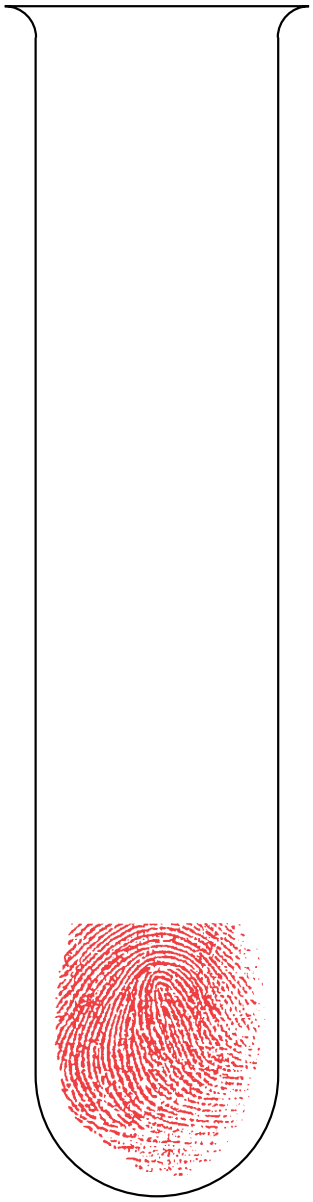
# Blood Stain Typing by Electrophoresis



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# Handbook for Rapid Blood Stain Typing Using Electrophoresis for the Crime Laboratory

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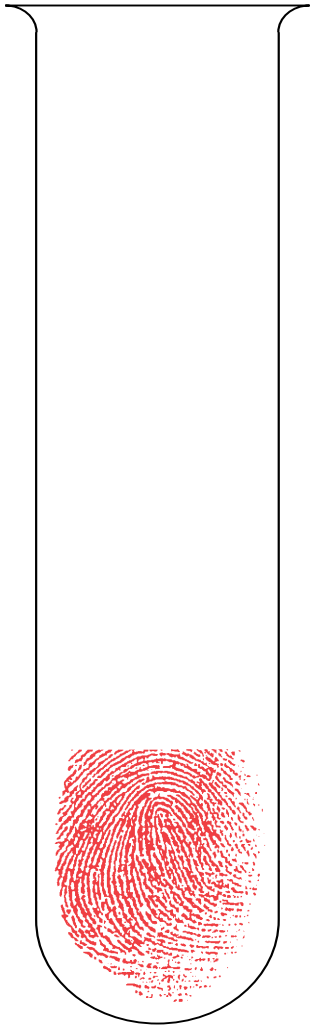

# Acknowledgements

The following students have provided valuable assistance in the development of these methods: Retha (Matthews) Edens[1], Alice Abbot[2], Randall Webster[3], Yvonne (Moll) Matthews[4] and Ralph Willis[5]. We especially appreciate Ms. Donna Pruneau[6] for reviewing and updating this manual (February, 1989). These procedures are in use in course FS552 (Blood and Body Fluids), an elective in the Science Division at Southeast Missouri State University, Cape Girardeau, Missouri. The authors would like to thank Helena Laboratories, Beaumont, Texas, for support of this project. Portions of this work were supported by the Research Council, Southeast Missouri State University, Cape Girardeau, Missouri. We would also like to thank Ms. Patty Butler[7] for typing the original draft of this handbook.

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# Section 1 General Information

## I. Introduction

In the past decade, progress in developing methodologies for typing blood factors using isoenzyme studies has made blood stain evidence one of the most important investigative tools available to forensic scientists. Originally, all procedures for isoenzyme typing involved electrophoresis on starch gels. At the present time, four major electrophoretic support media or utilized: starch gel, agarose, polyacrylamide gel and cellulose acetate. This presentation is a result of the research performed in our laboratories directed toward adaptation of isoenzyme typing of blood factors by electrophoresis on supported cellulose acetate membranes (CAM).

Traditionally, the only method for identifying human blood samples in the crime laboratory was typing by the ABO system. Using the ABO system, the most rare blood type encountered is AB, which is found in only 3% of the population. While the percentage of persons having blood type AB is very low, almost 50% of the human population has type O. It is apparent from these statistics that a system for more definite identification of blood systems is mandatory if blood typing is to be used in a crime laboratory.

In the early 1970s, research was initiated to improve the system for determining the type of human blood found on dried blood stains. In addition to the ABO system, other systems were utilized. The erythrocyte MN and Rh (primarily RhD) antigen systems were used. Protein studies involved hemoglobin and haptoglobin. Several enzyme systems were utilized; the most useful of these being phosphoglucomutase (PGM), adenylate kinase (AK), erythrocyte acid phosphatase (EAP), esterase D (EsD), and adenosine deaminase (ADA). Initial work was performed by Bryan J. Culliford of the Metropolitan Police Forensic Laboratory of London, England.[1] Determining blood types using a combination of the systems cited, greatly diminishes the occurrence of a particular type in the population. Although electrophoresis was the method of choice in determining the proteins present in a blood stain, the time involved became a prime concern since starch gel (the only media used at that time) electrophoresis procedures often required 15 to 30 hours for completion.

In the mid 1970s, workers at the University of Pittsburgh proposed a method for multiple enzyme separation on a single electrophoresis plate. This work was completed under the direction of B. Wraxall.[2] By covering a particular portion of the starch gel and developing each portion specifically for the desired enzyme, it was possible to identify several enzyme systems at the same time. Additional work by Wraxall developed a multiple systems using a starch/agarose gel.

Since that time, the research of Dr. B. Grunbaum and P.L. Zajac using the Beckman Micro-zone® System and adapting the starch gel procedures to Sartorious® cellulose acetate membranes has drastically reduced the time required. The Titan® III Cellulose Acetate Electrophoresis System from Helena Laboratories is now being used successfully by the Southeast Missouri Regional Crime Laboratory. Methods for identifying genetic markers are performed on supported cellulose acetate electrophoresis plates. The results are discernable and readily reproducible. Studies on all the enzyme systems can be completed in less than eight hours.

## II. Factors Influencing Results

Several variables influence the results obtained with an electrophoretic procedure. These include sample preparation and application, cellulose acetate plate preparation, pH and ionic strength of the buffers, voltage, amperage, and color development techniques. The cellulose acetate plates must be soaked in the individual system plate buffer prior to sample application. The soaking buffer is usually a dilution of the tank buffer. The correct dilution of plate buffer is essential for good resolution and for prevention of high amperage and excess heat. The plate must be soaked gradually from the bottom up to ensure saturation without air bubbles.

Air bubbles in the cellulose acetate impede the movement of the electric current through the plate. Once trapped in the plate, air bubbles cannot be removed and the plate must be discarded.

The pH of the tank, plate and reaction buffers is extremely important. All enzymes have an optimal pH for activity. Denaturation of the enzymes may occur if reagents with the incorrect pH are used. Improper reaction temperatures have the same effect. Correct ionic strength of the buffers is also very important.

