VALIDATION OF ABAcard™ HemaTrace® KITS

TO DETERMINE THE PRESENCE OF HIGHER PRIMATE HEMOGLOBIN IN BLOODSTAINS.

ABAcard ™HemaTrace Kit

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1. Introduction .

DNA amplification technology enables forensic scientists to profile a victim or suspect from small amounts of biological tissues found on evidence or at crime scenes. Blood is one of the sample types utilised for profiling, however if these samples fail to provide a DNA profile it can be important to determine that the sample was in fact human blood.

The current screening test for blood in the Evidence Recovery Section is the Hemastix Test Strip. Hemastix are used in Evidence Recovery as an indication that visible stains are the result of bloodstaining. However, Hemastix is not a human or blood specific test and other oxidising agents can provide false positives. Therefore this method can only suggest the presence of blood, particularly in the instance of weak Hemastix positive stains.

There are occasions where a sample is Hemastix positive, but no human DNA has been detected. In these cases it may be pertinent to determine if the sample is of human origin. To determine the species of origin of the sample an Ouchterlony Immunodiffusion test may be performed. These assays are carried out against a number of species anti-serums, including anti-human hemoglobin, to determine the species of origin of the suspected bloodstains.

This paper studies the validity and practicality for the use of the ABAcard™ HemaTrace test to complement the current presumptive Hemastix test, and as an alternative to the Ouchterlony method to confirm the presence of human (higher primate) hemoglobin. For most sample types the HemaTrace test can be carried out simply and efficiently in less than 30min compared to the time consuming and labour intensive Ouchterlony test. This validation also compares the HemaTrace Recommended Protocols with methods designed to optimise Evidence Recovery requirements.

#2. Materials and Methods .

1. ABAcard™ Hematrace® Test Kits
   - Kits contain 25 Test cards and one transfer pipette (sealed and desiccated in a foil pouch) and 25 tubes containing 2mls of Buffer.

2. Blood
   - Human blood — Provided by laboratory donors (ouch) from staff at the Adelaide Forensic Science Centre.
   - Animal Blood: — From Forensic Science –80C° freezer storage, Dr David Schultz of Adelaide Zoo Veterinary Department: and local Veterinary practices.
   - Bloodstains: — Bloodstains were prepared by depositing freshly taken blood on washed cotton cloth and air dried for 24hours.
   - Aged bloodstains — These were accessed from laboratory storage kept at room temperature.

3. Sample Extraction Procedure

Standard Extraction Procedures as detailed in the Technical Information Sheet (see Appendix 1) were generally adhered to, with some variation as listed in the following Validation Studies. All extractions were carried out at room temperature with the time period being the incubation time of the sample in the extraction solution.

4. Sample Test Methods

Standard Testing Procedures as detailed in the Technical Information Sheet (see Appendix 1) were adhered to.

- 150µl or 4drops were added to the test well as indicated
- Results were recorded at various intervals up to the maximum 10 minutes.

#3. ABAcard™ HemaTrace Test .

The HemaTrace test exploits antigen/antibody reactions and protein chromatography methods, targeting human hemoglobin (hHb) that is specific to red blood cells. The cards are compact, disposable, self-contained units that are individually packaged with disposable pipettes. The test has an optimum pH range of pH1 to pH9.

They have a stationary phase that is flanked top and bottom by absorbent membrane material. The bottom membrane is located in the sample well and allows the sample solution to migrate through the stationary phase. The stationary phase has been impregnated with mobile dye-tagged antibodies located near the sample well and immobilised antibodies located in the regions designated “T”(Test area) and “C”(Control area).

The mobile antibodies are monoclonal, anti-hHb conjugated-dye antibodies that to bind to human (higher primate) hemoglobin molecules forming a soluble, mobile complex.

The immobilised antibodies located in the “T”area are polyclonal anti-hHb-antibody antibodies that capture the mobile hHb-complexes as they migrate in the solution. Mobile monoclonal antibodies that are not bound to hemoglobin are not caught at this point and continue to move up the test screen.

