TRAINING PROGRAM FOR BLOOD IDENTIFICATION AT CRIME SCENES FOR CRIME SCENE SEARCH SPECIALISTS AND FORENSIC MOLECULAR GENETICISTS NORTH CAROLINA STATE BUREAU OF INVESTIGATION

Table of Contents

Appendices:

1. PURPOSE AND SCOPE

The purpose of this manual is to provide a consistent training program for presumptive identification of blood at crime scenes by Crime Scene Search Specialists and Forensic Molecular Geneticists of the North Carolina State Bureau of Investigation. This program provides individuals with the theoretical background and the working knowledge to conduct independent crime scene analysis and effective expert witness testimony in the field of presumptive tests for blood via phenolphthalein and luminol testing, and for the Forensic Molecular Geneticists in the identification of human blood. Heavy emphasis shall be placed on quality assurance of all tests performed, data integrity via thorough documentation, and excellence in obtaining consistent and congruous results.

The training program detailed in this document provides the following:

- Theoretical knowledge of the principles of presumptive tests for blood (phenolphthalein and luminol).

- Working knowledge of how to conduct presumptive tests for the presence of blood.

- Theoretical and working knowledge of the analytical procedures and techniques to identify human blood (Forensic Molecular Geneticists only).

- The ability to perform independent, accurate, and consistent forensic analysis on crime scenes.

- The ability to provide effective expert witness testimony that includes, but is not limited to the presentation of presumptive tests for blood, and the identification of human blood (Forensic Molecular Geneticists only), and the defense of analytical conclusions.

2. REQUIREMENTS FOR QUALIFICATION

2.1 Prerequisites

Forensic Molecular Geneticists - Individuals must possess a strong scientific background and have course work in biology, chemistry, biochemistry and genetics. Individuals must complete the following courses at NCSU or their equivalent: Genetics 501, 502 (now combined to form Genetics 513), and either Genetics 560 or Biochemistry/Genetics 561.

Crime Scene Search Specialists - Individuals must possess a four year college degree and must be graduates of the SBI Academy.

2.2 Competency Tests

Individuals must pass a series of well defined competency tests. These tests are to determine the trainee's ability to correctly use presumptive tests for the identification of blood, and to identify human blood (Forensic Molecular Geneticists only).

2.3 Written Examination

A written examination shall examine the trainee's understanding of the theoretical and working knowledge of presumptive tests for blood and how they are applied at the crime scene.

2.4 Internship

The trainee will undergo an internship program where they will conduct luminol tests for blood at a minimum of ten (10) crime scenes under the supervision of a qualified agent/examiner. This will include writing a report and submitting it to the Training Officer for evaluation.

Forensic Molecular Geneticists will undergo an internship program in the laboratory where they conduct human blood identification tests under the supervision of a qualified agent/examiner.

3. INSTRUCTIONS FOR THE TRAINING OFFICER

3.1 This program is designed to provide each trainee with the theoretical background and working knowledge to reliably analyze forensic evidence at crime scenes utilizing presumptive tests for blood, and for Forensic Molecular Geneticists tests to identify human blood. Every topic listed in this manual is equally important; a deficiency in one area can lead to the failure of a successful analysis and/or defense of the analysis in a court of law. Therefore, the training officer must pay very close attention to detail and ensure that all quality assurance guidelines are being followed for every sample processed in the training program. By ensuring each trainee maintains a high degree of concentration and awareness during the performance of his/her training, the proper techniques will be learned and later successfully applied to actual casework.

3.2 The order of topics listed in this manual are not necessarily in the chronological order that the tests will be performed. It may be necessary to learn and perform some techniques out of order. In this case, it is the responsibility of the training officer to provide the trainee with a clear explanation of any missing points or steps and later logically tie everything together.

3.3 It is the responsibility of the Training Officer to point out pertinent scientific literature and technical manuals included in the bibliography to the trainee so that they may become familiar with these readings.

3.4 It is the responsibility of the Training Officer to explain potential safety hazards to each Trainee BEFORE performing a task that may involve said potential safety hazard.

4. INSTRUCTIONS FOR THE TRAINEE

4.1 The trainee is required to keep files on all work completed. These files should include but are not limited to the Training Manual Log Sheet, worksheets, and copies of reports issued in cases they participate in. These files will be checked periodically by the Training Officer, Crime Scene Search Specialist Coordinator, or the Special Agent In Charge of the Molecular Genetics Section.

