Validation Study of the *Seratec HemDirect Hemoglobin Assay* for the Forensic Identification of Human Blood

Amanda Misencik[1] and Dale L. Laux[2]

Introduction

The *Seratec HemDirect* kit was first developed for the determination of occult blood in stool samples and in the early detection of colon cancer. However, this study examines the forensic use of the product in the identification of traces of human blood in forensic material. The *Seratec HemDirect Hemoglobin Assay* is a one-step chromatographic immunoassay test for the detection of hemoglobin. Hemoglobin reacts with a monoclonal murine anti-human hemoglobin antibody, which binds to form an antigen-antibody complex in the fiber pad after a sample has been loaded. Through a capillary effect in the membrane, the complex moves towards the test and control regions.

A second monoclonal murine anti-human hemoglobin antibody is immobilized at the test region on the membrane. When a sample containing human hemoglobin passes over, the antigen-antibody complex binds to the immobilized monoclonal antibody forming an antibody-antigen-antibody complex. The binding and formation of this complex is indicated by a red line in the test region. Additionally, the test features an internal control containing immobilized polyclonal goat anti-rabbit antibodies, which bind to gold-labeled rabbit-antibodies that were present in the fiber pad. This binding results in the formation of a red line in the control region and indicates a valid test.

The manufacturer lists the sensitivity of the *HemDirect* test at 40 ng/mL to 500 µg hemoglobin/mL. At higher concentrations a high dose hook effect is said to occur, resulting in a decrease in intensity of the test result line. The test is specific for human hemoglobin, but has shown cross reactivity with the blood of primates and ferrets [(1)] . Humans, some primates and ferrets share a common amino acid sequence (TNAVAHV) in the alpha chain of hemoglobin. This sequence can be responsible for the production of monoclonal antibodies, which are the primary component of an immunochromatographic test [(2)] . Previous studies have been executed using an immunochromatographic test in the determination of human blood, most notably Hochmeister [(2)] and Hermon [(3)], who tested the *Hexagon OBTI* kit, a precursor to the *Hemdirect Hemoglobin Assay* kit.

Methods

All tests were preformed using the *Seratec HemDirect Hemoglobin Assay*, test lot 15191 and buffer lot 10656.

[1]Student, Defiance College, Defiance, Ohio 43512
[2]Forensic Scientist, Ohio Attorney General's Office, Ohio BCI, Richfield, Ohio 44286

Specificity and Sensitivity

Hemoglobin (Sigma H7379-1G, Lot 039H7605) was prepared at a concentration of 1mg/mL. Using this hemoglobin standard, subsequent dilutions were prepared using deionized water, resulting in concentrations of 500, 250, 125, 62.5, 31.25, 16.5, 8.25, 4.13, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.0165 µg hemoglobin/mL. The hemoglobin dilutions were first tested by adding 100 µL to the test sample well and recording results after 10 minutes. The dilutions were additionally used in preparing 5.0µL samples on approximately 1x1mm cloth. The samples were dried, cut out, and extracted for 30 minutes in *HemDirect* buffer. Three drops (100 µL) of sample were added to the sample well and results were recorded after 10 minutes. A separate set of hemoglobin dilutions was prepared with concentrations of 80, 60, 50, 40, and 30 ng hemoglobin/mL. 100 µL of each dilution was added to the test sample well and results were recorded after 10 minutes.

Using whole blood (EDTA tube), dilutions with concentrations down to 1/500,000 were prepared with deionized water and 50 µL samples were added to cloth. The samples were dried, 1/4 portions were cut out, and then extraction was carried out in *HemDirect* buffer for 30 minutes. Three drops of the sample in buffer were added to the test sample well and results were recorded after 10 minutes. A separate set of dilutions from whole blood including: ½, 1/3, ¼, 1/5, 1/6, 1/8, 1/10, 1/12, 1/14, 1/16, 1/20, 1/24, 1/28, 1/30, 1/32, 1/40, 1/48, 1/56, 1/60, 1/64, 1/80, and 1/100 were prepared with deionized water. The dilutions were added to cloth in 50 µL samples and set to dry over night. A 1/4 portion of the sample was cut out and extracted in *HemDirect* buffer for 30 minutes. Three drops of each dilution was added to the test sample well and results were recorded after 10 minutes. The remaining samples on the cloth were used in the degradation study.