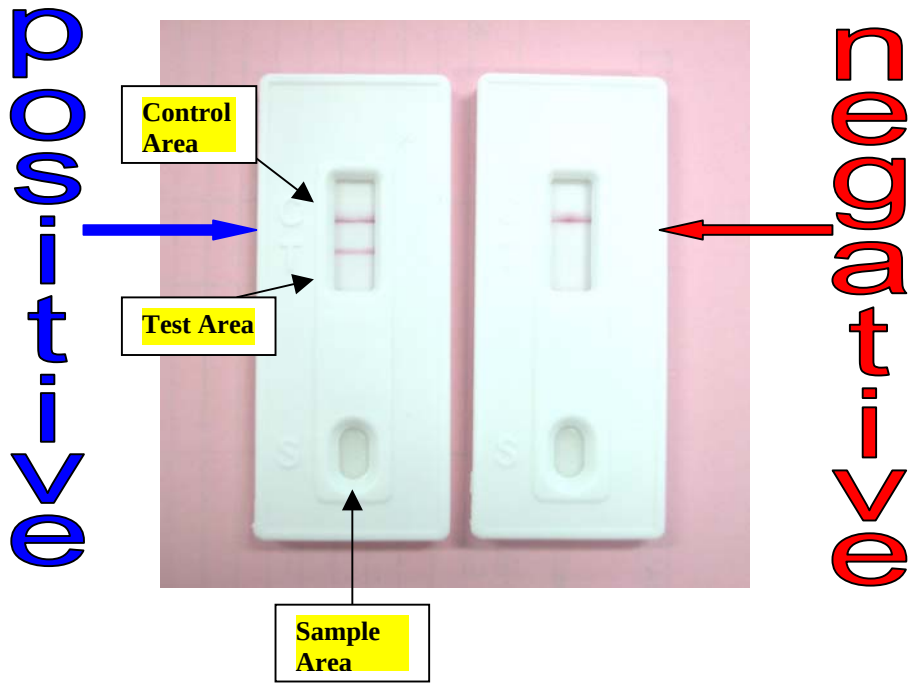
The immobilised antibodies located in the “C” area are polyclonal anti-immunoglobulin antibodies that will bind the excess mobile dye-tagged antibodies. The concentration of these molecules causes the appearance of the characteristic pink line in the control region to indicate that the test was successfully executed. If a line fails to appear in this region, the ABAcard HemaTrace test is faulty and should be recorded as invalid.

A positive result is recorded when there are two lines observed; one in the control area and one in the test area. This indicates that the sample hemoglobin levels are above 0.05µg/ml. A line observed only in the control area indicates that the concentration of hemoglobin in the sample is below 0.05µg/ml and is recorded as a negative result. (Refer to image below)

The fundamental action of the card is the 'lock and key' mechanism of the antigen/antibody interactions that is highly specific and selective giving the HemaTrace a high sensitivity level. Positive results may be recorded at any point up until the manufacturers recommended ten-minute time limit as possible false negatives are possible after this time.

The High Dose Hook Effect

False negative results may occur due to the prozone or "high dose hook effect". This can occur when samples have an excessively high concentration of hemoglobin molecules that inhibit the binding of the mobile hHb-antibody complexes to the stationary antibodies. The overloaded hHb becomes a competitive inhibitor for the anti-hHb-antibody complex, preventing binding with the immobilised antibodies in the "T" area.



HemaTrace Test Action

KEY:

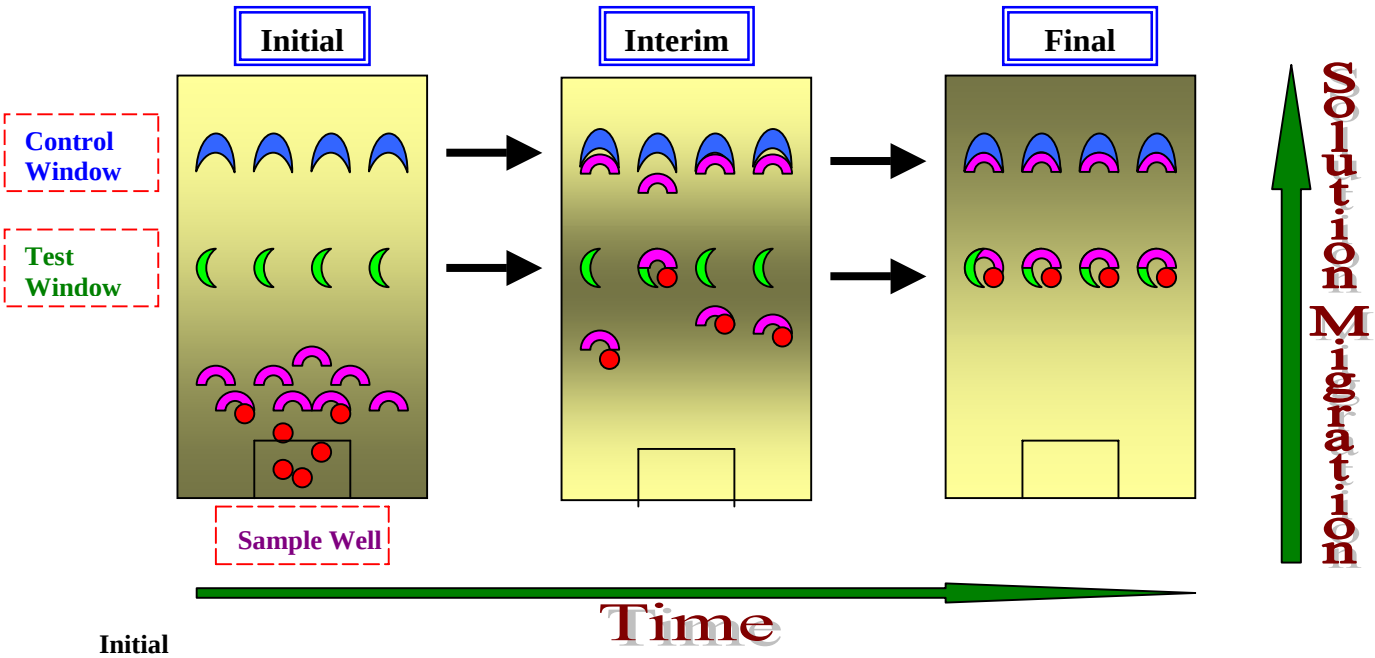
= Sample Solution

= Immobilised polyclonal anti-immunoglobulin antibodies

= Immobilised polyclonal anti-hHb-antibody antibodies

= Mobile monoclonal anti-hHb conjugated-dye complex

= Human Hb (hHb)



