Sample calculation:

   Given a specific activity of 870 units amylase/mg total protein (from vendor) with a total protein concentration of 30 mg/mL, then:

   $$\frac{870 \text{ units amylase}}{\text{mg total protein}} \times \frac{30 \text{ mg total protein}}{\text{mL of solution}} \times \frac{1 \text{ mL}}{1000 \text{ uL}} = 26.1 \text{ units amylase/uL}$$

   Use this value in the equation C1 x V1 = C2 x V2 where C1 and C2 are concentrations of solutions 1 and 2, while V1 and V2 are volumes of solutions 1 and 2. In this case, solution 1 is the vendor amylase stock solution while solution 2 is the 20 units standard in preparation:

   (26.1 units amymase/uL)(x uL) = (20 units/10uL)(1000 uL)

   solving for x = 77 uL

Therefore, to make 1 mL (1000 uL) of the 20 unit standard, mix 77 uL of vendor amylase standard into 923 uL of dH_2O. By design, the concentration of this solution is 20 units/10 uL.

4. Punch wells in the gel using the suction tube apparatus, leaving at least 1.5 cm between wells. Make sure that the holes that you create are completely clean of agar debris and residual liquid. This can be ensured by punching each hole twice in succession. Following this protocol taken together with an accurate dispension of agarose will guarantee an adequate amount of space for the loading of 10 uL each of standard, control, or sample into each well.

5. Fill wells (10 uL) with standards, blank, samples, and substrate controls (if applicable).

6. Incubate 5-8 hours at 37°C or 12-16 hours at room temperature; keep the plate in a humid chamber to avoid drying.

7. Pour a 0.01 N (100-fold dilution of a 1N stock) iodine solution onto the gel; clear areas indicate regions of amylase activity. Make sure not to overstain the plate. Do this by monitoring the plate as it is staining; pour off the iodine solution when a sufficient amount of staining has occurred so that all the standards are clearly visible.

8. Measure the diameter of diffusion for each sample and record on the worksheet. Photograph the results; ensure there is a scale in the photograph. Also, fill out the amylase spread sheet to determine whether the plate passes QC. The specifications are as follows: 5-8 and 6-10 mm of diffusion are allowed for the 0.02 and 0.002 unit standards, respectively. In addition, the amount of diffusion of the 0.02 unit standard must be greater than that of the 0.002 unit standard.

The interpretation of amylase results depends on the source of the sample:

Body cavity swabs (e.g., vaginal and anal) are positive if the diameter is equal to or greater than the diameter of the 0.02 U standard.

Samples not from a body cavity (e.g., penile swabs, cigarette butts, cups, etc.) are positive if the diameter is equal to or greater than the 0.002 U standard.

The location from which a "dried secretion" swab is taken affect the interpretation. Swabs taken essentially from a body cavity or similar place (e.g., introitus, etc.) are interpreted as if the sample is from a body cavity. Other locations (e.g., breast, thigh, penis, etc.) may need to be interpreted differently.

Entire items (blood spatter patterns, etc.) can be tested for amylase. Prepare a large plate on a bordered glass plate (scale up reagents) and allow to solidify; bring item (or area of item) into contact with the gel for 5 minutes. Follow steps 6-8 above to visualize any amylase pattern.

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Urea diffusion presumptive test for urine

Controls: urine, urea, and saline or water

Sample prep: 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.

Standard prep: 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 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
transmitted and oblique lighting

blank plates: *f* stop - 22, shutter speed - 1/30
oblique lighting only

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Takayama hemoglobin test

Controls: blood and saline or water

Perform the test in the organic fume hood because of the presence of pyridine.

For crusts or scrapings:

1. Place a blood crust or flake on a glass slide.
2. Add a cover slip. Allow a drop of reagent to flow under the cover slip and come into contact with the blood.
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 control and negative control 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 cover slip. Allow a drop of reagent to flow under the cover slip and come into contact with the blood.
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.