4.2 The readings assigned are very important. While it is not necessary to memorize reagent recipes, it is necessary to become familiar with the principles of each test and the protocol and be able to perform all duties independently. The trainee is expected to become familiar with the

literature that pertains to the forensic analysis of blood identification testing that is included in the bibliography.

5. SAFETY ISSUES

5.1 There are many potential hazards that exist at crime scenes. While the exposure to all hazards can be minimized or avoided, it is the responsibility of the Training Officer to ensure the Trainee is aware of all potential hazards. These potential hazards include but are not limited to the following:

Infectious Agents

A. Viral agents, including HIV and Hepatitis
B. Bacteria, including sexually transmitted diseases
C. Fungi
D. Parasites

5.2 Safety Procedures

5.2.1 All Individuals participating in this program have already received safety awareness training at the SBI Academy, including First Aid and Bloodborne Pathogen training. All agents have access to the DOJ Safety Manual.

6. ASEPTIC TECHNIQUE AND CONTAMINATION CONTROL

6.1 This SBI Molecular Genetics Section uses the Polymerase Chain Reaction (PCR) technology which allows very small amounts of DNA to be amplified over a million times. Because of the sensitivity of this technique, contamination control is a very serious issue that must be emphasized and practiced with every sample, starting with the identification of forensic evidence at crime scenes. The SBI Evidence Handling Procedures must be strictly followed.

6.1.1 All items used in the identification, transfer and isolation of forensic samples must be sterile and/or free of contaminate DNA.

6.1.2 Gloves must be worn at all times while handling samples. This is to protect both the agent/examiner and sample.

6.1.3 Scissors, tweezers, and other instruments used for collection shall be cleaned or sterilized before and after each use.

6.1.4 Possible sources of DNA contamination.

7. DOCUMENTATION

7.1 Goals

7.1 To provide protocols for the preparation of reagents and performance of tests to ensure consistent, reliable results.

7.2 To provide a thorough record of events for each case.

7.2 Approved Technical Procedures

Technical Procedures for the phenolphthalein, luminol, and anti-human hemoglobin test are found in Appendix IV of this manual. The agent/examiner shall not deviate from any procedure without permission from the Special Agent In Charge of the Molecular Genetics Section. Any deviation (purposely or by mistake) from the protocol shall be thoroughly documented on the worksheet or notes at the time of occurrence.

8. RECEIVING AND HANDLING OF EVIDENCE

8.1 Goals

8.1.1 To obtain a working knowledge of factors and conditions that influence the deterioration of evidence as it relates to packaging, handling, storage conditions, and time.

8.1.2 To develop a thorough understanding of evidence handling procedures.

8.1.3 To develop a thorough understanding of the necessity for detailed comprehensive notes and adequate labeling of evidential materials.

8.2 Tasks

8.2.1 Read and become thoroughly familiar with the SBI Evidence Handling Procedures regarding receiving, identifying, and handling of evidence; as well as specific guidelines for handling biological evidence within the Molecular Genetics Section.

9. ANALYTICAL TECHNIQUES

9.1 Goals

9.1.1 To develop a basic understanding of the methodology and theory of the presumptive chemical procedures used to identify blood; and for Forensic Molecular Geneticists the species from which the blood may have originated.

9.1.2 To develop skills that will allow the trainee to independently and successfully analyze blood evidence found at crime scenes.

9.1.3 To become familiar with the sensitivity and limitations of the procedures used.

9.1.4 To develop a cognizant understanding of contamination issues and the steps necessary to avoid contamination.

9.1.5 To understand the use of controls during each procedure.

9.1.6 To become familiar with and understand the function of any buffers, solutions, or reagents used.

9.1.7 To become familiar with all documentation required.

9.2 Tasks

9.2.1 Understand and perform quality control checks necessary on buffers, solutions, reagents and test kits used.

9.2.2 Perform testing on various sample types which allow for ample testing for each analytical procedure used. The training samples will represent materials commonly encountered at crime scenes.

The Training Officer will initially present the trainee with a short lecture on the analytical procedure in question. The trainee is then given samples to practice on. Once a block of instruction is completed, the trainee will receive a written test and a set of competency samples to analyze. The exact number of samples will be determined by the training officer in accordance with experience and ability of the trainee. Upon successful completion of the block of instruction, the trainee will start on the next block of instruction.

9.2.3 Blocks of instruction the trainee will complete include:

9.2.3.1 Phenolphthalein testing, a presumptive chemical test

9.2.3.2 Luminol testing - a presumptive chemical test

9.2.3.3 Species origin testing using anti-human hemoglobin - Forensic Molecular Geneticists only

10. REPORT WRITING AND TESTIMONY

10.1 Goals

10.1.1 To develop the skills necessary to effectively record results from crime scene analysts, and to provide expert witness testimony in a court of law.