Initial
The sample solution is added to the test well and migrates through the absorbent material carrying the solubilised hemoglobin molecules. When they reach the point of the adsorbed colour-tagged mobile antibodies a specific interaction between the two molecules forms a soluble complex that continues to migrate in solution through the stationary phase.

Interim
The unbound colour tagged antibodies have moved through the first immobilised antibodies to a second set of immobilised antibodies where they are captured. These molecules are smaller than the complexes so migrate faster. The subsequent concentration of these molecules will produce the first characteristic pink line in the “C” window.

Final
Immobilised antibodies trap the hemoglobin-antibody complexes, forming an antibody-hemoglobin-antibody sandwich. This concentration of dye particles displays a pink line in the “T” window. The colour tagged monoclonal antibodies will only be captured if attached to a hemoglobin molecule, therefore this second line will only appear in the presence of hemoglobin.

False negative results
May occur when the immobilised antibodies in the test region bind lone hemoglobin molecules inhibiting the binding of the hemoglobin-antibody complexes.

4. Validation Studies .

4.1 SENSITIVITY

- **SERIAL DILUTIONS: Liquid and Stains**

#1 Stain Samples

Aim: To test the sensitivity of the HemaTrace extraction procedure using known stain dilutions.

Variable: Dilution Factor

Method:

- Serial dilution of neat fresh blood was made using an initial volume of 750μl each of distilled water and blood. A 750μl aliquot of the sample was then added to 750μl of distilled water, this was repeated until the desired dilution factor was reached.
- 100μl aliquot of each dilution was pipetted onto washed cotton cloth and air dried for 48hrs.
- Each entire stain sample was extracted in 2mls of HemaTrace buffer for 5 minutes.
- Some dilutions re-extracted in 300μl of HemaTrace buffer for 5 minutes.
- 4 drops of sample extract solution was applied to test well.
- HemaTrace results were recorded at 2min and 10min intervals.
- Samples were Hemastix tested for comparison.

#2 Liquid Samples

Aim: To test the sensitivity of the HemaTrace test card using liquid samples.

Variable: Dilution factor

Method:

- Serial dilution of neat fresh blood was made using an initial volume of 750μl each of distilled water and blood. A 750μl aliquot of the sample was then added to 750μl of distilled water, this was repeated until the desired dilution factor was reached.
- The dilutions were placed directly into the test well, no buffer or further liquid was added.
- HemaTrace Results were recorded at 2min and 10min intervals.
- The dilutions were Hemastix tested for comparison.

➢ SAMPLE SIZE COMPARISON

Aim: To test the sensitivity of the HemaTrace cards in respect to sample sizes that may be encountered in routine casework.

Variable: Sample size

Method:

- Fresh human blood was dropped onto washed cotton cloth and air-dried at room temperature for 48hrs.
- Sample sizes tested were as follows: 10mm², 5mm², 2.5mm², 1mm², 2mm thread and a 1mm thread.
- Samples were extracted in the supplied 2ml HemaTrace Buffer for 5mins. No centrifugation was necessary.
- 4 drops of sample extract solution was applied to test well.
- HemaTrace test observations were recorded at 45secs, 2minutes and 10minutes.

➢ WASHED SAMPLES

Aim: To test the ability of the HemaTrace cards to detect hemaglobin in bloodstains after washing.

Variable: 2 different washing methods were used on control bloodstains, which were then HemaTrace tested.

Method:

- Fresh bloodstains, which had been air dried at room temperature for 24 hours and one year old bloodstains which had been stored at –20C were used in this trial. All stains were prepared on washed cotton cloth.
- 2 washing methods were used.
  1. A hot water wash where stains were agitated under running hot water for 2min.
  2. A tepid detergent wash where stains were soaked in 200ml of 20% laboratory detergent (Decon 90) solution for 30min.
- A $5mm^2$ sample size was extracted for 5 minutes in 2mls of HemaTrace buffer.
- 4 drops of sample extract solution was applied to test well.
- HemaTrace results were recorded at 2minutes and 10minutes.
- All extract solutions were Hemastix tested.

➢ AGED BLOODSTAINS

Aim: To test the viability of different extraction solvents for aged bloodstains.