A swab of whole blood (EDTA) was taken, dried, and a series of serial dilutions were prepared with *HemDirect* buffer. The whole blood swab was extracted in buffer and then dilutions were made with this solution. Dilutions of ½, 1/8, 1/24, 1/80, 1/240, 1/400, 1/800, 1/1600, 1/4800, and 1/9600 were prepared. 100 µL of the samples were added to the test sample well and results were recorded after 10 minutes. This test was used as a control for the environmental study, to estimate the dilutions of swabs that would test positive for hemoglobin.

Animal blood samples were obtained on slides from the Cleveland Metroparks Zoo from the following species: Chimpanzee (*Pan troglodytes*); Gorilla (*Gorilla gorilla*); Wolf (*Canis lupus*); Clouded Leopard (*Neofelis nebulosa*); Orangutan (*Pongo pygmaeus*); Siamang (*Hylobates syndactylus*); Zebra (*Equus burchelli*); Black Howler Monkey (*Alouatta caraya*); Hamadryas Baboon (*Papio hamadryas*); Domestic Goat (*Capra hircus*); Rabbit (*Oryctolagus cuniculus*); Merino Sheep (*Ovis aries*); Guinea Pig (*Cavia procellus*); Fennec Fox (*Vulpes (Fennecus) zerda*); Siberian Tiger (*Panthera tigris altaica*); Warthog (*Phacochoerus africanus*); Swamp Monkey (*Allenopithecus nigroviridis*); Wolf's Guenon (*Cercopithecus wolfi*); European Polecat/Ferret (*Mustela putorius*); Snow Leopard (*Uncia uncia*); Prevost's Squirrel (*Callosciurus prevostii*); Persian Leopard (*Panthera pardus saxicolor*); Mini Horse (*Equus caballus*); Patas Monkey (*Erythrocebus patas*); Mini Donkey (*Equus asinus*); and Lion (*Panthera leo*). The animal blood was prepared as smears on glass slides and samples were taken by swabbing a small portion, to obtain a blood sample covering the swab's tip. The swabs were dried and the tip

containing blood was cut off for extraction. The extraction was carried out for 30 minutes in *HemDirect* buffer. Three drops (100µL) of sample in buffer were added to the test sample well and results were recorded after 10 minutes.

Urine, semen, saliva, and perspiration were collected on cotton swabs from volunteers. The tip from each swab was cut off and extracted in 2 mL of *HemDirect* buffer for 30 minutes. Testing was done using 3 drops (100 µL) of sample in buffer and results were confirmed after 10 minutes.

Degradation

Two separate experiments were conducted to see the effects of the environment (heat, sunlight, rain, etc.) on exposed bloodstains. In the first, whole blood samples were swabbed onto different surface materials in five locations. The sites were (1) a metal dumpster with rust, (2) a hot air vent, (3) a metal doorframe, (4) brick/stone on the outside of a building, and (5) a wooden frame (Figures 1-5). Every week for a four week period, a swabbing from each location was taken. The sample covered the tip of a cotton swab, which was then cut off and set in *HemDirect* buffer for 30 minutes to extract. Three drops (100 µL) of sample was added to the test sample well for each and results were recorded after 10 minutes. After one and two weeks an additional test was done with the most reactive samples. Dilutions of 1/1000 were made using *HemDirect* buffer and 3 drops were added to the test sample well. Results were obtained after 10 minutes.