10.1.2 To develop a working knowledge of the terminology and presentation of analysis and results.

10.1.3 To become familiar with pertinent scientific literature.

10.2 Tasks

10.2.1 Read and understand pertinent scientific literature provided in the Bibliography (Appendix III). Trainees will be provided with copies of the literature.

10.2.2 Thoroughly understand and be able to accurately and concisely answer the questions in Appendix II.

10.2.3 To complete acceptable written reports on crime scenes attended with an experienced agent/examiner that is within the

constraints of accepted scientific practice and common sense.

10.2.4 Attendance at court to observe qualified agent/examiners testify.

11. INTERNSHIP PROGRAM

11.1 Goals

11.1.1 Once the trainee completes their competency tests, they are placed in a internship program with qualified agent/examiners. The purpose of this internship is to allow the newly qualified agent/examiner to work cases, but under the close supervision of a qualified examiner. The goal of this program is to allow the newly qualified examiner to work their first cases under the direct supervision of a qualified examiner and gain confidence in their analytical skills.

11.2 Tasks

11.2.1 The newly qualified agent/examiner will conduct the entire analysis of a more experienced agent/examiner's case, prepare the notes, and will write the report, just as if the case was theirs to work.

11.2.2 The more experienced agent/examiner will observe all results, initial the notes, and evidence, and review all work conducted. They will constructively critique the work conducted by the newly agent/examiner trainee and ask them questions about the crime scene that they could expect in court. The case will be assigned to the experienced examiner, and the experienced agent/examiner will sign the laboratory report. The crime scene notes or the laboratory notes cover sheet will contain a note that the case was worked as part of their internship program.

11.2.3 Trainees will undergo an internship program after their initial training. **This internship program will consist of a minimum of ten (10) crime scenes where luminol is used**

and which are processed under the supervision of an experienced agent/examiner. The Training Officer will complete an evaluation form on each trainee (see Appendix V). The evaluation form for Forensic Molecular Geneticists will be sent to the Special Agent In Charge of the Molecular Genetics Section. The evaluation form for Crime Scene Search Specialists will be sent to the Crime Scene Search Specialist Coordinator. The Training Officer can be an experienced Crime Scene Search Specialist or an experienced Forensic Molecular Geneticist (there is no reason that a Forensic Molecular Geneticist Trainee can't have an experienced Crime Scene Search Specialist as his/her Training Officer at a crime scene or vice versa).

11.2.4 Forensic Molecular Geneticists will undergo an internship program in the laboratory for the identification of human blood that will be no less than one month in length.

11.2.5 The Special Agent In Charge will decide when the Forensic Molecular Geneticist Trainee is ready for unsupervised crime scene work. The Crime Scene Search Specialist Coordinator will decide when the Crime Scene Search Specialist is ready for unsupervised crime scene work.

12. COMPETENCY TESTING

12.1. Goals

12.1.1 Upon successful completion of all blocks of instruction, the trainee will be given a series of competency tests that closely mimic forensic evidence. The trainee must score 100% accuracy on these tests. This test is the final one that the trainee must complete before being allowed to utilize the techniques covered in this block of training on crime scenes. This competency test is given upon completion of the laboratory training session and/or at the completion of the internship program. The competency test may be the processing of an actual crime scene under the supervision of a trained agent/examiner after completion of the internship program.

12.2 Tasks

12.2.1 Successful completion of the competency tests.

13. TRAINING RECORDS

13.1 Training records will be kept on all agent/examiners by the Special Agent In Charge of the Molecular Genetics Section or the Crime Scene Search Specialist Coordinator to include the Training Log (Appendix I), the written exam (Appendix II), the luminol evaluations (Appendix V) of not less than 10 scenes, and copies of the reports written in these cases by the trainee.

13.2 Final authority to certify the trainee as being competent to conduct independent testing using presumptive tests for blood at crime scenes will be made by the Special Agent In Charge of the Molecular Genetics Section or the Crime Scene Search Specialist Coordinator, depending on where the trainee is assigned.