Initials: RCJ **Date:** 1/15/19

Ouchterlony radial diffusion - species determination

Standards: Positive control, negative control, substrate controls (if applicable)

1. Extract approximately 5 x 5 mm stain or a portion of a swab in 100 uL water for 30 minutes at room temperature using the pipet tip and test tube method; the extract may need to be diluted to a pale straw color.

   Alternatively, a small, wet thread or small piece of swab can be inserted directly into the well.

2. Obtain a pre-made test tube of gel; heat in a boiling water bath until it is liquified.

3. Pour onto the hydrophilic side of a 2.5 x 3.5 inch piece of GelBond and let solidify.

4. Punch an array of wells consisting of a central well surrounded by four wells, using the template on the worksheet.

5. Apply anti-sera to the central well.

6. Apply the positive control to one of the surrounding wells.

7. Apply the sample(s) so that a stain extract is always next to a positive control.

8. Apply negative and substrate controls to the remaining wells; only one negative control is needed per gel.

9. Place the plate in a moisture chamber and incubate at 37°C overnight.

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. Stain: see "Coomassie Blue Staining", Section VIII, D,1.

12. 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.

    The presence of a spur may mean the presence of a closely related species. Further analysis, including testing dilutions of the sample extract or testing against other anti-sera, may be necessary.

Initials: RCJ **Date:** 5/15/19

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

1. Prepare samples and gels as described in steps 1-4 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 9-12 above.

5. Any positive result needs to be confirmed by Ouchterlony or crossover electrophoresis using all appropriate controls and standards.

Initials: RG **Date:** 5/5/99

Crossover electrophoresis - species determination

Standards: Positive control, negative control, substrate controls (if applicable)

1. Extract approximately 5 x 5 mm stain or a portion of a swab in 100 uL water for 30 minutes at room temperature using the pipet tip and test tube method; the extract may need to be diluted to a pale straw color.

   Alternatively, a small, wet thread or small piece of swab can be inserted directly into the well.

2. Obtain a pre-made test tube of gel; heat in a boiling water bath until it is liquified.

3. Pour onto the hydrophilic side of a 2.5 x 3.5 inch piece of GelBond and let solidify.

4. Punch small wells (about 1-2 mm) in rows using the template on the worksheet. Punch enough wells for controls, samples, and substrate controls.

5. Apply anti-sera in the left-hand wells; the antibody will travel towards the cathode.

6. Apply samples in the right-hand wells; the antigen will travel towards the anode.

7. Place plate in electrophoresis tank with the anti-sera closest to the anode.

8. Electrophoresis is at 120V for 20 minutes.

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. A sharp precipitin band (not a cloud) is a positive.

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Christmas tree stain for spermatozoa

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 and picric indigo carmine

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

Initials: [illegible] **Date:** 6/15/01

P30 enzyme linked immunosorbent assay (ELISA)

Phosphate buffered saline (PBS):

To prepare 1 liter, dissolve 5 tablets in 1 liter of deionized water. This can be stored at 4°C for up to 2 weeks. Put initials of the preparer on label along with the date of make (DOM) and the date of discard (DOD).

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

Dilute 20 ml of casein stock solution to 1 liter with PBS.

One plate uses approximately 800 mL.

Standards: P30 antigen and phosphate buffered saline

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

Standards prep: Prepare a 1 ug/ml solution of P30 antigen in PBS-BSA. Divide into 60-100 uL aliquots and freeze at -20°C (-80°C for long term storage).

Plates Immulon II microELISA plates

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

Store at 4°C.

Rabbit polyclonal anti-human PSA

Store at 4°C.

Goat anti-rabbit IgG alkaline phosphatase conjugate

Store at 4°C.

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

When needed, reconstitute with 1 mL of deionized water. Store at 4°C.

Initials: RH **Date:** 6/15/01

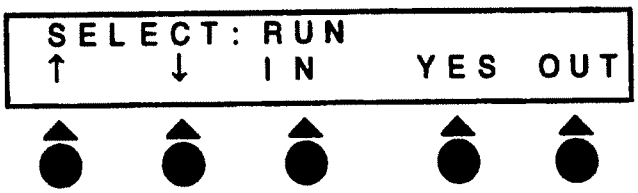
Instructions for Bio-Rad 1575 washers (#1 & #2)

The Bio-Rad 1575 plate washer has a cover that can be swivelled to an opened or closed position. This cover should be in the closed position while the plate washer is in use to safeguard against sample contamination, and avoid possible injury.