Proper voltage and amperage are extremely important to good electrophoretic resolution. Voltage should remain constant, allowing the amperage to start low (about 2 milliamperes per plate) in order to avoid overheating the system. The temperature of the electrophoresis plate rises as the amperage rises. If the temperature exceeds the optimal range, the enzyme will denature.

The amount of time required for electrophoresis varies with the enzyme system under study. None of our procedures require more than one hour.

The technique used for developing the cellulose acetate plate after completion of electrophoresis is critical. The two basic types of development are colorimetric and fluorometric. After development, results must be recorded and filed. All plates are photographed with black and white Polaroid film.

## III. Sample Extraction and Storage

To preserve enzymatic activity, any materials containing dried blood stains should be frozen. At the time of testing, cut a 0.5 cm x 0.5 cm piece of stained material from the original sample and place in a well with 2 to 3 drops of solvent. Distilled water is routinely used as the solvent; however, Cleland's reagent must be used for phosphoglucomutase and erythrocyte acid phosphatase assays. Samples extracted with purified water may be soaked overnight in a humidity chamber at 4°C. Samples extracted in Cleland's reagent (dithioerythritol; available from Sigma) are stable for 3 to 5 hours and should not be stored overnight. The procedure from making Cleland's reagent is included in the PGM procedure.

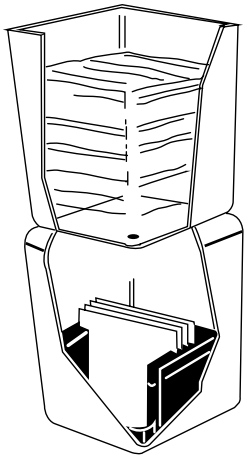
# IV. Materials

Titan III Cellulose Acetate System available from Helena Laboratories.

| **Hardware** | **Cat. No.** |
|---|---|
| TITAN® plus Power Supply (110V) | 1504 |
| Zip Zone® Chamber | 1283 |
| Super Z Applicator Kit | 4088 |
| Development Weight | 5014 |
| Titan Photo VuBox Camera | 5048 |
| Bufferizer | 5093 |
| IOD Incubator, Oven Dryer (110V) | 5116 |
| Micro-Hood | 8009 |
| **Consumables** | |
| Titan® III-Lipo Cellulose Acetate (60 x 76 mm) | 3900 |
| Electra® HR Buffer | 5085 |
| Supre-Heme Buffer | 5802 |
| Electra® B1 Buffer | 5016 |
| LD Vis Isoenzyme Reagent | 5909 |
| Hemolysate Reagent | 5125 |
| Blotters | 5034 |
| Zip Zone® Chamber Wicks | 5081 |
| Development Slides | 5008 |
| Titan Plastic Envelope | 5052 |
| Titan Identification Labels | 5006 |
| Helena Marker | 5000 |

# V. Electrophoresis Techniques

## A. Preparation of Titan III-Lipo Plates

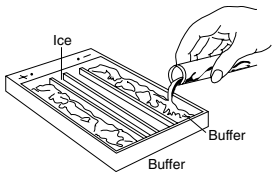


1. Properly code the required number of Titan III-Lipo plates by marking on the glossy, hard side with a magic marker. It is suggested that the mark be placed in one corner of the plate so that it is always aligned with sample #1.

2. Soak the plates for 20 minutes in the individual system plate buffer. The soaking buffer is usually a dilution of the tank buffer. The plates must be soaked gradually from the bottom up to ensure saturation without trapping air bubbles in the cellulose acetate.

   The plates may be soaked in the Bufferizer (available from Helena Laboratories, Cat. No. 5093) or they may be soaked in a laboratory beaker using the principle of the Bufferizer. Place the plates in the beaker in a vertical position and allow the plate buffer to slowly flow down the side of the beaker from a separatory funnel or directly from the buffer container. Alternately, fill a beaker with buffer and slowly and steadily immerse the plates (vertical position) into the buffer.

## B. Preparation of the Electrophoresis Tank (Zip Zone Chamber)

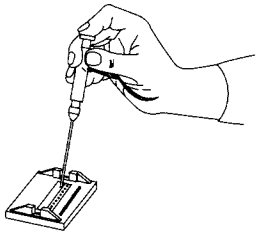



1. Prior to using the tank, fill the inner chamber halfway with water and keep frozen until the tank is used.

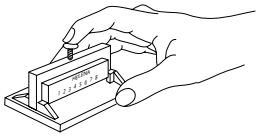
2. When preparing for electrophoresis, pour 50 mL of tank buffer into the cathode chamber and a 1:1 dilution (25 mL tank buffer/25 mL $H_2O$) in the anode chamber. (Except when stated otherwise; check specific procedures.)

3. Wet two Zip Zone® Chamber Wicks in the tank buffer and drape one over each support bridge. Be sure they make contact with the buffer and that there are no air bubbles under the wicks. Do not allow the wicks to touch the ice in the inner chambers. Cover the tank until used.

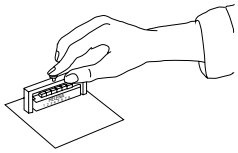
## C. Sample Application

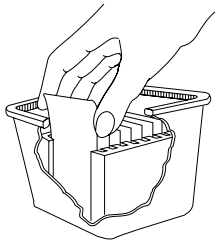


1. Fill the wells of the sample well plate with blood stain extract using a small capillary pipette.

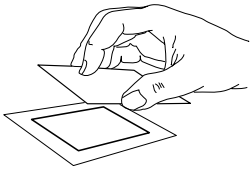


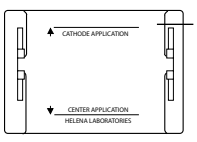
2. Prime the applicator by depressing the tips into the sample wells 3 or 4 times. Apply this loading to a piece of blotter paper. Priming the applicator makes the second loading much more uniform. Do not load the applicator again at this point, but proceed quickly to the next step.





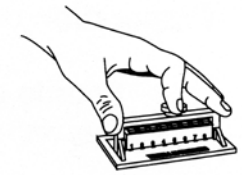
3. Remove the wetted Titan III plate from the buffer and blot once firmly between two blotters. Place the plate in the aligning base, cellulose acetate side up, aligning the top of the plate with the black scribe line marked “cathodic application”. The identification mark should be aligned with sample #1. Most procedures require a cathodic application with the exception of phosphoglucomutase (PGM) which requires an extreme cathodic application.