APPENDIX I. NORTH CAROLINA STATE BUREAU OF INVESTIGATION:
Crime Scene Training Manual Log Sheet

Training Area	Date Completed Trainee's Initials	ing Officer's Initials
ıcation		
Safety		
1. Bloodborne Pathogens		
Presumptive Tests for Blood		
1. Lecture		
2. Readings		
Human Blood Identification Forensic Molecular Geneticists only		
1. Lecture		
2. Readings		
Quality Control and Documentation		
1. Use of controls		
Receiving and Handling Evidence		
1. Read SBI Evidence Procedures		
alytical Training		
Aseptic Technique and Contamination Control		
1. Cleaning of Equipment		
2. Protective Equipment		
3. Sources of contamination		
Training Area	**Date Completed**	**ing Officer's**

	Trainee's Initials	Initials
Blood Identification		
1. Presumptive Testing- Phenolphthalein		
1.1 Read and Understand Procedure		
1.2 Demonstration of Test		
1.3 Supervised Testing of Known Samples		
1.4 Competency testing		
2. Presumptive Testing - Luminol		
2.1 Read and Understand Procedure		
2.2 Demonstration of Test		
2.3 Supervised Testing of Known Samples		
2.4 Competency Testing		
3. Species Origin Testing- Forensic Molecular Geneticists only		
3.1. Read and Understand Procedures		
3.2. Demonstration of Test		
3.3 Supervised Testing of Known Samples		
3.4 Competency Testing		
Training Area	**Date Completed Trainee's Initials**	**ing Officer's Initials**

port Writing and Testimony		
ble to Draft a Satisfactory Report		
Witness Expert Testimony		
amiliar with Court Procedures		
mpetency Testing		
Successful Completion of Practical Competency Tests		
Successful Completion of Final Written Test		
se Internship Program		
Successful Completion of a Case Internship Program		
rtification		
All required training steps and records are complete		
Memo of successful completion of training has been issued and is attached		

APPENDIX II. NORTH CAROLINA STATE BUREAU OF INVESTIGATION:

1. Describe the basis for presumptive tests for blood in general.
2. Name two classes of substances that react with the presumptive tests other than blood.
3. Why is a three step procedure for the presumptive identification of blood better than a one step blood testing procedure?
4. When, why, and how is luminol used instead of the other chemical tests?

5. What substances will cause a false positive reaction with luminol?

General Questions For Forensic Molecular Geneticists

1. Explain the high dose hook effect. Why are we worried about it in these tests. How can one overcome this problem?
2. Describe the principle of the Heme Trace test cards and how they work. Make sure your explanation includes a discussion on how the C and T lines are created or visualized during the test run and how to interpret the results based on the C and T lines appearing (or not appearing).
3. You have gotten a positive result on a Heme Trace Card. What two other pieces of data (or observations) are required for you to identify human blood as being present?
4. Does the Heme Trace Card give specific results for human blood (absent all other results or testing methods)?

APPENDIX III. NORTH CAROLINA STATE BUREAU OF INVESTIGATION: Background and Reference Information

1. Bloodstain Identification

1.1 Presumptive tests

Presumptive tests, or catalytic tests, for blood center on the erythrocyte portion of the formed elements. Each of these red blood cells carries approximately 280 million molecules of hemoglobin, each possessing four heme units. A heme compound is represented by an iron center coordinated with four nitrogen containing compounds, each of which bind to one oxygen molecule to transport it in the circulatory system. This heme group acts like a peroxidase, an enzyme which can catalyze the oxidation of an organic compound by utilizing peroxide. While heme is actually a conjugated protein, and not an enzyme, it acts like a peroxidase to dissociate hydrogen peroxide into two hydroxyl free radicals, which are highly reactive and tend to oxidize organic substances. In catalytic testing, these organic compounds are color reagents which change in color when transforming from the reduced to the oxidized state. This technique allows for a quick visual screening of blood but should not be judged as a confirmation of the presence of blood. Presumptive tests are designed to be used in conjunction with confirmatory tests for blood if enough sample is available.

1.1.1 Luminol

Background

In 1928 Albrecht reported on the chemiluminescent properties of the compound 3-aminophthalhydrazide. In 1935 Hundress, Stanley, and Parker published a method for the preparation of the compound and named it luminol. In 1937 Specht published his findings on the application of lumionol to the forensic field. It was through his studies that the use of luminol as a chemiluminescent test for the presence of blood was advocated.

Luminol is a chemiluminescent presumptive test for the presence of blood. Although the exact mechanism of conversion from the reduced to oxidized state is not well understood, it is generally recognized that two tautomeric forms of the oxidized structure create the chemiluminescent qualities.

Once sprayed on a surface, a bluish light emission may be detected at concentrations as low as 0.1 PPM. Luminol is employed when no visible

blood is detected or other less sensitive presumptive tests have failed. It is also primarily used for large areas such as cars and houses.