Rinsing and priming

1. The on/off switch is located on the right-hand side of the lower back of the washer. Turn plate washer on.

2. After initialization (concurrent with a quick flash of a Bio-Rad version #), the **main menu** appears and looks like this:



The circles with the triangles above represent the soft keys that you will be pressing to access various functions on the machine. In this menu, soft keys corresponding to **"In"** and **"Out"**, when pressed instruct the plate washer to move the plate holder in and out of the washer, respectively. Soon after turning on the plate washer, the plate holder automatically sets itself in the out position.

3. Fill the **Rinse** bottle with deionized water, close the lid, and attach the tube with the blue colored line to the top of the container. Also, make sure that the stopper connected to the yellow and red colored lines is snugly connected to the **Waste** bottle (if not, the plate washer will not aspirate, which can result in flooding of the plate holder).

4. Prime the washer with deionized water (from the **Rinse** bottle). Do this by pressing the very first soft key corresponding to the upward arrow in the **main menu**. Now you will see the **Prime/Rinse** menu. Press the soft key corresponding to **"YES"** and the washer will proceed to prime itself. This step is important to ensure proper vacuum pressure and for the washer to dispense correct volumes. Always make sure to re-prime the washer whenever changing the blue colored tube from the **Wash** bottle to the **Rinse** bottle and vice versa.

5. Fill the **Wash** bottle with PBS-casein solution. Remove the blue-striped tube from the **Rinse** bottle and attach it to the **Wash** bottle. Repeat the priming procedure 1 time using the PBS-casein solution from the **Wash** bottle. You are now ready to run the plate washer.

Initials: RS **Date:** 6/15/01

PLATE PREPARATION:

COATING THE PLATES

1. Prepare an appropriate dilution of mouse monoclonal anti-human PSA by adding the antiserum to PBS as determined by the QC of the current lot of antiserum. Each plate requires about 5 ml of diluted antiserum. **Always make dilutions in glass, not in plastic.**

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. For 4 plates add 2.5 uL MOPC to 20 mL PBS.

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	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA
D	PBS	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA
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	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA
H	PBS	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA	αPSA

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

4. Cover plates with Parafilm and label "Coating" with initials and date. 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 for up to 2 weeks.

Initials: RE **Date:** 6/15/01

Running plate washer programs

1. To start the blocking procedure, insert a coated plate into the plate washer lengthwise, so that plate well #A1 is located on the upper right portion of the plate

2. Proceed with the first portion of the blocking procedure in which the contents of the wells are aspirated, washed 2 times, and then filled with PBS-casein. The program that does this is called **BLOCK1**.

3. To access the **BLOCK1** program, press the soft key that corresponds to **"YES"** while in the main menu. You will now be in the **run menu**. While in the run menu, scroll to the **BLOCK1** program by pressing the soft keys that correspond to the upward and downward arrows. Before proceeding, you should see **"RUN: BLOCK1"** on the first line of the display. Press the **"YES"** key. You will then see **"LAST STRIP 12"** on the first line of the display. Press the **"YES"** key again. Usually you will then get one more prompt (**"CONNECT THE WASH R9"**) on the first line of the display. Press the **"YES"** key once again. The program will then run on its own, leaving the wells filled at the end of the program.

4. Incubate the plate for 15-20 min. as described in the P30 protocol. This can be done by leaving the plate in the washer OR removing the plate from the washer if you need to block more plates. To remove plate, first return to the **main menu** by pressing the **"ESC"** key. While in the main menu press the **"OUT"** key and then remove your plate.