Variable: Extraction method

Method:

- Samples consisted of bloodstains on cotton cloth stored at room temperature in laboratory conditions. A 12year old bloodstain and a 26year old bloodstain were tested.
- Hemastix tests were performed on each stain sample.
- $2mm^2$ sample size was used for all tests.
- HemaTrace aged stain extraction protocol:
    1. 2-5 min soak in 2-3drops of 5% ammonia solution.
    2. Allow ammonia to evaporate.
    3. Add 300µl Hematrace buffer and apply this solution to the test well
- All other extractions were soaked for 30minutes in the supplied 2ml Hematrace buffer.

➢ SUPERNATANT SENSITIVITY

Aim: To test the viability of using the discarded supernatant from DNA blood extraction samples.

Variable: Sample (hemoglobin) Concentration

Method:

- Samples 1 and 2 were derived from extractions of fresh bloodstains on FTA paper using 10mM NaOH.
- Samples 3 to 8 were derived from actual casework extractions using distilled water.
- Volume of all samples consistent with initial wash for DNA extraction supernatant excess, approximately 950µl.
- All supernatants were Hemastix tested.
- Hematrace results were recorded at 2minutes and 10minutes.
- The supernatant colour was recorded.
- In each test, 4 drops of neat supernatant were placed directly in the Hematrace test well.

4.2 SPECIFICITY

➢ SPECIES CROSS REACTIVITY

Aim: To test the species specificity of the HemaTrace Cards.

Variable: Bloods from various animal species, some of which may be commonly encountered in forensic casework.

Method:

- Animal samples were derived from –80Cº stores and fresh bloodstains that were prepared on washed cotton cloth.
- $2mm^2$ samples of each animal bloodstain, with the exception of pig and ferret which were 1 drop liquid.
- Each sample was extracted for 5 minutes in 2mls of HemaTrace buffer. HemaTrace results were recorded at 10 minutes.
- All samples were Hemastix tested for comparison.

➢ OUCHTERLONY SENSITIVITY COMPARISON

Aim: To compare the sensitivity of Ouchterlony Immunodiffusion test with the HemaTrace test sensitivity.

Variable: Dilution factor of fresh liquid blood samples.

Method:

- Serial dilutions of fresh human blood were prepared.
- These dilutions were tested against anti-human hemoglobin and anti-human serum using Ouchterlony.
- Ouchterlony results were recorded after 24 hours and compared to the serial dilutions tested using HemaTrace cards.

- **CASE WORK EXAMPLES**

Case 1: Blue Denim Shirt from Suspect

In this case a person was violently assaulted causing severe bleeding. The suspect had washed the clothing items worn at the time of the offence. Pale brown staining was located on the front and sleeves of the shirt.

3 samples were taken for HemaTrace testing as follows:

1. Two 5mm spots of pale brown staining from below the right-front pocket
2. $15mm^2$ of pale brown staining from the right front of the shirt, just above bottom seam.
3. $25mm^2$ of pale brown staining from the right elbow.

Case 2: Bath water from a murder scene

Water from a bath, which contained clothing belonging to a deceased and which was Hemastix positive, was HemaTrace tested. It was possible the suspects had washed their hands in this bath water.

Case 3: Staining on a piece of linoleum.

Police had information that a murder had been committed in a house. Strong Hemastix positive staining was detected on the back and upper surface of the kitchen linoleum flooring which had been under the skirting boards. There was a strong odour of cat urine associated with the stained areas. A portion of the Hemastix positive area was Hematrace tested.

Case 4: T-Shirt from Deceased Victim

The decomposing victim was found in summer at his home 2-3 weeks after his murder. A blood like stain from the t-shirt that was the victim was wearing at the time of discovery was Hematrace tested.

5. Results .

Sample Size **(SEE TABLE 1)**

Neat blood stain samples ranging from $1cm^2$ down to a 1mm length of cotton thread all produced positive HemaTrace results when extracted in the full 2ml of HemaTrace buffer. As the sample size increased the time to result decreased and the intensity of the test line increased. In contradiction to this trend the speed and strength of the result from the $1cm^2$ sample appeared slower and slightly weaker than the smaller samples. This also proved to be the case with a sample of liquid blood that was placed into the 2ml buffer and tested. It is thought therefore that the variation was a consequence of the 'high dose hook' effect noted in the limitation section of the HemaTrace Technical Information Sheet (See Appendix 1).

Further investigation into these samples suggests that there is a visual correlation between the buffer extract's colour and its hemoglobin concentration. For example, a strong visual colour (dark-orange to pink/red) corresponds to a high concentration of hemoglobin and possible weaker, slower results due to the 'high dose hook' effect. (Refer Tables 1&7 and Picture 6.2).

Aged Stains **(SEE TABLE 2)**

Tests using aged bloodstains indicate that the supplied HemaTrace buffer produces indistinguishable results when compared with the more time-consuming HemaTrace ammonia protocol (See Appendix 1). The initial sample obtained using the ammonia extraction method had an elevated pH level that produced a slower result in comparison to other extraction methods. The pH of this sample was reduced using glacial acetic acid and the test was repeated improving the time of the result. The HemaTrace buffer proved to be a reliable extraction solution.