The analyst should be particularly aware that false positives mayoccur on purified vegetable peroxidases, some metals, bleach, and chemicals. Therefore, care should be taken when interpreting the test results. Patterns visualized during the use of luminol should be photographed, if possible.

Luminol Photography:

Luminol reactions must be photographed in total darkness so that the only light to strike the film comes from the luminol reaction. To photograph the reactions follow the steps below:

1. Load a Nikon 35 mm camera with Tri-X Pan film and set the film speed (ASA) at 400.

2. Use a 50 mm f2 lens and set the aperture to its widest opening. Adjust the shutter speed to the “B” setting.

3. Mount the camera on a tripod in such a way that the lens is facing the object being photographed. Adjust the tripod length so that the entire viewfinder frame is used for the object photographed.

4. Place a six inch ruler with fluorescent tape at one inch intervals next to the pattern being photographed so that a 1:1 photograph can be made for direct comparison to a known object.

5. Use a shutter release cable. To maintain enough light, and thus a visual pattern, lightly spray the area with luminol while the shutter is open. Avoid over-spraying the area, spray just enough to see the pattern.

6. To ensure a good quality photograph, use varying exposure times. These exposure times will vary depending on how strong the reaction is.

<u>References</u>

Gaensslen, Ren (1989) <u>Sourcebook in Forensic Serology, Immunology, and Biochemistry, 2nd ed.</u>, National Institutes of Justice, pp. 112-114

Lytle LT, Hedgecock DG. (1978) Chemiluminescence in the visualization of forensic bloodstains, *Journal of Forensic Sciences*, 23: 550-562

Proescher F, Moody AM (1939) Detection of blood by means of chemiluminescence. *The Journal of Laboratory and Clinical Medicine* pp 1183-1189

White EH, Roswell DF (1970) The chemiluminesence of organic hydrazides. *Journal of the American Chemical Society* 3:54-62

Zweidinger RA, Lytle LT, Pitt cG (1973) Photography of bloodstains visualized by luminol. *Journal of Forensic Science* 18: 296-302

1.1.2 Phenolphthalein test

Background

The phenolphthalein test is a presumptive catalytic test for the presence of blood. The heme portion of hemoglobin possess a peroxidase-like activity which catalyzes the breakdown of hydrogen peroxide into free hydroxyl radicals. These hydroxyl radicals then oxidize the reduced phenolphthalin, producing a pink color.

To maintain this pink color, the reaction is carried out in alkaline conditions. If the pH is lowered to acidic conditions, the solution will again become colorless, but is in the form of phenolphthalein. The working solution is maintained in zinc to keep phenolphthalin in the reduced state. Use care in the preparation of the phenolphthalin, since flammable gas may be generated. For this reason an electric heating mantle is used. Also be cautious since zinc dust, in the presence of water, may act as a fire catalyst.

This test is particularly useful because there are less known false positives than other presumptive tests. The literature reports that certain plants including horseradish, tomato, turnip, and Jersulem artichoke possess elevated levels of peroxidase which may give a positive reaction with phenolphtalin. This false reaction may be eliminated by heating the filter paper to 100^{o}C for 30 minutes, which destroys the peroxidase activity. The literature also reports that bacteria which possess a high catalase activity may give a false positive reaction. If a pink color appears after the addition of phenolphthalin to the filter paper, but before the addition of the

hydrogen peroxide, then the presence of an oxidant is indicated. Any reaction that occurs more than 5 seconds after the addition of the hydrogen peroxide is considered a false positive and is not recorded. Metals and rust do not interfere with this testing. However, it may be slightly less sensitive than some other catalytic tests.

References

Gaensslen, RE (1989) Sourcebook in Forensic Serology, Immunology, and Biochemistry, 2nd ed., National Institutes of Justice, pp 103-105

Lee, HC (1982) Identification and Grouping of Bloodstains, in R. Saferstein, ed., Forensic Science Handbook, Vol. 1, Prentice Hall, Englewood Cliffs, N.J., pp 272-276

Blake, ET, Dillon DJ (1973) Microorganisms and the presumptive tests for blood. *Journal of Police Science and Administration* 1: 395-400

Higaki RS, Philp WMS (1976) A study of the sensitivity, stability, and specificity of phenolphthalein as an indicator test for blood. *Canadian Society of Forensic Sciences* 9: 97-102

Training Tasks:

1. Testing the effects of washing and heating on blood detection

Use fresh blood and make a stain on clean cotton sheeting. Subject a portion of the stain to each of the following conditions:

a. Wash in a washing machine and dry in the dryer
b. Wash in cold water in a sink, air dry
c. Wash in hot water in the sink, air dry
d. Wash by hand with soap, air dry
e. Soak in water overnight, air dry
f. Dry in a dryer, but don not wash first
g. Char on a microscope slide
h. Heat to 100^{o} C
i. Iron the stain

2. Sensitivity Testing

Take a fresh liquid bloodstain and make serial dilutions. Make stains from the dilutions on clean cotton sheeting and then air dry them. Test each stain with phenolphthalein.