5. If you have removed your plate for the incubation step, place plate back into the plate washer by using the **"IN"** and **"OUT"** keys from the main menu. The last step of the blocking procedure requires aspiration of the wells. This is achieved by the **BLOCK2** program. Access the **BLOCK2** program from the **run menu** as described in step 3 for the **BLOCK1** program. Before proceeding, you should see **"RUN: BLOCK2"** on the first line of the display. Run the **BLOCK2** program by pressing the **"YES"** key.

6. The washing program required for the P30 run for plate washers #1 and #2 has been named **"WASH"**. The **WASH** program can be accessed from the **run menu** as described for the **BLOCK1** program above. Before running the **WASH** program, make sure that **"RUN: WASH"** appears on the first line of the display. Press the **"YES"** key to run the **WASH** program.

7. When finished using the plate washer, remove plate from the washer. Replace it with a dummy wash plate that is provided at each plate washing station. Remove the blue colored tubing from the **WASH** bottle and attach it to the **RINSE** bottle. Prime the machine with deionized water; access the priming command from the **main menu** as described above. Also, run the **WASH** program so that the lines are thoroughly flushed with deionized water.

8. Turn off plate washer. Discard liquids from the **WASH** and **WASTE** bottles and rinse thoroughly with water. Replace covers onto these bottles and leave them near the plate washer.

Initials: BCJ **Date:** 6/15/01

9. When the plate washer sits idle for about 10 min., it will display a **"PLEASE RINSE"** message. When you are ready to use the washer again, press the **"YES"** key to return to the **main menu.**

Initials: RCJ **Date:** 8/15/01

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 480 uL wash buffer (PBS-casein).

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

2. **Standards** Prepare a 2ng/ml standard by first preparing a 10 ng/ml solution followed by a 5-fold dilution of this solution as follows:

   a. 10 ng/mL: 50 uL P30 (1 ug/mL) + 5 mL PBS-casein

   b. 2 ng/mL: 1000 uL 10 ng/mL P30 standard + 4.0 ml of 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	S2	S4	S6	S8	S10	S12	S14	S16	S18
B	PBS	W	2ng	S2	S4	S6	S8	S10	S12	S14	S16	S18
C	PBS	W	2ng	S2	S4	S6	S8	S10	S12	S14	S16	S18
D	PBS	W	2ng	S2	S4	S6	S8	S10	S12	S14	S16	S18
E	PBS	W	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19
F	PBS	W	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19
G	PBS	W	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19
H	PBS	W	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19

PBS = phosphate buffered saline
W = PBS-casein
S = samples

Initials: RJ **Date:** 6/6/07

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 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. For 2 plates add 20 uL antiserum to 20 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 1/2500 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate by adding 5 uL antiserum to 12.5 mL PBS. For 2 plates add 10 uL antiserum to 25 mL PBS.

9. Apply 100 uL of the 1/2500 dilution of goat 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, aliquot 20 mL of alkaline substrate buffer (ASB) and allow to come to room temperature, about 30 minutes. For each two plates, dissolve one 20 mg p-nitrophenyl phosphate tablet (PNPP) in the 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 PNPP 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.

Initials: JH/S **Date:** 2/13/02

READING THE PLATE

BioRad 3550 Plate Reader

1. Turn on the plate reader and the computer/monitor. Allow the plate reader to warm up (≈15 minutes). Enter Windows 3.1 by typing ***WIN*** at the prompt and then press **ENTER**.

2. During plate reader warm-up, enter the **BIO-RAD Data Collector** program (in the **Plate Reader** program group or window) by highlighting it's iconic symbol and pressing **ENTER** (or by rapidly clicking twice on the icon with the arrow).

3. Place a floppy diskette in drive A. Enter a file name (5 characters maximum) for the data to be saved under (*e.g.*, ***RUN01, 0000A, 01018,*** *etc.*). This name is arbitrary and will be changed later. It is only important that you remember the name you have given it.

4. Check the reader model to make sure it is correct (should read **Model 3550**).

5. Check the data file type (should read **Microplate Mgr (*.LOT)**).

6. Change the data directory by pressing **F2**. Select a new path by pressing **F3**. Enter the letter for disk drive A: by typing *A* and then pressing **ENTER**. Press **ENTER** when asked for the directory. Press **ENTER** again to confirm that the path is correct.