Serial Dilutions **(SEE TABLE 3 & 4)**

The first group of stain samples tested resulted in positive tests for all stain concentrations up to and including the 1:8192 sample concentration. This was less sensitive than expected from other articles, so the stain samples were re-tested with an altered extraction method. The second group of stain samples were extracted using a reduced HemaTrace buffer volume and subsequently the sensitivity was improved four fold with positive results observed up to the 1:32768 sample concentration.

Liquid samples derived from the same serial dilution as the stain samples were tested neat without the addition of buffer and produced positive HemaTrace results up to 1:262144 concentration. These results correspond to those sited in Kristaly et al that recorded a positive HemaTrace result at 1:100,000 but not at 1:1,000,000, however they differ from the results in Swander & Stites which recorded positives HemaTrace results at 1:16777216.

A comparison of the stain and liquid dilution results indicates that the difference in extraction methods had a significant impact upon the sensitivity of the HemaTrace tests.

Washed Samples **(SEE TABLE 5)**

Positive results were obtained for all four tests, however the detergent washed samples produced weaker results than hot rinsed samples that were identical in origin. It was thought that this was due to either the more efficient washing process using the detergent or an altered pH due to the detergent, however the pH of the washed samples (pH 7) was within the range noted in the ABAcard™ Technical Information Sheet (see Appendix.1).

Supernatant Sensitivity **(SEE TABLE 6)**

Seven out of eight supernatants produced fast positive results. All supernatants were Hemastix tested and were shown to be varying strengths. The HemaTrace negative supernatant was also Hemastix negative. It was observed that the Hemastix results were not indicative of the HemaTrace results.

Species Specificity **(SEE TABLE 7)**

The Ferret samples (neat and diluted) produced a false positive HemaTrace result as expected from the ABAcard™ Technical Information Sheet: Limitations (see Appendix.1). As the HemaTrace test is Higher Primate specific not human specific the monkey sample also produced an expected positive result. The monkey solution appeared slower and weaker than expected from its solution colour that had a dark pink visual appearance. It was concluded that this was due to a slight 'high dose hook' effect (See Sample Size Study).

All other species tested produced negative results, which is consistent with the papers read for this study (See References) that tested a number of species similar to those used in this validation as well as other novel species samples.

Forensic Cases **(SEE TABLE 8)**

The HemaTrace tests were used to analyse forensic cases that were suitable candidates for the study. Case 1 did not produce a positive result as expected from the Hemastix results, although the best samples had been taken for casework testing. Case 2 was not expected to give a positive result as any possible sample would have been significantly diluted by the volume of bath water. Case 3 was tested a number of times but did not produce a positive which was in line with observations made by evidence recovery analysts. Case 4 was expected to produce a positive as the sample was known to be concentrated neat blood and tissue fluid although the quality of the sample was in question to body decomposition processes and possible hot environmental exposure.

Ouchterlony Comparison **(SEE TABLE 9)**

Ouchterlony gels were run as a sensitivity comparison starting with the initial dilution of 1:2 descending to 1:4096. Both the Anti Human Serum and the Anti Human Hb Ouchterlony gel series dropped out at 1:2048 with the both serums giving a positive result for 1:1024 dilution. These results correspond with the sensitivity noted in Swander & Stites.

6. Discussion .

The HemaTrace® ABAcards™ are highly sensitive one-step sandwich immunoassays that are compact, disposable and user friendly. This one-step detection method has been used for a variety of commercial tests, such as pregnancy tests, and in this case has been adapted to detect human hemoglobin in bloodstain samples. The HemaTrace tests produce reliable results from a range of bloodstains encountered in Evidence Recovery for example samples ranging from $1cm^2$ to 1mm thread, aged stains, DNA extraction supernatant and denatured samples. They are an expedient way to confirm that doubtful stains found to be Hemastix positive are derived from human blood.

One of the more significant limitations of the HemaTrace test that is noted in the ABAcard™ Technical Information Sheet (see Appendix.1) is the "High Dose Hook Effect". Excessive quantities of human hemoglobin molecules can bind non-specifically to the solid phase antibodies. This steric hindrance obstructs the desired specific binding of hHb-antibody complexes. Two step immunoassays prevent this by washing excess molecules away in-between binding steps (Fernando & Wilson). However with the one-step immunoassays both the mobile molecules are mixed together thus causing a competitive binding environment.

Hemastix tests are based upon the oxidising properties of the Heme group in Hemoglobin, subsequently other oxidising agents such as bleach and compounds that have peroxidase-like properties can also produce false positives. The SANGUR validation study (Russell Cook) which tested the Hemastix sensitivity produced positive results for liquid dilutions of whole blood up to and including 1/500000 (HemaTrace 1/250000) and 1/10000 (HemaTrace 1/32000)in similar stain dilutions.