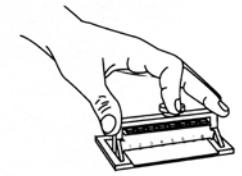





Placement of plate for extreme cathodic application

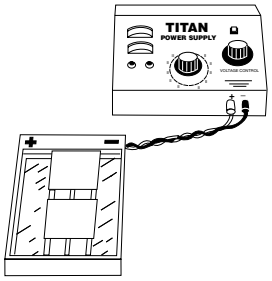
4. Apply the sample to the plate by depressing the applicator tips into the sample wells 3 or 4 times and promptly transferring the applicator to the aligning base. Press the button down and hold for 5 seconds. Make a second or third superimposed application by repeating this step. Three applications of .25 µL each is sufficient for most isoenzyme studies.





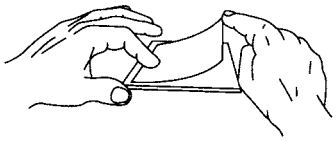
## D. Electrophoresis of the Sample Plate

1. Quickly place the plate, cellulose acetate side down, in the electrophoresis tank. Place a weight (plastic rod, glass slide, etc.) on the plate(s) to insure contact with the wicks. Cover the chamber securely.
2. Electrophorese according to the instructions of the appropriate procedure.

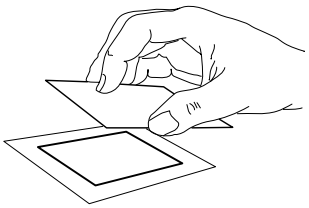



## E. Development

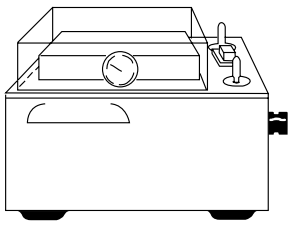
1. For each sample plate used, a reaction plate must also be soaked according to the soaking method described in Step A. The reaction plate is usually soaked in the reaction buffer. See individual procedures, Section 2.
2. Remove each plate from the reaction soaking buffer 5 minutes before completion of electrophoresis and blot gently. Place the plate, cellulose acetate side up, onto a glass development slide and place on a level counter top or a leveling table.
3. Pour approximately 2.0 to 5.0 mL of reaction mixture onto the plate. Tilt the slide until the reaction mixture covers the plate. Cover each plate with a small box (if the reaction mixture is light sensitive) and allow to stand for five minutes.



4. After completion of electrophoresis, remove the sample plate from the electrophoresis tank and blot lightly to remove excess buffer.



5. Carefully layer the sample plate, cellulose acetate side down, onto the reaction plate. Place a blotter over the sandwiched plates. Remove excess substrate from the sandwich by drawing the edge of the aligning base across the plate several times. Blot away excess substrate.
6. Place the plates between moistened blotters and place on the top of a preheated Micro-Hood or in a Helena IOD or other laboratory incubator at 37°C. Place a preheated development weight on top of the blotters.



7. After the appropriate incubation period, prepare the plates for evaluation as described in individual procedures (Section 2). After development, all plates must be dried before reading either by air drying or with a light stream of warm forced air. Both plates, the sample plate and reaction plate (negative), can be read. In most instances, dried plates may be stored indefinitely for future reference if placed in a Titan Plastic Envelope or small plastic storage bag.

## VI. Results and Discussion

At the present time, the following procedures are being performed routinely in SEMO Regional Crime Laboratory using the cellulose acetate membrane (CAM) system:

Phosphoglucomutase (PGM)
Erythrocyte/Vaginal/Seminal Acid Phosphatase (EAP/VAP/SAP)
Adenylate Kinase (AK)
Group Specific Component (Gc)

**Other procedures which are currently performed on CAM are:**

Glucose-6-Phosphate Dehydrogenase (G-6-PD)
Lactate Acid Dehydrogenase (LDH)
Carbonic Anhydrase II (CA-II)[5]

Isoenzyme typing of blood using cellulose acetate membranes as a support medium for electrophoretic separation has proven to be a relatively inexpensive and rapid method for identifying blood stains according to polymorphic proteins and enzymes. Using these procedures, determinations can be performed using microliter volumes of specimen (stain extract).

Esterase D (EsD), Glyoxylase-I (GLO-I) and Peptidase A (Pep A) determinations are performed with starch-agarose gel due to superior separation and visualization. Haptoglobin determinations are performed utilizing a gradient polyacrylamide system (overnight). If the haptoglobin procedure is run at 300 volts, it can be completed in four hours. It is possible for a trained analyst to run all systems, including species, ABO typing and haptoglobin in eight hours.

## VII. References


1. Culliford, B.J., The Examination and Typing of Bloodstains in the Crime Laboratory, Law Enforcement Assistance Administration, Washington, D.C., 20402, Stock # 2700-0083.
2. Wraxall, Bryan G.D., Ed., *Bloodstain Analysis System Procedures Manual*, Serological Research Institute, Emeryville, California, 1977.
3. Grunbaum, B.W., Selvin, S., Pace, N., Black, D.M., J of Forensic Sci, 23, 577, 1979.
4. Grunbaum, B.W., Ed., *Handbook for Individualization of Forensic Blood and Bloodstains*, University of California, Berkeley, California, 1980.
5. Noppinger, K.E., Morrison, R.D., J of Forensic Sci, 26:1, 176-180, 1981.

# Section 2 Typing of Dried Blood Stains With Cellulose Acetate

## Part 1: Isoenzymes

### Phosphoglucomutase (PGM)

1. Tank Buffer: Tris/maleic acid buffer 0.075M; pH 7.4
   6.05 gm Tris: Tris (hydroxymethyl) aminomethane
   5.81 gm Maleic acid
   1.46 gm EDTA (Free Acid): Ethylenediaminetetraacetic Acid
   3.47 gm $MgCl_2 \bullet 6\ H_2O$
   Dissolve in 900 mL of distilled water and adjust to pH 7.4 with 40% NaOH.
   Q.S. to final volume of 1000 mL with distilled water.
   Cathode Buffer: 50 mL of tank buffer
   Anode Buffer: 15 mL tank buffer + 35 mL distilled $H_2O$

2. Plate Buffer: 30 mL tank buffer + 270 mL distilled $H_2O$

3. Preheat incubator and development weight to 37°C.

4. Sample Preparation and Application
   Cleland's Reagent (0.01N)
   154 gm/dL Dithiothreitol + 100 mL $H_2O$. Adjust pH to 8.0 with 6M NaOH.
   Soak blood stains for 15 minutes in fresh Cleland's reagent. Postmortem blood may have to be serially diluted with Cleland's reagent until a readable result can be achieved. Run known type 2-1 standard on each plate. Make one or two applications, extremely cathodic.

   NOTE: The extract made in Cleland's reagent is stable for 3 to 5 hours.

5. Electrophoresis: 250 volts for 30 minutes; Initial amperage = 2 mA/plate.

6. Reaction Buffer: pH 8.0
   3.64 gm Tris
   Dissolve in 500 mL distilled water. Adjust to pH 8.0 with concentrated HCl.