Figures 1-5. Locations of samples for degradation study

The second degradation study was performed using the whole blood (EDTA) samples that had been prepared on clothing in the specificity and sensitivity study. Four strips of cloth containing the whole blood and dilutions of ½, 1/3, ¼, 1/5, 1/6, 1/8, 1/10, 1/12, 1/14, 1/16, 1/20, 1/24, 1/28, 1/30, 1/32, 1/40, 1/48, 1/56, 1/60, 1/64, 1/80, and 1/100 were set on a metal air vent outside. For a four week period, a quarter portion of the faintest stain was collected for testing. Extraction was done using *HemDirect* buffer for 30 minutes and results were obtained after 10 minutes. Testing was repeated with the next dilution until a positive result was reached indicating the minimum concentration that could be detected.

Aged Blood Stains

Blood samples that had been stored at room temperature from the years 1974, 1984, and 1989 were obtained. The blood was on strands of fabric and was extracted by setting in *HemDirect* buffer overnight in the refrigerator, for a total extraction time of approximately 24 hours. For testing, 3 drops (100 µL) of the sample in buffer was added to the test sample well and results were recorded after 10 minutes.

Reproducibility

From the aged blood extractions that had been prepared, two of the samples were obtained to test the reproducibility of the test results. Type O and Type AB blood from the year 1974 were used. For each blood sample, four separate testing cassettes were used and to each cassette, 3 drops (100 µL) were added to the test sample well. Results were obtained after 10 minutes.

Results

Specificity and Sensitivity

The *HemDirect* kit was found to be specific for human blood and hemoglobin in all tests performed. In the tests using animal blood, positive results were observed in higher primate species and in the ferret, including some weak positive test lines. When testing other body fluids it was determined that trace amounts of blood will test positive. In regard to this study, saliva and semen were samples that tested positive for hemoglobin. In all tests, perspiration always showed a negative result.

Regarding sensitivity, the manufacturer lists 40 ng hemoglobin/mL as detectable and in this study, a weak positive at this concentration was observed. A positive test line for a weak concentration of hemoglobin was detected in a 62.5 ng hemoglobin/mL dilution made from hemoglobin (Sigma H7379-1G, Lot 039H7605) standard. Whole blood (EDTA) from a swab was diluted to 1/9600 with positive results being observed. In fresh whole blood (EDTA) stains made on clothing, samples diluted to 1/100 react positively. In reference to the high dose hook effect, the onset of this result was observed at concentrations of 500 ug and 1 mg hemoglobin/mL. For these concentrations, the strength of the test line had weakened and samples with a higher concentration of hemoglobin could lead to false negatives. Additionally, whole blood can not be added to the test device as a sample; because of its viscosity, the sample is unable to pass through the membrane.

Species	Animal Identification Number	Result
Chimpanzee	851113	+
Chimpanzee	840711	+
Gorilla	940815	+
Gorilla	940818	+
Orangutan	840408	**weak**
Orangutan	930321	-
Orangutan	881111	+
Siamang	880211	**weak**
Hamadryas Baboon	790408	-
Ferret	010329	+
Ferret	980635	**weak+**
European Polecat (Ferret)	010329	-
Black Howler Monkey	991105	-
Swamp Monkey	M11201	-
Patas Monkey	980586	-
Wolf	961203	-
Clouded Leopard	880911	-
Zebra	900307	-
Domestic Goat	M10521	-
Rabbit	980595	-
Warthog	900404	-
Merino Sheep	991230	-
Guinea Pig	M40105	-
Fennex Fox	M50305	-
Siberian Tiger	MM0319	-
Snow Leopard	MM0329	-
Prevost's Squirrel	970208	-
Wolf's Guenon	M21006	-
Persian Leopard	970822	-
Mini Horse	870503	-
Mini Donkey	MM0327	-
Lion	930302	-

Table 1. Results from animal blood testing

Degradation

In the study of blood samples on exterior surfaces, all samples were positive after two weeks of exposure to the environment. However, after four weeks of exposure, significant deterioration was seen in four of the five samples. Table 2 contains the results of the testing. In regard to collection of test samples, those obtained from metal surfaces were difficult to swab and yielded weaker results as the time interval increased. Wood and brick proved to be the best surfaces for sample collection and yielded continuous positive results during the four week period.