Although the sensitivities of the two tests are similar the Hemastix is not always indicative of the HemaTrace result. Negative Hemastix results did not always

correspond to negative HemaTrace was results as observed in the supernatant study.(See Table 6)

A sample size study was carried out to determine an optimum sample size in relation to casework items encountered in Evidence Recovery. This study considered neat bloodstains and found that samples ranging from $1cm^2$ to a 1mm thread gave strong reliable results. During the casework study, Case 1 did not give the expected positive result though it was known that the victim had lost a large volume of blood and visible washed stains produced weak Hemastix positive results(See Table 8). Further to this the first group of stain samples in the serial dilution study produced significantly less sensitive results with positives only observed up to 1:8092 concentration. A reduction in extraction buffer volume improved these results four fold. (See Table 3 & 4)

As a part of this validation, efforts were made to induce the "High Dose Hook Effect" to determine its impact on forensic casework. All HemaTrace tests from the highly concentrated samples gave genuine positive results. When these samples were diluted and re-tested the results were observed to be faster and stronger than the original tests. This was confirmed by further diluting other suspect samples such as the monkey and re-testing the extracts, which again improved the time and strength of the results.

The high concentration samples indicated a visual correlation between their buffer colour (dark orange to pink/red) and the strength and speed of their HemaTrace results. Therefore buffer colour should be considered along with other case observations when analysing the HemaTrace test results to ensure that results are reliable (for example: a negative HemaTrace result from an obviously bloodied solution). It should be noted though that a colourless sample solution is not an indication that the sample is hemoglobin negative.

Various extraction methods, including the recommended protocol noted in the HemaTrace Technical Information sheet (See Appendix1), were tested using neat aged bloodstains to determine the optimum extraction protocol for poor quality

samples. Results indicated that the supplied HemaTrace buffer sufficiently extracted the hemoglobin from these stains with an extended extraction time.

The results of the neat samples indicated that the HemaTrace tests were remarkably sensitive and it was expected that this would prove to be the same in the serial dilution study, however the initial stain samples were disappointing with positive results only being recorded up to 1:8192. The stain samples were re-tested with an altered extraction method that used less HemaTrace buffer and subsequently this increased the HemaTrace sensitivity for the stain samples to 1:32768.

Liquid samples derived from the same serial dilution as the stain samples were tested neat without the addition of buffer and produced positive HemaTrace results up to 1:262144 concentration. The results of liquid dilution were up to ten times more sensitive than the stain dilution samples therefore the variation in the extraction method, ie buffer volume, had a significant impact upon the sensitivity of the HemaTrace tests. Theses results highlighted the buffer volume as a factor in protocol design (See Tables 3& 4).

Further evidence of the HemaTrace sensitivity was observed through the accidental contamination of a negative control. The supplied pipette was placed upon the sampling area and the trace blood dust was sufficient to produce a positive result from the neat buffer sample. This result was confirmed by testing the sampling area with a Hemastix that produced a strong positive result.

A Species specific study was carried out using a range of animal samples both domestic and novel. The specificity of the HemaTrace cards is adequate for real forensic casework as in the majority of cases it is not necessary to know which species the blood originated from but merely if it is human (higher primate) in origin.

Tests were carried out on washed stains to study the specificity and sensitivity of the HemaTrace test under these conditions. This validation was limited and did not include more extensive tests such as washing machine cleaning or bleaching. Whilst the positive results are promising, a comparison to Forensic Case Sample 1(see table

8) which did not produce a positive HemaTrace result, indicates that protocols for washed samples need to be independent of protocols for neat stain samples.

In cases that yield very little physical evidence, DNA testing must be given priority over all other evidence recovery methods. It is useful then to determine if the supernatant from the initial washing of the sample is suitable for HemaTrace testing. This supernatant is routinely tested in Biology with Hemastix. The Hemastix results from the corresponding supernatant's were not a reliable indication of the possible HemaTrace results.

As a basis for our HemaTrace protocols we compared those recommended in the Technical Information Sheet with those used in Swander & Stites and Kristaky et al. All extractions were carried out at room temperature with our initial sample sizes under $1cm^2$. The methods were then refined with each validation according to sample type and the results observed when altering sample size, extraction time and buffer volume.

7. Conclusion .

The HemaTrace test is a viable and effective presumptive test method suitable for use in the Evidence Recovery department of the Adelaide Forensic Science Centre to confirm the presence of human (higher primate) hemoglobin in samples. The High Dose Hook effect will not be a limitation in the use of the HemaTrace test for evidence recovery purposes, as no false negatives were observed due to this limitation, despite efforts to induce the effect. However, negative test results should be considered in conjunction with observations regarding the visual colour of the sample solution (strong colour indicates high hemoglobin concentration) and background case information.

Positive HemaTrace results conclusively indicate the presence of human hemoglobin in the tested sample irrespective of the strength of the result. False positives will not be a serious consideration as ferret and higher primates will not be common considerations to the majority of crime scenes or evidence and will generally be referred to in case notes.

There is a strong indication from the serial dilution study that the buffer decreases the concentration of the hemoglobin in the sample and therefore decreases the HemaTrace test sensitivity. In the instance of forensic casework, care must be taken in the consideration of buffer volume when extracting weak stains (eg: small or washed). False negatives from these types of samples that produce weak sample solutions, will be the main concern in the use of HemaTrace test, therefore careful attention must be given to the sample type and the optimum testing procedure, to minimise the possibility of false negative results.