7. Turn the printer on and ensure it is in the offline mode. Read the plate, by pressing the "start' button on the plate reader, when the reader is ready. When the data has been displayed print onto plane paper by pressing the **FUNCTION** key and then the print button on the reader. Transfer data to disk by pressing the **FUNCTION** key and then the **+/-** key on the reader **(make sure there is a floppy diskette in the drive!)**. Check to make sure the data is transferring to the diskette by observing that the green A: drive light is on.

8. More than one plate may be read. After the data has been transferred for the first plate, simply insert the next plate and read this plate. When the data has been displayed, it can be transferred to diskette by pressing the **FUNCTION** key and then the **+/-** key on the reader **(make sure the floppy diskette is still in the drive!)**. Check to make sure the data is transferring to diskette by observing that the green drive light is on. The computer will automatically assign the next plate with a sequential filename.

9. After transferring the data, the diskette may be removed and the computer shut down.

Initials: HH **Date:** 2/13/02

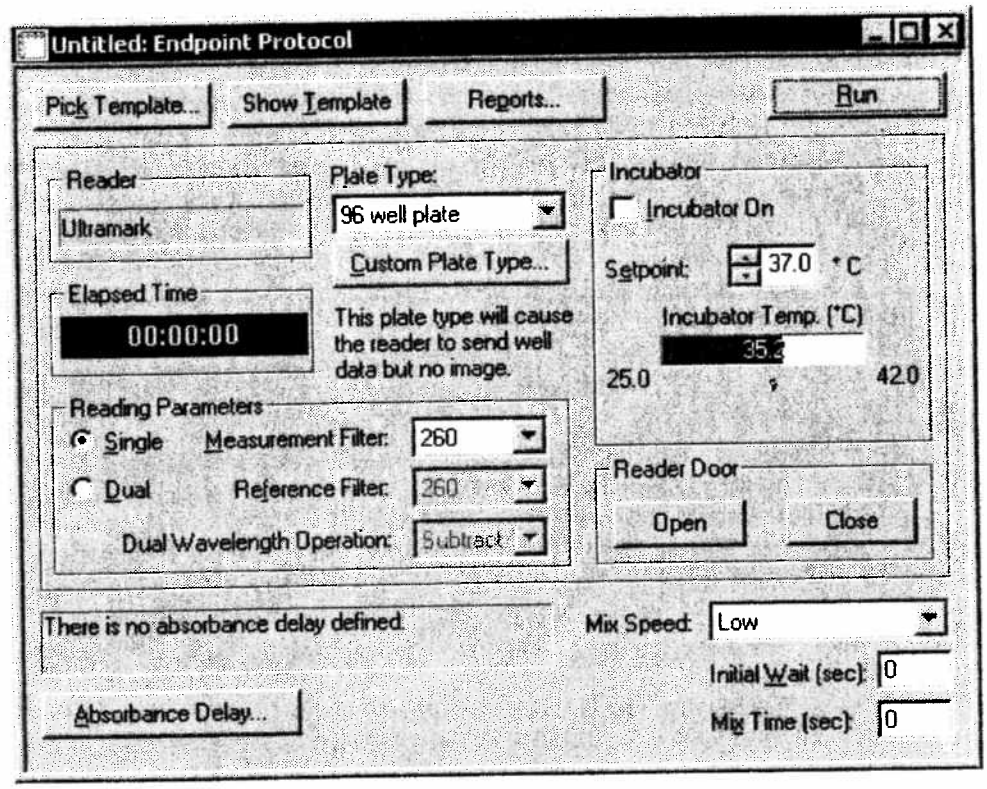
BioRad Benchmark Plate Reader

1. Turn on the computer by pushing the power button on the lower left of the computer screen. Allow Windows NT software to boot up. Turn on the Benchmark plate reader and let it warm up. This will take approximately 3 minutes following an initial self diagnosis that requires about 1 minute. When the plate reader has warmed up, its screen should read as follows:

PLATE READING:
M=2:405, R=4:655
Mixing = ON (05s)
Incu.= OFF

2. From this point on, you will be controlling the plate reader from the computer it is attached to. Double click on the **Microplate Manager 5.1** icon. From **File** in the main menu select **Open.** The Microplate Manager File Window will open. For the field labeled **Files of Type:**, click on the arrow and choose **Endpoint Proto [.epr]**. The Endpoint protocol **Forensic-p30.epr**.for P30 will appear. Highlight it and select **Open**.