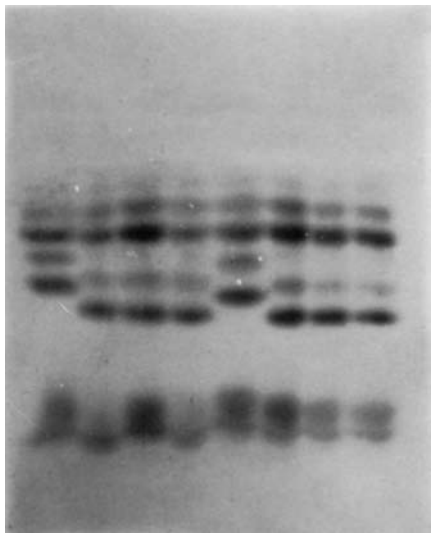
7. Reaction Plates: Soak plates in reaction buffer approximately 15 minutes prior to completion of electrophoresis.

8. PGM Reaction Mixture
   a) Prepared PGM mixture in 3.0 mL of reaction buffer or
   b) 30.0 mg Glucose-1-Phosphate*
      0.3 mg Glucose-1, 6-Diphosphate*
      20.0 units Glucose-6-Phosphate Dehydrogenase
      2.5 mg PMS: Phenazine Methosulfate (Light sensitive)
      2.5 mg MTT: Thiazoyl Blue (Light sensitive)
      2.0 mg NADP: Nicotinamide Adenine Dinucleotide Phosphate
      5.0 mL reaction buffer
      Stir mixture until dissolved. Pour on reaction plates about 5 minutes before completion of electrophoresis.
      *Available together from Sigma Chemical Company; -D-Glucose 1-Phosphate Disodium Salt, cat. no. G-1259

9. Incubation: 15 minutes at 37°C, with weight on top or until blue bands appear.

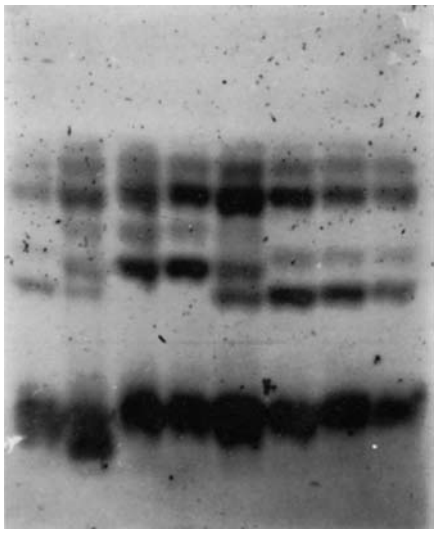
10. Visualization: Separate plates and soak 5 minutes in 5% acetic acid. Blot, dry and read using transmitted light.

## Figure 1 – Phosphoglucomutase (PGM)




**Electrophoretic patterns for two types of PGM are demonstrated as follows:**
**Type 1-1: Samples 2, 3, 4, 6, 7 and 8**
**Type 2-2: Samples 1 and 5**

## Figure 2 – Phosphoglucomutase (PGM)




**Electrophoretic patterns for three types of PGM are demonstrated as follows:**
**Type 1-1: Samples 1, 6, 7 and 8**
**Type 2-2: Samples 3 and 4**
**Type 2-1: Samples 2 and 5**

## Erythrocyte/Vaginal/Seminal Acid Phosphatase (EAP/VAP/SAP)

1. Tank Buffer: Citrate Phosphate pH 5.5
   4.42 gm Sodium Citrate
   3.36 gm Dibasic Sodium Phosphate ($Na_2HPO_4 \bullet H_2O$) (2.98 gm anhydrous)
   0.372 gm EDTA (Free Acid)
   Dissolve in 900 mL of distilled water and adjust to pH 5.5 with phosphoric acid.
   Q.S. to final volume of 1000 mL with distilled water.
   Cathode Buffer: 50 mL of tank buffer
   Anode Buffer: 25 mL tank buffer + 25 mL distilled $H_2O$

2. Plate Buffer: 60 mL tank buffer + 232 mL distilled $H_2O$

3. Preheat incubator and development weight to 37°C.

4. Sample Preparation and Application
   Cleland's Reagent (0.01N)
   154 gm/dL Dithiothreitol + 100 mL $H_2O$. Adjust pH to 8.0 with 6M NaOH.
   Soak blood stains for 15 minutes in fresh Cleland's Reagent. Run known type BA as standard. Make three cathodic applications.
   NOTE: The extract made in Cleland's reagent is stable for 3 to 5 hours.

5. Electrophoresis: 200 volts for 60 minutes; Initial amperage = 2 mA/plate.

6. Reaction Buffer
   5.25 gm Citric Acid (anhydrous)
   2.0 gm Sodium Hydroxide
   Dissolve in 500 mL of distilled water. Adjust to pH 5.0 with sodium hydroxide or citric acid depending on pH.

7. Reaction Plates: Soak plates in reaction buffer approximately 15 minutes prior to completion of electrophoresis.

8. EAP Reaction Mixture
   2.0 mg 4-methylumbelliferyl phosphate
   5.0 mL reaction buffer
   0.75 mL glycerol (final solution is 15% glycerol vol/vol)
   Pour on reaction plates about 5 minutes before completion of electrophoresis.

9. Incubation: 15 minutes at 37°C, with development weight on top.

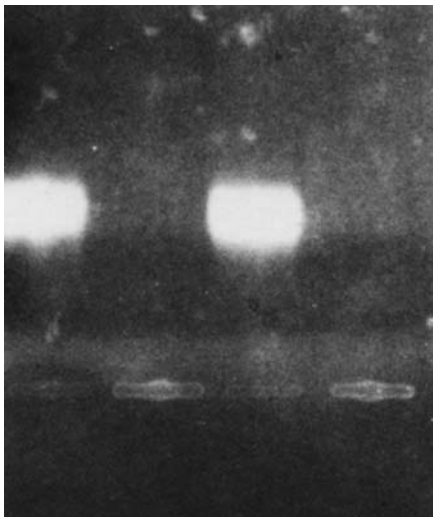
10. Visualization (Fluorescence): Separate and dry plates. Mist lightly with 0.1N NaOH. Read under U.V. light box. Store plates in plastic bags. Bands can be regenerated by misting with 0.1N NaOH.