The HemaTrace cards can exclude a sample from consideration but cannot shed light on blood sample origins if they prove to be negative for human hemoglobin.

In these cases, where necessary, the Ouchterlony test will still be required to determine the non-human species of origin.

The protocols for the use of the HemaTrace ABAcards in Evidence Recovery have been designed based upon the results and observations of the validations with specific consideration to the type of evidence encountered and the information required.

In most real forensic cases it is sufficient to include or exclude samples based upon the results of the HemaTrace tests. This test should reduce the non-human stains sent to biology for DNA profiling reducing costs and time. As the HemaTrace test is up to ten times more sensitive than the current Ouchterlony test it will be a valuable addition to the Evidence Recovery apparatus.

8. Recommended Protocols .

Neat Stains, FREDS & Aged Stains

(Neat Stains: Sample size <5mm^2 in 2ml buffer for 5min @ room Temp)

(FREDS: Sample size <5mm length in 2ml buffer for 5min @ room Temp)

(Aged Stains: Sample size <5mm^2 in 2ml buffer for 1hr @ room Temp)

1. Cut out a sample using sterile scissors or scalpel on a clean surface. The sample size should be between 5mm^2 and 2mm^2, however smaller fragments are sufficient to produce a result.
   - **FREDS: use 2-5mm length of thread**
2. Place the sample into the provided container of 2ml HemaTrace buffer allowing the sample to extract for 5minutes at room temperature.
   - **AGED STAINS: extract for 1hr @ room temperature**
3. If the sample is not to be tested immediately store the solution at 4ºC until required.
4. Check the buffer's visual appearance ensuring the colour is no darker than a light orange/pink colour to avoid the "high dose hook effect". Colourless solutions will still result in positive results if the sample contains sufficient hHb.
5. If the solution is deemed too strong dilute the sample 1:4 with de-ionised water to a minimum total volume of 200µl in an eppendorf tube before testing.
6. Using a sterile pipette extract 150µl of sample solution and place it into the small sample well at the bottom of the test marked "S".
7. Begin recording the time allowing a maximum of 10minutes to observe a result.
8. Record positive if two separate pink lines appear in the window next to the "T" and "C" labelled areas respectively. If a single line appears in the uppermost area of the window labelled "C" record a negative result. In the absence of any lines in the window the test must be deemed invalid. Any line regardless of colour strength can be interpreted as valid.

NB: A Decrease in quality or size of a sample should relate to a decrease in volume used (Minimum Buffer volume 300µl).

8. Recommended Protocols .

Washed Stains

(Sample size <1cm^2 in 300µl buffer for 30min @ room temp: centrifuge 3min)

1. Cut out a sample using sterile scissors or scalpel on a clean surface. The sample size should be up to 1cm^2 where possible.
2. Label an F-Eppendorf and using a sterile pipette place a maximum of 300µl of HemaTrace buffer from a new container into the tube.
3. Place the sample into the F-Eppendorf and allow the sample to extract for 30min at room temperature.
4. Transfer the sample using sterile tweezers to an eppendorf filter and return the filter with the sample to the tube.
5. Centrifuge the sample tube for 3min ensuring the tube is not inverted after the spin.
9. If the sample is not to be tested immediately store the solution at 4ºC until required.
10. Remove the eppendorf filter and using a sterile pipette extract 150µl of the sample solution
11. Place the aliquot into the small well at the bottom of the HemaTrace card marked with an "S".
12. Begin recording the time allowing a maximum of 10minutes to observe a result.
13. Record positive if two separate pink lines appear in the window next to the "T" and "C" labelled areas respectively. If a single line appears in the uppermost area of the window labelled "C" record a negative result. In the absence of any lines in the window the test must be deemed invalid. Any line no matter regardless of colour strength can be interpreted as valid.

NB: A Decrease in quality or size of a sample should relate to a decrease in volume used (Minimum Buffer volume 300µl).

8. Recommended Protocols .

SWABS

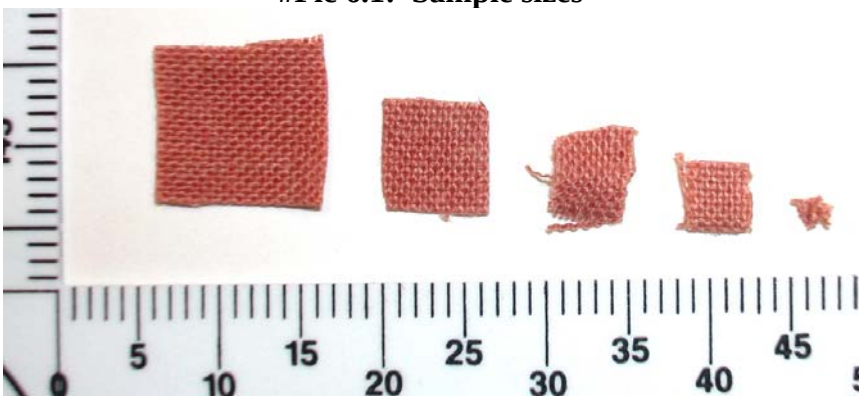
(Sample size <5mm^2 300µlml buffer for 1hr @ room temp: centrifuge 3min)