3. Specificity Testing

Collect the following vegetables and rub them thickly over filter papers and test with phenolphthalein.

Horseradish	Pineapple	Red Grapes	Red Cabbage	Cantaloupe
Radish	Celery	Onions	Spinach	Lettuce
Carrots	Broccoli	Tomatoes	Peas	Raisins
Mushrooms	Artichoke	Turnip	Ketchup	Cucumber

Also collect and test cola stains, chicken blood, beef blood (from meats).

1.3. Human (or Species) Origin test - Anti-human Hemoglobin Test Forensic Molecular Geneticists Only

Background

The precipitin test is one method of distinguishing between human and animal blood. It utilizes the biological properties of antibody-antigen complex formation to allow a visual representation of a reaction. Antibodies are very large molecules and are represented by five classes of immunoglubulins, IgG, IgA, IgM, IgD, and IgE. An antigen is a substance which has the ability to produce an immunological response when introduced into a foreign animal. The antibodies are produced by a host animal when the animal is injected with a foreign protein containing

antigens and the host becomes sensitized. The immune system of the host recognizes the foreign antigen and produces antibodies to react with it in a very specific manner.

In common forensic testing, the antibodies to human antigens are raised in rabbits which results in rabbit anti-human antiserum. Dr. Uhlenhuth in 1901, presented evidence of the specificity for human antigens to only agglutinate with complementary antibodies. Therefore, if the antibodies in the anti-human antiserum comes in contact with human antigens, the specificity of the reaction allows for the formation of the human antigen-antibody complex and prohibits the formation of other non-specific complexes. In addition to testing for human antigens, the same test can be performed on a variety of animals. For example, goat anti-swine antiserum can be used to determine if a blood sample originated from a pig.

It should be noted that there is some anti-sera cross reactivity between the antigens in a closely related species. For example, ant-ram sera may cross react with goat and cow antigens. In humans, some monkeys or higher primates may produce a response. The Cappel rabbit antiserum to human serum will produce (I) a positive reaction in a 1:3 dilution of monkey blood in a 24 hour test (ii) a positive reaction up to a 1:100 dilution for human blood in a 24 hour test and (iii) a positive reaction up to 1:1000 for human blood in a 48 hour test.

The antigen-antibody reaction occurs in two steps. The first is called sensitization where the antigen and antibody form weak bonds, probably a combination of electrostatic, hydrophobic, and vander Waals interactions, and coordinate themselves for phase two. In this step, the complex is formed and the lattice structure begins to multiply which is representative of precipitation. This visualization step creates a white band which can be recognized for analysis.

Several methods have been developed to monitor the formation of the antigen-antibody complex including the Ring Test, Single Diffusion in One or Two Dimensions, or Double Diffusion in One or Two Dimensions, and the ABA OneStep Heme Trace Card. The latter is also referred to as the Ouchterlony, or immunodiffusion, method. While the different tests exhibit a range of sensitivity, some general characteristics are the same due to the antigen-antibody interaction mechanism.

A blood sample can fail to produce a precipitin band, also known as a false negative. This may occur if the sample is degraded due to age, heat, sunlight, chemical treatment with detergents, aluminum oxide, pulverized iron ore, or when mixed with some types of soil. Also note that the complex is best formed when the antigen and antibody are present in approximately equal concentrations. Either component present in excess can result in a weak reaction or a false negative.

With certain tests, several substances have been documented to exhibit a reaction which may mimic a true antigen-antibody reaction, also known as a false positive. These include aluminum and iron chlorates, aluminum chromate, salts of alkyl sulfonates and alkyl sulfates, peroxide, some dilute acids and bases, tannic acid, and spruce bark extract.

The ABA OneStep Heme Trace Card detects an antigen-antibody reaction. A small extract of a stain is placed into a well and the sample migrates across a strip to the test and control areas. If anti-human hemoglobin is present in the specimen, it will react with the mobile monoclonal antihuman hemoglobin antibody and a complex is formed. A polyclonal antihuman hemoglobin antibody is immobilized on the membrane in the test area which captures the complex so than an antibody-antigen-antibody sandwich is formed. The dye particle-hemoglobin complex concentrate in the test area. When the hemoglobin concentration in the sample is excessive, the pink dye particles will form a pink colored band. The hemoglobin antibody-dye conjugates cannot bind to the antibody in the test area, but are captured by an immobilized anti immunoglobulin antibody present in the control area. A pink band in this area indicates that the test performed properly. Therefore, a pink line must be formed in both the test and control areas for a positive result.