## Figure 3 – Erythrocyte Acid Phosphatase (EAP)


**Electrophoretic patterns for six types of EAP are demonstrated as follows:**

**Type BB: Samples 1 and 3**
**Type BA: Samples 2 and 8**
**Type DB: Samples 4**
**Type CB: Samples 5**
**Type CA: Samples 6**
**Type AA: Samples 7**

## Figure 4 – Vaginal/Seminal Acid Phosphatase




**Electrophoretic patterns for two types of vaginal/seminal fluid acid phosphatase are demonstrated as follows:**

**Type 1-1: Samples 1 and 3, seminal stain**
**Type 2-2: Samples 2 and 4, vaginal stain**

## Adenylate Kinase (AK)

1. Tank Buffer: Citrate Phosphate pH 5.5
   4.42 gm Sodium Citrate
   3.36 gm Dibasic Sodium Phosphate ($Na_2HPO_4 \bullet H_2O$) (2.98 gm anhydrous)
   0.372 gm EDTA (Free Acid)
   Dissolve in 900 mL of distilled water and adjust to pH 5.5 with phosphoric acid.
   Q.S. to final volume of 1000 mL with distilled water.
   Cathode Buffer: 50 mL of tank buffer
   Anode Buffer: 25 mL tank buffer + 25 mL distilled $H_2O$

2. Plate Buffer: 60 mL tank buffer + 232 mL distilled $H_2O$

3. Preheat incubator and development weight to 37°C.

4. Sample Preparation and Application: Two to three cathodic applications of distilled water extract of stain. If possible, run a known 2-1 AK on the test plate to help clarify readings.

5. Electrophoresis: 200 volts for 60 minutes; Initial amperage = 2 mA/plate.
   ADA and AK may be run in same tank, utilizing separate plates for each system.

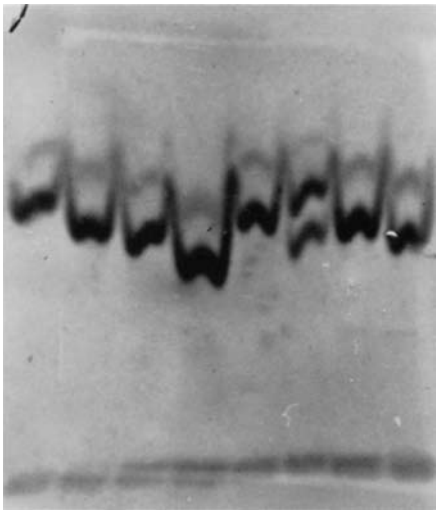
6. Reaction Buffer: pH 7.9
   9.0 gm Tris
   8.92 gm $MgCl_2 \bullet 6H_2O$
   Dissolve in 500 mL distilled water. Adjust to pH 7.9 with 6M HCl.

7. Reaction Mixture
   150.0 mg Glucose
   10.0 mg ADP (Adenosine-5' diphosphate)
   10.0 mg NADP
   5.0 mg PMS (light sensitive)
   5.0 mg MTT (light sensitive)
   14 IU Hexokinase*
   6 IU Glucose-6-Phosphate Dehydrogenase*
   5 mL Reaction buffer
   NOTE: Add MTT and PMS just before pouring on reaction plate and work in the dark because both chemicals are light sensitive. Filter reaction mixture onto the reaction plate.
   *Available together from Sigma Chemical Company; Hexokinase-Glucose-6-Phosphate Dehydrogenase, cat. no. H-8629.

8. Incubation: 5 minutes at 37°C or until blue bands appear.

9. Visualization: Separate plates and soak for 5 minutes in 5% acetic acid. Blot well, dry plates and read blue bands under transmitted light.

## Figure 5 – Adenylate Kinase (AK)




**Electrophoretic patterns for two types of AK are demonstrated as follows:**

**Type 1-1: Samples 1, 2, 3, 4, 5, 7 and 8**
**Type 2-1: Sample 6**

## Adenosine Deaminase (ADA)

1. Tank Buffer: Citrate Phosphate pH 5.5
   4.42 gm Sodium Citrate
   3.36 gm Dibasic Sodium Phosphate ($Na_2HPO_4 \bullet H_2O$) (2.98 gm anhydrous)
   0.372 gm EDTA (Free Acid)
   Dissolve in 900 mL of distilled water and adjust to pH 5.5 with phosphoric acid.
   Q.S. to final volume of 1000 mL with distilled water.
   Cathode Buffer: 50 mL of tank buffer
   Anode Buffer: 25 mL tank buffer + 25 mL distilled $H_2O$

2. Plate Buffer: 60 mL tank buffer + 232 mL distilled $H_2O$

3. Preheat incubator and development weight to 37°C.

4. Sample Preparation and Application
   Cleland's Reagent (0.01N)
   154 gm/dL Dithiothreitol + 100 mL $H_2O$. Adjust pH to 8.0 with 6M NaOH.
   Soak blood stains for 15 minutes in fresh Cleland's Reagent. Run a known Type 2-1 as a standard. Make four or five cathodic applications.
   NOTE: The extract made in Cleland's reagent is stable for 3 to 5 hours.

5. Electrophoresis: 200 volts for 60 minutes; Initial amperage = 2 mA/plate

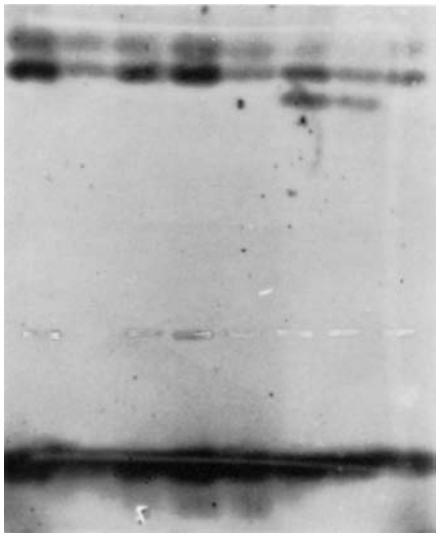
6. Reaction Buffer: pH 7.9
   9.0 gm Tris
   8.92 gm $MgCl_2 \bullet 6H_2O$
   Dissolve in 500 mL distilled water. Adjust to pH 7.9 with 6M HCl.

7. Reaction Mixture
   10.0 mg Adenosine
   0.1 units (4 µL) Xanthine Oxidase
   0.5 units (0.05 µL) Nucleoside Phosphorylase
   5.0 mg PMS (light sensitive)
   5.0 mg MTT (light sensitive)
   3 mL Reaction buffer
   NOTE: Add MTT and PMS just before pouring on reaction plate and work in the dark because both chemicals are light sensitive. Filter reaction mixture onto reaction plate.

8. Incubation: 15 minutes at 37°C or until blue bands appear.

9. Visualization: Separate plates and soak 5 minutes in 5% acetic acid. Blot well, dry and read results under transmitted light.
   NOTE: If upper band is darker, there is a possibility of oxidation. Use mercaptoethanol rather than Cleland's reagent to extract the dried stain.

## Figure 6 – Adenosine Deaminase (ADA)




**Electrophoretic patterns for two types of ADA are demonstrated as follows:**

**Type 1-1: Samples 1, 2, 3,4, 5 and 8**
**Type 2-1: Samples 6 and 7**

## Carbonic Anhydrase II (CA-II) and Hemoglobin (For Negroids)

1. Tank Buffer: TAPS, 0.075M, pH 8.4
   9.12 gm TAPS (Tris (Hydroxymethyl) Methylaminopropane Sulfonic Acid)
   Dissolve in 500 mL distilled water. Adjust to pH 8.4 with 40% NaOH(sodium hydroxide).
   Monitor pH closely.