Sample Location	1 Week	2 Weeks	3 Weeks	4 Weeks
(1) Dumpster	+	+	**weak**	**faint +**
(2) Vent		+	**weak**	-
(3) Doorframe		**weak**	-	
(4) Brick	+	+	+	-
(5) Wood	+	+	+	+
1/1000 dil. Dumpster	+	-		
1/1000 dil. Brick	**weak +**			
1/1000 dil. Wood	**weak +**	-		

Table 2. Results from degradation study of blood samples

Whole Blood Dilution	1 Week	2 Weeks	3 Weeks	4 Weeks
Whole Blood				+
½			+	**weak +**
1/3			-	
¼			-	
1/5		**weak +**		
1/6				
1/8		-		
1/10				
1/12		-		
1/14		-		
1/16		-		
1/20	**weak +**			
1/24				
1/28				
1/30				
1/32				
1/40	-			
1/48				
1/56				
1/60	-			
1/64				
1/80	-			
1/100	-			

Table 3. Results from degradation study of blood samples on clothing. The shaded areas were not tested but assumed to be positive based on the results from the stronger dilution testing as a weak positive .

The study using whole blood (EDTA) stains on clothing showed positive results for whole blood over a four week period. However, the stain dilutions prepared showed significant deterioration after one and two weeks of environmental exposure. By weeks three and four, only whole blood and a ½ dilution of whole blood showed positive results. It's hypothesized that

large amounts of rainfall and heat lead to the breakdown of hemoglobin on the cotton fabric. Table 3 contains results from the four week period when the test was performed.

Aged Blood Stains

Of the four aged blood stains tested from 16, 21, and 31 years ago, all tested positive for hemoglobin. The 24 hour extraction period in buffer worked sufficiently to obtain a sample for testing.

Reproducibility

The Type O blood from 1974 tested as a weak positive when originally tested. When the same sample was added to four separate test cassettes, a weak positive result was observed in all of them with no variation. The Type AB blood from 1974 tested as positive when originally tested. The sample was then tested in four separate test cassettes and the same positive results were observed in all of them with no variation.

Discussion

The results from this study show that the Seratec HemDirect Hemoglobin Assay is an applicable test kit for the determination of human blood in the forensic field. The HemDirect kit is human or primate specific, although has a cross-reactivity with ferret blood, and has a broad sensitivity range for detecting varying concentrations of hemoglobin. The test kit is efficient in detecting aged blood samples at least thirty years old and consistently yields the same result.

Acknowledgements

We wish to thank the Cleveland Metroparks Zoo for providing the animal blood samples used in this research, particularly Linda DeHoss at the Animal Hospital for her assistance in the procurement of the samples.

References

1. Product Literature. Seratec HemDirect Hemoglobin Assay. Seratec Diagnostica Gesellschaft fur Biotechnologie mbH, Ernst-Ruhstrat-Str. 5, D-37079 Gottingen, Germany, 2004.
2. Rowley BO. Commentary on Hochmeister. Validation studies of an immunochromatographic 1-step test for the forensic identification of human blood. J Forensic Sci 1999; 44:1323.
3. Hochmeister MN, Budowle B, Sparkes R, Rudin O, Gehrig C, Thali M, Schmidt L, Cordier A, Dirnhofer R. Validation studies of an immunochromatographic 1step test for the forensic identification of human blood. J Forensic Sci 1999; 44: 597-602.
4. Hermon D, Shpitzen M, Oz C, Glattstein B, Azoury M, Gafny R. The use of the hexagon obti test for the detection of human blood at crime scenes and on items of evidence. Part 1: validation studies and implementation. J Forensic Ident 2003; 53: 566-575.
5. Domestic ferret issues in California, California's plants and animals. California Department of Fish and Game, 29 March 2005. http://www.dfg.ca.gov/hcpb/species/nuis_exo/ferret/ferret_issues_1.shtml
6. PIR-International Protein Sequence Database. National Biomedical Research Foundation, Georgetown University Medical Center (DC), 2005. http://pir.georgetown.edu