References

Gaensslen, RE, (1989) Sourcebook in Forensic Serology, Immunology, and Biochemistry, 2nd ed., National Institutes of Justice,. pp. 43-56

Lee, HC, (1982) Identification and Grouping of Bloodstains, in R. Saferstein, ed., Forensic Science Handbook, Vol. 1, Prentice Hall, Englewood Cliffs, N.J., pp. 283-297

Hochmeister, MD et al (1999) Validation studies of an immunochromatographic 1-step test for the forensic identification of human blood. *Journal of Forensic Sciences* 44:597-602

Spear, TF and Khoskebari, N (1999) The evaluation of ABAcard Hema Trace. Tieline 22:42-45

Kristaly, A and Smith, DAS (1999) Validation of the OneStep ABAcard HemaTrace for the rapid forensic identification of human blood.

Training Tasks:

1. Test a wide variety of human and animal bloods that will be provided by the training officer.

APPENDIX IV. NORTH CAROLINA STATE BUREAU OF INVESTIGATION: Approved Technical Procedures

PHENOLPHTHALIN

1. Reagents:

   Preparation of Stock Solution

Phenolphthalein	4 grams	(Fisher -79)
Sodium Hydroxide pellets	40 grams	(Sigma S 5881)
Zinc dust	20 grams	(Fisher Z-15)
Distilled water	1000 ml	
Absolute Ethyl Alcohol	Bring up to 1200 mls (USI Chemicals)	

   Add each reagent of the stock solution to a 5000 ml round bottom refluxing flask. Attach the condensing column to the flask and heat the flask with an electric heating mantle. Reflux the solution for approximately two to three hours, until the solution is colorless. After allowing the solution to cool down; decant the liquid into a measured container and use absolute ethyl alcohol to bring the total volume to 1200 mls. Add enough zinc dust to cover the bottom of a dark bottle and pour the phenolphthalein solution into the bottle. Label, date the bottle and store it in the refrigerator at 4^{o}C. Phenolphthalein solution shelf life is 6 months when kept in a refrigerator. Aliquots kept at room temperature expire after 30 days.

   An aliquot of phenolphthalein solution is kept at each analyst's bench. A fresh aliquot is prepared the first working day of each month.

   Additional reagents needed for the test include:
   Absolute Ethanol (USI Chemicals) and 3% Hydrogen Peroxide {prepared from stock 30% H_2O_2} (Fisher H-327).

2. Sample description:

   The blood stain may or may not be visible to the naked eye.

3. Standards and controls:

   Standards should include a known blood stain (positive control) and a known blood-free sample (negative control). These controls will be run each day of use and recorded in the field or laboratory notes.

4. Procedure:

   To conduct this test, either rub the suspected stain with a folded piece of filter paper or a clean cotton swab. Add the following reagents in order; one drop of ethanol, one drop of phenolphthalein, and one drop of 3% H_2O_2 onto the sample rubbing. A positive reaction is indicated by the development of a pink color within 5 seconds. Reactions occurring after 5 seconds, or before the addition of the hydrogen peroxide are inconclusive.

   NOTE - Phenolphthalein is only a presumptive test for blood and can give reactions for substances other than blood.

5. References:

   Blake ET, Dillon DJ (1973) Microorganisms and the presumptive tests for blood. J Police Science and Administration 1: 395-400

   Higaki RS, Philp WMS (1976) A study of the sensitivity, stability and specificity of phenolphthalin as an indicator test for blood. Can Soc Forensic Sciences 9: 97-102

   Gaensslen RE (1983) Sourcebook in forensic serology, immunology and biochemistry. The National Institute of Justice, Washington, DC p 103-105

6. Safety Precautions

   Use care during refluxing of phenolphthalein solution.

manuals\2000manuals\procedures\bodyfluid\phenol.wpd

LUMINOL TEST (Albrecht Reaction)

1. REAGENTS:

   5 - Amino - 2,3 dihydro - 1,4 phthalazinedione or 3 aminophthalhydrazide (0.5 g)(Aldrich 12,307-2)
   Sodium carbonate (Na_2CO_3) 25g (Fisher S-263)
   Sodium perborate ($NaBO_3$ $4H_20$) 3.5g (Aldrich 24,412-0)
   Water (distilled) 500mls

   WORKING REAGENT:

   Weigh out each chemical separately. Place the luminol (5-amino-2,3 dihydro - 1,4 pthalazinedione) and sodium carbonate (Na_2CO_3) in a labeled ziplock bag. Place the sodium carbonate perborate ($NaBO_3$) in a separate labeled ziplock bag.