2. Plate Buffer: Same as tank buffer.

3. Preheat incubator and development weight to 37°C.

4. Sample Preparation and Application: Prepare a distilled water extract of the stain.
   Run a known type 2-1 as standard. Make 5-6 center applications.

5. Electrophoresis: Constant amperage of 9.0 mA per plate for 45 minutes. Voltage is approximately 400.
   It is very important to keep the electrophoresis chamber very cool during the run. The tank buffer must be kept cold until ready for use. To keep the chamber at a low temperature, another lid or metal pan is filled with ice and placed on top of the closed electrophoresis tank.
   NOTE: The hemoglobin bands are easily seen as distinct red bands on the plate within 10-15 minutes after starting electrophoresis. Hemoglobin variants can be determined while the plate is still in the tank and are more distinct before staining for CA-II.

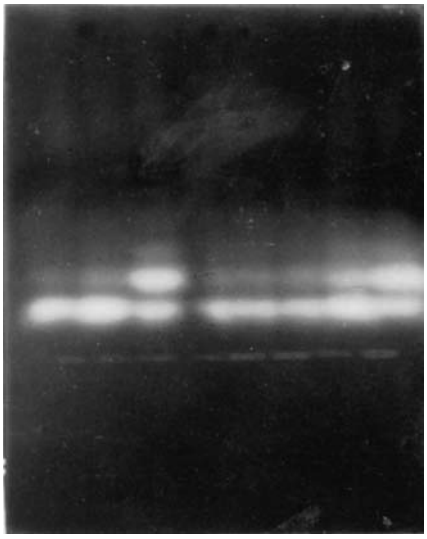
6. Reaction Buffer
   3.60 gm $Na_2HPO_4$, dibasic
   0.05 gm $MgCl_2 \bullet 6H_2O$
   Dissolve in 250 mL distilled water. Adjust to pH 7.5 with HCl (1.0N).

7. Reaction Mixture
   a. 8.0 mg Fluorescein diacetate
      1.0 mL Acetone
      20 mL reaction buffer
   b. 0.4 gm agarose, Sigma Type II
      20 mL distilled water
   c. Heat agarose until it is dissolved. Cool to 60°C. Dissolve fluorescein diacetate in 1.0 mL acetone and immediately add 20 mL of reaction buffer. Add this mixture to the cooled agarose. Pour the agarose/fluorescein diacetate mixture into flat Petri dishes; makes 3 to 4 plates. Store in refrigerator.
   d. Place the cellulose acetate plate onto the agarose/fluorescein diacetate gel, cellulose acetate side down.

8. Incubation: 20 to 30 minutes at 37°C or longer if necessary.

9. Visualization: CA-II bands are bright yellow fluorescent bands on a dark background.

## Figure 7 – Carbonic Anhydrase (CA-II)




**Electrophoretic patterns for two types of CA-II are demonstrated as follows:**

**Type 1-1: Samples 1, 2, 4, 5 and 6**
**Type 2-1: Samples 3, 7, and 8**

## Lactate Dehydrogenase (LDH)

1. Tank Buffer: Electra HR Buffer (Helena Cat. No. 5805), pH 8.8.
   Tris barbital-sodium barbital buffer. Dilute to 1750 mL with distilled water.

2. Plate Buffer: Same as tank buffer.

3. Preheat incubator and development weight to 37°C.

4. Sample Preparation and Application: Apply two cathodic applications of distilled water extract of stain. Seminal stains may have to be serially diluted until a readable result is achieved.

5. Electrophoresis: 180 volts for 30 minutes; Initial amperage = 8 mA each.

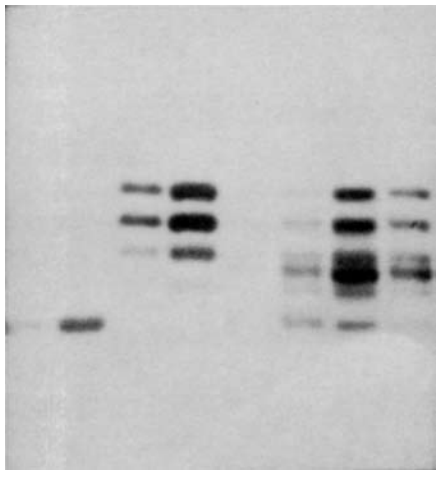
6. Reaction Plates: Soak plates in PGM reaction buffer about 15 minutes before completion of electrophoresis.

7. LDH Reaction Mixture
   5 mL PGM reaction buffer
   25 mg Calcium Lactate
   2 mg NAD (Nicotinamide Adenine Dinucleotide)
   2.5 mg MTT (light sensitive)
   2.0 mg PMS (light sensititve)
   Or use LD VIS Isoenzyme Reagent, Cat. No. 5909, Helena Laboratories

8. Incubation: 15 minutes at 37°C with development weight on top.

9. Visualization: LDH pattern appears as blue bands. View under transmitted light.

### Figure 8 – Lactic Acid Dehydrogenase (LDH)




**LDH electrophoretic patterns from various sources are illustrated as follows:**

**Liver: Samples 1 and 2**
**Heart: Samples 3 and 4**
**Mixture of menstrual blood and seminal fluid: Samples 5, 6, 7, and 8**

## Glucose-6-Phosphate Dehydrogenase (G-6-PD)

1. Tank Buffer: Supre-Heme Buffer (Helena Cat. No. 5802), pH 8.4.
   Tris-EDTA Borate buffer. Dilute to 1000 mL with distilled water.

2. Plate Buffer: Same as tank buffer.

3. Preheat incubator and development weight to 37°C.

4. Sample Preparation and Application: One cathodic application of distilled water extract of stain.

5. Electrophoresis: 350 volts for 20 minutes.

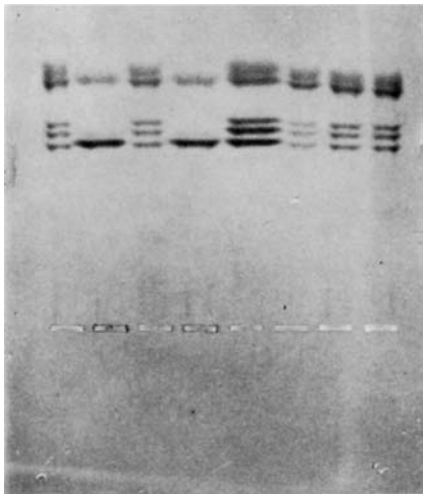
6. Reaction Buffer: Undiluted tank buffer.

7. G-6-PD Reaction Mixture
   a. G-6-PD Reagent, Helena Cat. No. 5620 or
   b. 10.0 mg Glucose-6-Phosphate
      6.0 mg NAD (Nicotinamide Adenine Dinucleotide)
      7.0 mg MTT (light sensitive)
      7.0 mg PMS (light sensititve)
   Reconstitute reagent with 3 to 5 mL of undiluted tank buffer. This makes sufficient reagent for 3 to 4 reaction plates.

