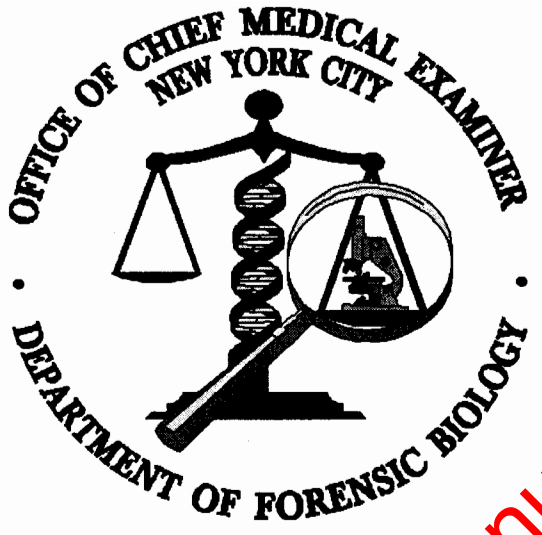
TABLE OF CONTENTS

DATE EFFECTIVE	VERSION	PAGE
01-07-2005	5.0	1 OF 1



BIOCHEMISTRY METHODS MANUAL
VERSION 5.0

Effective date: January 7, 2005

APPROVED BY			
Title	**Print Name**	**Signature**	**Date**
Deputy Director/ Technical Manager	**Howard J. Baum, Ph.D.**	Howard J. Baum	**January 7, 2005**

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1. 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, and saliva prior to further analysis.

2. PROCESSING OF POSTMORTEM SPECIMENS

1. Retrieve the postmortem specimens. Specimens from the Manhattan office must be picked up from the refrigerator in the autopsy room. Specimens from the satellite offices 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 and make a note of any discrepancies.

3. For discrepancies, a multi-purpose form letter is available for the following situations and must be sent via facsimile to the satellite office or the pathologist (if known):

   Unlabeled specimen(s)
   Specimen(s) collected but not listed on transport sheet

4. Place the transport sheets into the appropriate files (by borough) when finished.

5. Place a blank transport sheet and two plastic ties into each container after they have been emptied. Place the red containers out in the hallway by the service elevator.

6. Retrieve the autopsy case worksheets and daily case census sheets for all five boroughs. This paperwork can be found in the following locations:

Manhattan	Communications department (Forensic Biology tray)
Brooklyn	FB interdepartmental mailbox or FB main office
Bronx	FB interdepartmental mailbox or FB main office
Staten Island	FB interdepartmental mailbox or FB main office
Queens	Toxicology office

7. Arrange the autopsy case worksheets and daily case census sheets in numerical and/or chronological 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
   - Possible homicides and CUPPI's
   - Any case in which sexual assault evidence has been collected
   - Unidentified decedents
   - Any case in which evidence from the NYPD or DA's office has been submitted

- Motor vehicle accidents (MVA's) in which there is an investigation (hit and run)
- Any case that involves child abuse or suspected child abuse

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

8. Compare each autopsy case worksheet with the specimens received to ensure that all of the specimens designated for Forensic Biology have been received. A multi-purpose form letter is available for the following situations and must be sent via facsimile to the satellite office or the pathologist (if known):

    Unlabeled specimen(s)
    Specimen collected but not received by Forensic Biology
    Blood sample (or other suitable sample) not collected for Forensic Biology

9. Remove all autopsy case worksheets for cases that will be given an FB case number and separate the specimens from those that will not be accepted.

10. **For cases that will NOT be assigned an FB case number**: all autopsy case worksheets and daily case census sheets must be placed into their designated folders (by borough) for retention. Place all paperwork into the appropriate folders in numerical and/or chronological order.

    All specimens must be placed into refrigerated storage into designated racks (by borough) in numerical order (by day). These samples must also be entered into the MB log book grouped by borough in numerical order (by day).

11. **For cases that will be assigned an FB case number**: check the Forensic Biology log book and/or 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.

12. Assign the remaining specimens new FB case numbers and enter the appropriate information into the FB logbook.

    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 (only for blood and sexual assault evidence that is not part of a kit).

13. 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, signature of the individual who received them, and the location the specimens were received ("autopsy" for Manhattan and "sealed red container #n" for the red containers).

14. Autopsy case worksheets and evidence packaging worksheets are affixed to the right side of the folder. Chain of custody forms are affixed to the left side of the folder.