3. The protocol window shown below will appear:



Initials: HL **Date:** 2/13/02

The default settings should read as follows:

Reader:	**Benchmark**
Reading Parameters:	**Dual**
Measurement Filter:	**405**
Reference Filter:	**655**
Dual Wavelength Operation:	**Subtract**
Incubator On:	**deselected**
Initial Wait:	**0 sec**
Mix Time:	**5 sec**

4. Place your microtiter plate into the plate reader.

5. Click **Run** located at the upper right corner of the protocol window. The **Fill Labels** window appears. Type your initials, date, and plate name into the appropriate fields and click **OK**. Name plates by date as follows: ***021202a*** (where ***a*** designates the first plate, if more than one) The plate analysis takes about 20 seconds

6. When the analysis is finished the **Raw Data** window appears. Save the Raw Data onto a floppy disk by doing the following: Go to **File** from the main menu and select **Export**. Change the (i) **Save In:** box to read **3 ½ Floppy (A:\)**, (ii) the **Save As Type:** box to **Tab delimited (.txt)** and (iii) **File Name** to your plate name. Click **Save**.

7. When you are finished with the Microplate Manager software, go to **File** in the main menu and select **Exit**. Click **NO** when prompted to save changes to your plate.

8. When done, shut down the computer and turn off the plate reader.

Initials: QC **Date:** 6/15/01

CALCULATION OF P30 ELISA Results

Manual calculations

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. Subtract the mean value for column 1 from each value in all remaining columns.

2. 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 value = 2(AVE) + 3(SD)

3. Subtract the PT value from the values in columns 3-12.

4. For all standards and samples, calculate the average of the duplicate samples for both the MOPC and monoclonal PSA coated wells.

5. 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

Value	Value
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

Initials: RC **Date:** 6/15/01

plate threshold = 2(0.089) + 3(0.020) = 0.238
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 0.028
F3 0.034 average of E3 and F3 = 0.031
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

Initials: HB **Date:** 2/13/02

Spreadsheet calculations

1. Open the *Quattro Pro* program by going into the **Start** menu window by clicking once on the button in the lower left of the screen. Move the arrow to the **Corel WordPerfect Suite 8** menu. Click once on the **Corel Quattro Pro 8** menu.

2. Pull down the **File** menu and click once on **Open...**. Click once on the box directly to the right of **Look in:** and then click once on **Vol1 on 'OCME1' (G:)**. Open the following file by rapidly clicking twice on each subdirectory as it appears (*i.e.*, **Users**, **Fbiology**, **P30elisa**, *etc.*).

   G:\USERS\FBIOLOGY\P30ELISA\WRKSHEET\P30OLD for BioRad 3550 Plate reader.

   G:\USERS\FBIOLOGY\P30ELISA\WRKSHEET\P30NEW for BioRad Benchmark Plate reader

   IMPORTANT: It is critical to use the proper spreadsheet template for the plate readers designated above. Using the wrong template may yield incorrect results!

3. A message will appear that states "Open file for read only?". Press **ENTER**. The P30 spreadsheet will appear (this a **READ-ONLY** file; it cannot be altered). A series of "tabs" lettered A through R will be at the bottom of the page. Using the mouse, click once on the **B** tab.

4. The cursor should be at the **A1** box position. If not, position the cursor in the **A1** block using the mouse (simply click once on the **A1** box; if it is not visible, use the scroll bars/buttons on the right and bottom sides of the screen to bring that box into view).