8. Incubation: 20 minutes at 37°C.

9. Visualization: Separate plates and soak in 7.5% trichloroacetic acid (TCA) for 2 to 3 minutes. Rinse in 5% acetic acid for 5 minutes. Blot, dry plates and read under transmitted light.

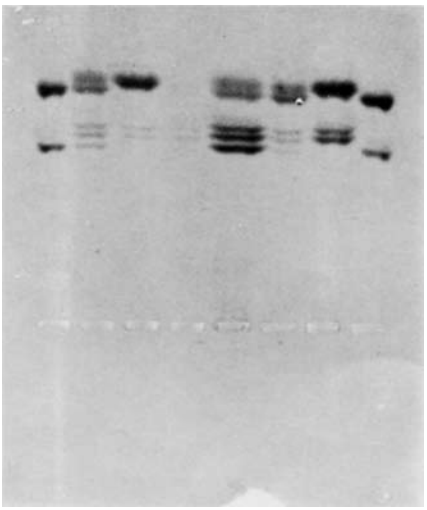
## Figure 9 – Group Specific Component (Gc)




**Electrophoretic patterns of two types of Gc are demonstrated as follows:**

**Type 2-1: Samples 1, 3, 5, 6, 7 and 8**
**Type 2-2: Samples 2 and 4**

## Figure 10 – Group Specific Component (Gc)




**Electrophoretic patterns of three types of Gc are demonstrated as follows:**

**Type 1-1: Samples 3, 6 and 7**
**Type 2-2: Samples 1 and 8**
**Type 2-1: Samples 2, 4 and 5**

## Part II. Proteins

### Group Specific Component (Gc)

1. Tank Buffer: Tris/Glycine, pH 8.4
   21.8 gm Glycine
   4.5 gm Tris
   Dissolve in 900 mL water. Adjust to pH 8.4 with NaOH. Q.S. to a final volume of 1000 mL with distilled water.

2. Plate Buffer: Same as tank buffer.

3. Sample Preparation and Application: Extract blood stains with 3 drops of 6M urea (3.6 gm: 10 mL $H_2O$). Place all of extract in a small plastic centrifuge tube. Fill the tube halfway with chloroform. Vortex to mix. Centrifuge the mixture in a microcentrifuge at high speed. The chloroform washes down the debris in the stain.
   Extraction of stains with Hemolysate Reagent (Helena Cat. No. 5125) will eliminate aging bands which appear above the Gc bands. This will allow BC typing to be performed on old stains.*

4. Electrophoresis: 500 volts for 10 minutes; Initial amperage = 2 to 4 mA/plate.

5. Visualization (Immunofixation)
   a. After electrophoresis, the plates are covered with human Gc antiserum diluted 1:4 with physiological saline (2 mL serum + 6 mL saline). Allow plate to soak for 20 minutes.
      The diluted antiserum can be reused and is stable about 1 month after dilution, depending on the number of times used. Store in the refrigerator.
   b. Remove excess serum from plate and soak overnight in physiological saline (0.85%).
   c. Stain for 5 minutes in 0.2% Ponceau S (v/v in distilled water).
   d. To destain, place plates in a solution of 5% acetic acid, agitate for 15 minutes. Pour out this solution and add fresh acetic acid, agitate for another 15 minutes until the background becomes white. Plates may be washed overnight. The plates are then dried; the bands are dark pink on a white background.

      Two suggested sources for Gc antisera are:

      **Atlantic Antibodies**
      10 Nonesuch Road
      P.O. Box 1032
      Scarborough, ME 04074
      (207) 883-4154

      **In Vitro Research Sources, Inc.**
      P.O. Box 110
      Benson, MD 21018
      (301) 877-7110

      Figures 9 and 10 on page 22 illustrate the electrophoretic patterns of Gc.

### Haptoglobin (Hp)

The stain extract prepared for the Gc procedure may be used for haptoglobin determination on polyacrylamide gradient gel. However, before using the extract, prepare a 1:1 mixture with 40% sucrose.

*Personal Communication: Willard (Bud) Stuver, Supervisor, Serology/Biology Section, Metro-Dade Police Crime Laboratory, Miami, FL 33125.

# Section 3 Typing Dried Blood Stains Using Agar Starch Gels

## Isoenzymes

### Esterase D (EsD) and Glyoxylase I (GLO I)

This procedure should be performed within two days of receiving sample. Maximum sample age is 10 days. After 10 days the sample may be too deteriorated to produce a readable result.

1. Tank Buffer: HEPES, pH 7.5
   5.95 gm HEPES 0.05M (N-2 hydroxyethyl piperazine-N-2 ethanesulfonic acid)
   Dissolve in 400 mL distilled water. Adjust to pH 7.5 with 40% NaOH. Q.S. to final volume of 500 mL with distilled water.

2. Gel Buffer: 1:14 dilution of tank buffer, pH 7.4.
   Dilute 10 mL of tank buffer with 140 mL distilled water.

3. Starch/Agar Gel
   1% Agarose, Sigma Type V (low EEO)
   2% Hydrolyzed starch (Connaught Labs, Willowdale, Ontario, Canada).
   Available from Fisher, Cat. No. S-676.
   Dissolve 0.3 gm agarose and 0.6 gm starch in 30 mL gel buffer. After dissolving, de-gas the solution prior to pouring into 8 cm x 10 cm x 2mm deep plastic plates. Gel must sit overnight before use.

4. Sample Preparation and Application
   a. Soak dried blood stains for 15 minutes in 0.1N Clelend's reagent, pH 8.0. Run a known type 2-1 as a standard (GLO and EsD).
   b. Eight wells (1 mm x 4 mm, 3 mm apart) are cut in the gel 2 cm from the cathodic end with a metal template. Apply 5 µL of blood in each well using a micropipet.

5. Electrophoresis: 250 volts for 30 minutes.
   It is very important to keep the electrophoresis chamber very cool during the run. The tank buffer must be kept cold until ready for use. To keep the chamber at a low temperature, another lid or metal pan is filled with ice and placed on top of the closed electrophoresis tank. Plate is run face down on sponge wicks. Amperage at start 8 to 9 mA; at the end, about 10 mA.

6. Reaction Buffer
   a. EsD: 0.41 gm sodium acetate, anhydrous (0.05M). Dissolve in 100 mL distilled water. Adjust to pH 6.5 with 1% acetic acid.
   b. GLO I: 2.42 gm $NaH_2PO_4$, anhydrous (0.2M)
   1.31 gm $Na_2HPO_2$, anhydrous (0.072M)
   Dissolve in 100 mL distilled water, adjust to pH 6.2 with phosphoric acid.