15. Label each specimen with the corresponding FB case number and PM number. The purple (or red) cap of each vial of blood must be labeled with the FB case number.

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

17. Processing of postmortem blood requires that the blood processing section of the evidence packaging worksheet be completed. The "vial labeled" section must contain the ME case number which should be present on each vial of postmortem blood.

18. After two (2) months of storage, the blood and other tissue samples that were signed into the MB logbook should be discarded. The discard date **must** be entered in the MB logbook for each discarded blood sample. Purple-topped vials **must** be discarded in a plastic BIOHAZARD "sharps" container.

19. After one (1) year, all non-blood postmortem items (such as tissue) may be discarded provided that a dried bloodstain exists (only in cases that have been signed into the laboratory). Bones are typically retained indefinitely. The chain of custody **must** reflect the samples discarded, date of discard, and the individual who discarded the samples.

3. BLOODSTAIN PREPARATION FROM WHOLE BLOOD

DATE EFFECTIVE	VERSION	PAGE
01-07-2005	5.0	1 OF 2

Staincards are prepared from all vouchered blood samples and from post-mortem blood samples under the following circumstances:

- *All homicide victims*
- *All unidentified persons, regardless of cause of death*
- *All persons whose cause of death is listed as "pending"*

1. Find the case files and obtain the blood vials; sign the chain of custody forms.

2. Prepare the UltraSTAIN™ cards by affixing a pre-printed FB case number sticker (if available) and writing in the following:

   - Subject's name
   - Date
   - Initials of person preparing the stain
   - FB number, if no sticker is available

   Wear latex gloves when handling these cards.

3. Preparation of the bloodstain **must** be witnessed by another laboratory staff member. After each stain is made, the witness must initial the stain card and the evidence packaging worksheet. The witness must confirm that the processor is handling the correct blood vial and stain card BEFORE the stain is made.

4. 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. It is advisable that a new KimWipe™ be used to open each vial stopper. 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. The stain cards must be allowed to dry overnight before storage.

7. Package the air-dried stains into a 4x6" KAPAK™ bag; do not seal. The stains are stored frozen for approximately one year, then stored at room temperature. Continue the chain of custody to reflect their final location.

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

9. Place all case files that contain **any** sexual assault evidence in the designated area so that they may be processed. Place all cases files that contained any evidence from the NYPD or DA's office back from where they were retrieved (either "cases to be called on," "cases to be assigned," or the assigned analyst). Place all remaining case folders in the Forensic Biology office so that they may be filed.

10. Disposal of blood and blood vials:

    For non-vouchered blood, the remainder of the liquid blood and the blood vial will be discarded in approximately two months. Purple-topped vials **must** be discarded in a plastic BIOHAZARD "sharps" container.

    For vouchered blood, the remainder of the liquid blood is discarded immediately and the empty vial rinsed with 10% bleach. The empty vial is packaged for return to the Evidence Unit.

4. KASTLE-MEYER (KM) PRESUMPTIVE TESTING FOR BLOOD

DATE EFFECTIVE	VERSION	PAGE
01-07-2005	5.0	1 OF 1

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 water, then rub over the stained area while still wet.

2. Apply a drop of water.

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. Each lot/aliquot of reagent must be tested against a positive and negative control at least once per day.

5. ACID PHOSPHATASE PRESUMPTIVE TEST FOR SEMEN

DATE EFFECTIVE	VERSION	PAGE
01-07-2005	5.0	1 OF 1

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. Each lot/aliquot of reagent must be tested against a positive and negative control at least once per day.

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. Each lot/aliquot of reagent must be tested against a positive and negative control at least once per day.

6. 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 100uL water for 30 minutes at room temperature using the pipet tip and test tube method. For samples that have been analyzed with P30 ELISA, use the extracts prepared in that procedure.

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 to prepare the remaining 2, 0.2, 0.02, and 0.002 unit standards by doing ten-fold serial dilutions. This is easily accomplished by first adding 900 uL of dH_2O to each of 4 microcentrifuge tubes. Then transfer 100 uL of your 20 unit standard into one of the tubes containing 900 uL of dH_2O. This is your 2 unit standard. Continue making the remaining dilutions in the same manner.

   When doing the serial dilutions, make sure to mix each standard before each 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 according to the Amylase Diffusion Worksheet (10 uL each well) with standards, negative control, and samples. The first two wells are reserved for the 0.02U and 0.002U amylase standard, the negative control is added to the third well, and the remaining wells are filled with samples.