   Just prior to use, add the contents of both bags into the distilled water and shake until all of the reagents have dissolved and are in solution. Transfer the solution into a hand pump spray bottle.

2. SAMPLE DESCRIPTION:

   The sample size may vary from trace amounts which cannot be seen all the way up to large quantities of blood which could be present as well as smears, wipes and other patterns which may be left.

3. STANDARDS AND CONTROLS:

   A penny or blood stain is sprayed to ensure that the chemicals are working properly. Note: Luminol is only a presumptive test and can give a reaction for things other than blood.

4. PROCEDURE:

   Before proceeding make sure permission has been given via consent or a search warrant. Check the spray bottle to ensure a fine mist is being expelled. Usually one starts at a place where the assault has occurred. Avoid walking over an area that has already been sprayed. Taking this precaution will eliminate unnecessary tracking up of the crime scene.

Always spray in front of you and walk backwards while spraying, keeping others behind you. Look for areas where a brightly lit reaction occurs for 5 to 20 seconds. Test these areas by taking a filter paper rubbing of the area and doing a phenolphthalein test on the rubbing.

Record only the results that give a positive reaction to both the phenolphthalein test and the luminol test.

The luminol reactions obtained may be photographed with a fluorescent ruler present for later comparison.

5. REFERENCES:

Zweidinger RA, Lytle LT, Pitt, CG (1973) Photography of bloodstains visualized by luminol. J. Of Forensic Sciences 18: 296-302

Proescher F, Moody Am (1939) Detection of blood by means of chemiluminescence. The Journal of Laboratory and Clinical Medicine 1183-1189

Blake ET, Dillon DJ (1973) Microorganisms and the presumptive tests for blood. J Police Science and Administration 1: 395-400

6. SAFETY PRECAUTIONS

Use gloves when handling powder and liquid solutions.

manuals\2000manuals\procedures\bodyfluid\luminol.wpd

Anti-Human Hemoglobin Testing

2. Pipette 3 drops (150µl) of Hema Trace Extraction Buffer into 1.5ml centrifuge tube.

3. Using sterile scissors, cut at least a 1/4in. fiber from your sample and place into the centrifuge tube using sterile forceps.

4. Then allow the sample to extract for a minimum of 5 min.

5. For weak or older samples, analysts may desire to use a larger quantity of material and a longer extraction time.

6. After completing the extraction process, pipette 3 drops (150µl) of liquid buffer into the well marked S on the OneStep ABAcard.

7. When there is a positive reaction, two lines will appear, one line in the area marked C for control and one in the area marked T for test. If the reaction is negative then only one line appears in the area marked C. In order to determine that the test is negative a full ten minutes must pass after the liquid is added to the card. If no lines appear the test must be repeated.

8. Beware of a high dose hook effect. If a sample gives a negative or extremely weak positive reaction, and your preliminary (phenolphthalein) testing indicates you would expect a strongly positive result, repeat the testing in the following manner. Add 3 drops of Hema Trace Extraction Buffer to the prior extract tube, pipette the fluid up and down a couple of times to mix the fluid thoroughly, and re-run the test. If necessary, one could repeat the dilutions again in this manner, or do a serial dilution of a new extract.

9. Since validation studies have shown that anti-human hemoglobin reactions were obtained from body fluids other than blood (e.g. urine), then in order for one to identify human blood, both the phenolphthalin and anti-human hemoglobin test must be positive.

manuals\2000manuals\procedures\bodyfluid\antihb1.wpd

APPENDIX V. NORTH CAROLINA STATE BUREAU OF INVESTIGATION: Luminol Evaluation Form

Trainee _____

Training Officer _____

Date _____

Location_____

Agency File No. _____

Upon completion of a luminol crime scene with a trainee, please check the areas that were successfully performed by that individual.

___Adequate visual search prior to luminol.

___Collected blood prior to luminol, if required.

___Collected blood using proper techniques.

___Checked to determine all reagents react properly.

___Proper phenolphthalein application.

___Proper spraying technique.

___Sprayed area in an organized manner.

___Proper interpretation.

___Wrote acceptable report.

Comments:

cc: Trainee
SAC Molecular Genetics Section or Crime Scene Search Specialist Coordinator