5. Pull down the **Tools** menu and move the arrow to the **DataTools** menu. Click once on **Combine Files...**. The **Combine Files** window will pop up. Click once on the small button with the little yellow folder directly to the right of the **File name** window. The **Open File** window will appear. Click once on the box directly to the right of **Look in:** and then click once on **3½ Floppy (A:)**. (NOTE: This may be out of view. Simply scroll up using the small scroll bar in the right of the window.)

6. Also while in the **Open File** window click once on the arrow directly to the right of **File Type**. Scroll to the end of the list and click once on **All Files (*.*).**

7. You should now be able to see icons representing the raw data files that were generated by the plate reader. Rapidly click twice on the file that needs to be examined (or click once to highlight the file and press **ENTER**). This window will now close and another will still be open. Press **OK**. The saved data from the plate reader should appear on the page in the upper left corner.

Initials: HL **Date:** 2/13/02

8. Below the data are a series of bold headings for the following: name, date, plate (number), QC # (for QC purposes only), and the sample series (3E-H, 4A-D, 5E-H, and so on). Using the mouse, click on the box to the immediate right of each heading and enter with the keyboard the appropriate information.

NOTE:

a) The date **MUST** be entered in the following format: ***June 21, 2002***
b) **Plate:** refers to the plate number (*i.e.*, 1, 2, *etc.*)

9. Enter the sample series in the following manner: ***FB02-0000 vaginal***

10. When all the information has been entered, click once on the **A** tab at the bottom of the page with the mouse. All the information should now be in the pre-made template. Print the page by pulling down the **File** menu and clicking once on **Print...**. When the **PRINT** window appears, click once on the **Print** button (or press **ENTER**).

11. The file should now be saved. Pull down the **File** menu once more. Click once on **Save As...**.

12. Click on the **File name:** window and enter the correct filename. The filename should be the date entered as such: ***062102a*** (where ***a*** designates the **first** plate, if more than one). Also check that the file is being saved in the proper location; **Wrksheet** should be present in the **Save in** window. If not, click on the arrow directly to the right of the **Save in** window. Save file in the **Wrksheet** folder via this path:

G:\USERS\FBIOLOGY\P30ELISA\DATA\WRKSHEET

13. To do calculations for a second plate, close the file by pulling down the **File** menu and clicking once on **Close**. Repeat steps 2 through 12.

14 When you are finished, exit the program by pulling down the **File** menu and click once on **Exit**.

Initials: RCJ **Date:** 7/30/01

Interpretation of results

The interpretation of P30 results depends on the source of the sample:

1. Body cavity swabs (e.g., oral, vaginal and anal) are positive if the result is greater than the 2 ng standard.

2. Samples not from a body cavity (e.g., panties, etc.) are positive if the result is greater than 0.05 absorbance units.

3. The location from which a "dried secretion" swab is taken affect the interpretation. Swabs taken essentially from a body cavity or similar place (e.g., introitus, etc.) are interpreted as if the sample is from a body cavity. Other locations (e.g., breast, thigh, etc.) may need to be interpreted differently.

If P30 results are close to the 2 ng level (for body cavity swabs) or the 0.05 level (for other samples), a slide should be prepared from the sample and a sperm search done.

Off scale P30 values are indicated by "HIGH" in the results table of the P30 ELISA spreadsheet. All "HIGH" values are interpreted as positive results.

Initials: RCJ **Date:** 5/15/19

Interpretation of results

The interpretation of P30 results depends on the source of the sample:

1. Body cavity swabs (e.g., oral, vaginal and anal) are positive if the result is greater than the 2 ng standard.

2. Samples not from a body cavity (e.g., panties, etc.) are positive if the result is greater than 0.05 absorbance units.

3. The location from which a "dried secretion" swab is taken affect the interpretation. Swabs taken essentially from a body cavity or similar place (e.g., introitus, etc.) are interpreted as if the sample is from a body cavity. Other locations (e.g., breast, thigh, etc.) may need to be interpreted differently.

If P30 results are close to the 2 ng level (for body cavity swabs) or the 0.05 level (for other samples), a slide should be prepared from the sample and a sperm search done.