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 the clear areas and record on the worksheet. Photograph the results; ensure there is a scale in the photograph.

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 affects 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.

7. UREA DIFFUSION PRESUMPTIVE TEST FOR URINE

DATE EFFECTIVE	VERSION	PAGE
01-07-2005	5.0	1 OF 2

Controls: Urine, urea, and saline or water

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.

Standard 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 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.

8. CHRISTMAS TREE STAIN FOR SPERMATOZOA

DATE EFFECTIVE	VERSION	PAGE
01-07-2005	5.0	1 OF 1

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 that 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).

9. P30 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Solution Preparation:

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). Replenish as uses. For each plate or pair of plates you will need 2 bottles (1 liter).

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

1. Thaw casein @ room temperature.
2. Dilute 20 ml of casein stock solution to 1 liter with PBS.
3. One plate uses approximately 800 ml.

Standards: P30 antigen and phosphate buffered saline
Prostate specific antigen (PSA) is another name for P30 antigen.

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)

Store at 4°C.

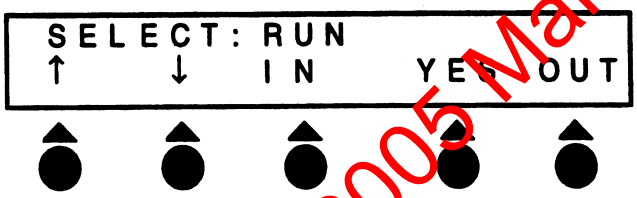
Instructions for Bio-Rad 1575 washers

The Bio-Rad 1575 plate washer has a cover that can be swiveled to an opened or closed position.

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.

Running plate washer programs

BLOCK 1

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.

BLOCK 2

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.

WASH

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 the "test" 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.

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.**

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.

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

	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

4. Cover plates with Parafilm and label "Coating" with initials and date. Incubate overnight at 4°C.

BLOCKING THE PLATES:

Prime/Rinse washer

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 (see Block 1 procedure).

2. Aspirate the final wash and blot dry. Plates that are not used immediately should be wrapped in plastic and stored at 4°C; they can be stored for up to 2 weeks (see Block 2 procedure).

SAMPLE PREPARATION:

Prepare samples and standards on the day of use.

1. Stains/swabs Using the pipette 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).

Refrigerate 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) + 5.0 mL PBS-casein
b. 2 ng/mL: 1000 uL of 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

2. Cover the plates with Parafilm and incubate at room temperature for 1 hour. Prime/Rinse washer.

3. Aspirate contents of wells. Wash the plate three times with PBS-casein (see WASH procedure). 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 4 uL antiserum to 12 mL PBS. For 2 plates add 8 uL antiserum to 24 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. 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. Store in dark place during preparation.

11. Aspirate contents of wells. Wash the plate three times with PBS-casein. Make sure that there is no excess buffer remaining in the wells.

12. Apply 100 uL of PNPP substrate solution to each well of columns 1-12.

13. Cover the plates with Parafilm and incubate for 1 hour at 37^{o}C. Uncover and read at 405nm.

READING THE PLATE

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. A protocol window will appear. 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 letter of plate used 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. On the plate reader, press **START/STOP** soft key once and then **PAGE(+)** key three times (3x), and then press **ENTER**. You can now close the Raw Data window.

8. 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.

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

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

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

Spreadsheet calculations

1. Open the *Microsoft Excel* 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 **All Programs** menu. Click once on the **Microsoft Excel** icon.

2. Pull down the **File** menu (Top Left corner) 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\P30xl.xls

3. The P30 spreadsheet will appear (this a **READ-ONLY** file; it cannot be altered). Two lettered A and B 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 **Data** menu and move the arrow to the **Import External Data** menu. Click once on **Import Data...**. The **Select Data Source** window will pop up. 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. 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 **Finish.**

7. The **Import Data** window will pop up. The **Existing Worksheet** option should have a black dot to the left, and **=A1** should appear in the window below it. Press **OK**. The saved data from the plate reader should appear on the page in the upper left corner.

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**.

NOTE: **Files should be deleted after supervisory review to avoid cluttering up network drive space.**

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 2ng 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.

10. OUCHTERLONY RADIAL DIFFUSION – SPECIES DETERMINATION

DATE EFFECTIVE	VERSION	PAGE
01-07-2005	5.0	1 OF 2

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^{o}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.

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.

11. 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.