7. Reaction Mixture and Visualization
   a. EsD: 4 mg MU-Acetate (4-methylumbelliferyl acetate)
   1.0 mL acetone (spectral grade)
   5 mL reaction buffer
   Dissolve MU-acetate in about 1.0 mL of acetone and immediately add 10.0 mL of the reaction buffer. Soak into Whatman 3 MM paper 4 cm x 10 cm. Lay this paper over a portion of the gel from the origin toward anode. Leave at room temperature for about 5 minutes. Remove the paper after incubation at room temperature and read EsD by using UV light.

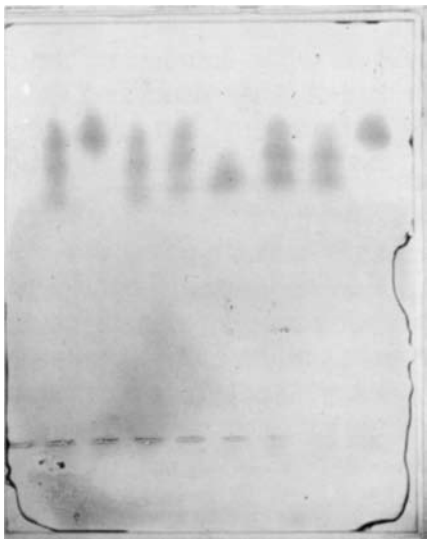
The EsD bands appear as white fluorescent bands on a dark background. Use an EsD 2-1 as a control. After visualizing EsD, mark the position of the number 2 band of the EsD pattern. The gel is covered with GLO I reaction mixture beginning with this position and incubated again.

b. GLO I: 20 mg reduced Glutathione
   50 µL 40% Methyl glyoxal
   7.5 mL GLO I reaction buffer

Soak mixture into Whatman paper cut to fit the size of the gel from the second band of the EsD pattern to the edge of the plate (approximately 6.5 cm x 10 cm). The filter paper is placed on the gel beginning at the cathode side, gently rolling down the rest of the paper, being sure no air bubbles remain under the paper. Incubate 30 to 40 minutes at 37°C.

Heat 0.2 gms agarose (Sigma Type II) in 20 mL distilled water until dissolved. Allow the agarose to cool to 60°C and then add 2 drops of iodine solution. (Iodine solution contains 1.65 gms of KI, 2.94 gms of $I_2$ dissolved in 30 mLs of water.) Mix the agarose/iodine mixture. This mixture will be a light orange color. After the electrophoresis gel has been incubated as above, the agarose/iodine mixture is quickly poured onto the gel starting at one corner of the gel allowing it to flow over the entire gel surface. The phenotype appears as blue bands on a light yellow background. The heterozygote (2-1) has three blue bands present and should be used as a control. A second agarose/iodine mixture may be applied to the gel if the blue bands appear weak in the first gel. This is done after removing the first agarose/iodine gel.

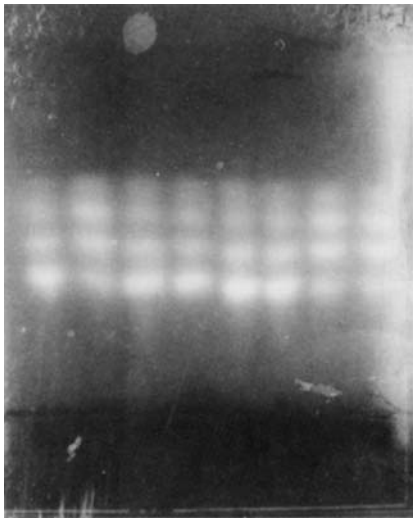
## Figure 11 – Glyoxylase-I (GLO-I)




**Electrophoretic patterns of three types of GLO-1:**

**Type 1-1: Samples 5**
**Type 2-1: Samples 1, 3, 4, 6 and 7**
**Type 2-2: Samples 2 and 8**

## Figure 12 – Esterase D (EsD)




**Electrophoretic patterns of two types of EsD:**

**Type 1-1: Samples 1, 3, 4, 5 and 6**
**Type 2-2: Samples 2, 7 and 8**

## Peptidase A (Pep A)(For Negroids)

1. Tank Buffer: Same as for GLO-I and EsD

2. Gel Buffer: Same as for GLO-I and EsD

3. Starch/Agar Gel: Same as for GLO-I and EsD

4. Sample Preparation and Application: Soak dried blood stains for about 15 minutes in distilled water and apply to gel in the same manner as GLO-I and EsD. Run a known type 2-1 as a standard.

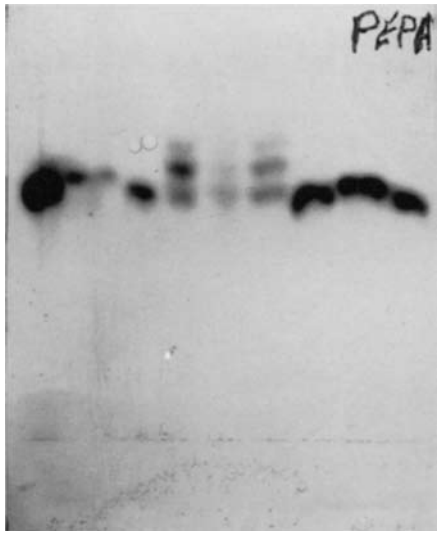
5. Electrophoresis: 250 volts for 45 minutes. Amperage at the start about 10 mA, at the end about 6 mA.

6. Reaction Buffer (Same as PGM reaction buffer)
   3.64 gm Tris, pH 8.0
   Dissolve in 500 mL of distilled water. Adjust to pH 8.0 with concentrated HCl.

7. Reaction Mixture
   40 mg L-valyl-L-leucine
   20 mg L-amino acid oxidase (crude snake venom)
   10 mg MTT (light sensitive)
   4.0 mg PMS (light sensitive)
   10 mL reaction buffer

8. Visualization: Overlay 0.2 gm agarose (Sigma Type II) dissolved in 10 mL of reaction buffer.
   Heat the agarose until dissolved. Allow the agarose to cool to 55°C before adding the reaction mixture. This is quickly poured over the gel. Incubate the gel for 1 hour at 37°C.
   The phenotypes appear as blue bands on a light yellow background.

## Figure 13 – Peptidase A (PepA)




**Electrophoretic patterns of three types of Pep A:**
**Type 1-1: Samples 1, 2, 3, 7, 8 and 9**
**Type 2-1: Samples 4 and 6**
**Sample 5 is a suspected 1 + 2-1 mix from a vaginal/seminal sample**

## Section 4 Final Notes

Titan® III and the Super Z Applicator System are fast, easy and practical for forensic investigation of many different proteins and enzymes. The examples in this booklet will give the investigative laboratorian specific techniques for some assays and the equipment and reagents are easily modified for other uses.

Please contact the authors at SEMO Regional Crime Laboratory or Helena Laboratories if you need assistance or have information to share.

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