Initials: RCJ **Date:** 5/5/99

Isoelectric focusing (IEF) - general guidelines

Plate preparation:

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

Gel preparation:

2. Described in detail in "Preparation of ultrathin polyacrylamide gels" and Quality Manual.

Sample preparation:

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

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

5. 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.

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

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

   A. extract in 0.05 M DTT and use that extract for all analyses; there will be a blue discoloration at the PGM origin

   OR

   B. 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:

8. Use a silicone applicator strip to apply samples at the origin specified by the method. It is generally preferable to use the 1x7 mm sample slots.

9. 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.

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

11. 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, ½, 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.

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

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

Gel assembly:

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

15. 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.

16. Moisten filter paper wicks, 6 mm wide and as long as the gel (not the plate), with the appropriate electrolyte. The wicks should be uniformly wet, but not dripping or too dry.

17. Place wicks on gel to give the wick distance specified by the method. The wicks **must** be parallel and not touching the tape.

18. 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.

19. 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:

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

21. 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.

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

20W.

Overlays:

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

24. 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.

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

Incubation:

26. 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.

Photography:

27. 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.

28. The lab generally uses two types of film - fast (3000 ASA, Polaroid type 667) and slow (80 ASA, Polaroid type 664). A slow film requires more light; therefore, a larger aperture and/or a longer exposure time.

    Suggested settings are:

Type 667	ASA 3000	UV photo	non-UV photo
	f-stop	11	22 or 32
	shutter	1/8	1/60
Type 664	ASA 100	UV photo	non-UV photo
	f-stop	5.6	8
	shutter	B (10 sec)	1/30

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

filter.

30. 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).

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

Results:

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

33. 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:

-, NR	no visible activity
inc	activity, but no clear bands
( )	possible type, not clear enough to call

34. 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:

35. All electrophoresis results **must** be called **INDEPENDENTLY** by a second reader, using an "Electrophoresis Review Worksheet".

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

Initials: RG **Date:** 5/5/11

Preparation of Ultrathin Polyacrylamide Gels

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 gel solution (see appropriate IEF reagent sheets in the Quality Manual). 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 over 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.

5. 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.

6. 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.

7. To use the plates, separate them immediately before sample application by placing the 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.

8. 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 apply a sample in that position.

Initials: RA **Date:** 5/15/99

Erythrocyte acid phosphatase (ACP) by IEF

Gel prep: 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: BA

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 prep: For lysates, mix 5 uL of washed packed red blood cells with 100 uL 0.05 M DTT; store frozen if the sample may be typed again.

For stains, extract an approximately 3x3 mm stain in 50 uL 0.05 M 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.

Run: 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.

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 if forced to be lower. This means that the voltage may not be 2000V at the beginning.

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

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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.

Interpretation: 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. 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.

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.

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Esterase D (ESD) by IEF

Gel prep: 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 prep: 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.

Run: 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).

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 if forced to be lower. This means that the voltage may not be 2000V at the beginning.

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Reaction mix: 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.

Interpretation: 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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Phosphoglucomutase (PGM) by IEF

Gel prep: 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 prep: 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.

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: Place gel on platen at 4 °C. Apply the electrodes and cover. Conduct

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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 if forced to be lower. This means that the voltage may not be 2000V at the beginning.

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

Dissolve dry ingredients (available pre-measured in the freezer) in reaction buffer; add 200 uL meldola blue (1 mg/mL) and 75 uL glucose-6-phosphate dehydrogenase enzyme.

Mix in 15 mL overlay agarose (1% Sigma type I in deionized water, melted) and pour onto plate between the origin and the hemoglobin. Incubate at 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: 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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Hemoglobin (Hb) by IEF

Gel prep: Prepare the gels as described, using pH 3-10, 6-8, and 7-9 ampholytes

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 prep: 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: 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.

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 if forced to be lower. This means that the voltage may not be 2000V at the beginning.

Photograph the bands.

Interpretation: 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.

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Coomassie blue staining of gels

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.

Initials: RCJ Date: 5/15/97

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