1. Cut out a portion of the swab (¼ or ½) sample using sterile scissors or scalpel on a clean surface.
2. Label an F-Eppendorf and using a sterile pipette place a maximum of 300µl of HemaTrace buffer from a new container into the tube.
3. Place the sample into the F-Eppendorf without a filter and allow the sample to extract for 1hr at room temperature.
4. Transfer the sample using sterile tweezers to an eppendorf filter and return the filter with the sample to the eppendorf tube.
5. Centrifuge the sample tube for 3min ensuring the tube is not inverted after the spin.
6. If the sample is not to be tested immediately store the solution at 4ºC until required.
7. Remove the eppendorf filter and using a sterile pipette extract 150µl of the sample solution.
8. Place the aliquot into the small well at the bottom of the HemaTrace card marked with an "S".
9. Begin recording the time allowing a maximum of 10minutes to observe a result.
10. Record positive if two separate pink lines appear in the window next to the "T" and "C" labelled areas respectively. If a single line appears in the uppermost area of the window labelled "C" record a negative result. In the absence of any lines in the window the test must be deemed invalid. Any line no matter regardless of colour strength can be interpreted as valid.

NB: A Decrease in quality or size of a sample should relate to a decrease in volume used (Minimum Buffer volume 300µl).

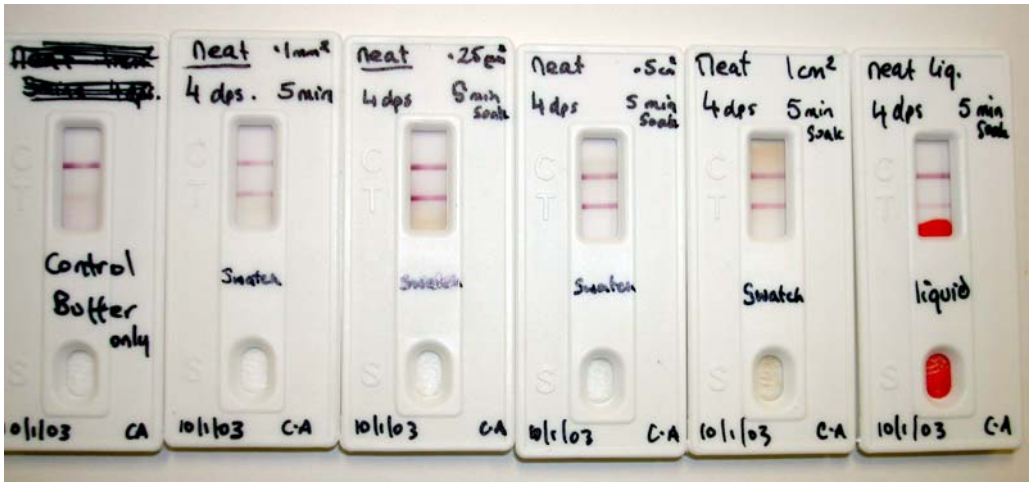
9. Images .

#Pic 6.1: Sample sizes

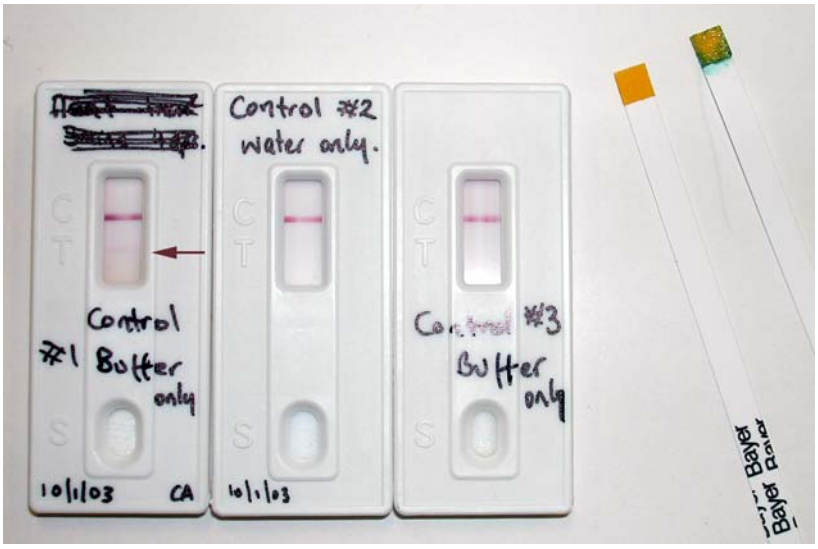


 # Pic 6.2: Solution colours

#Pic 6.3 Hematrace results



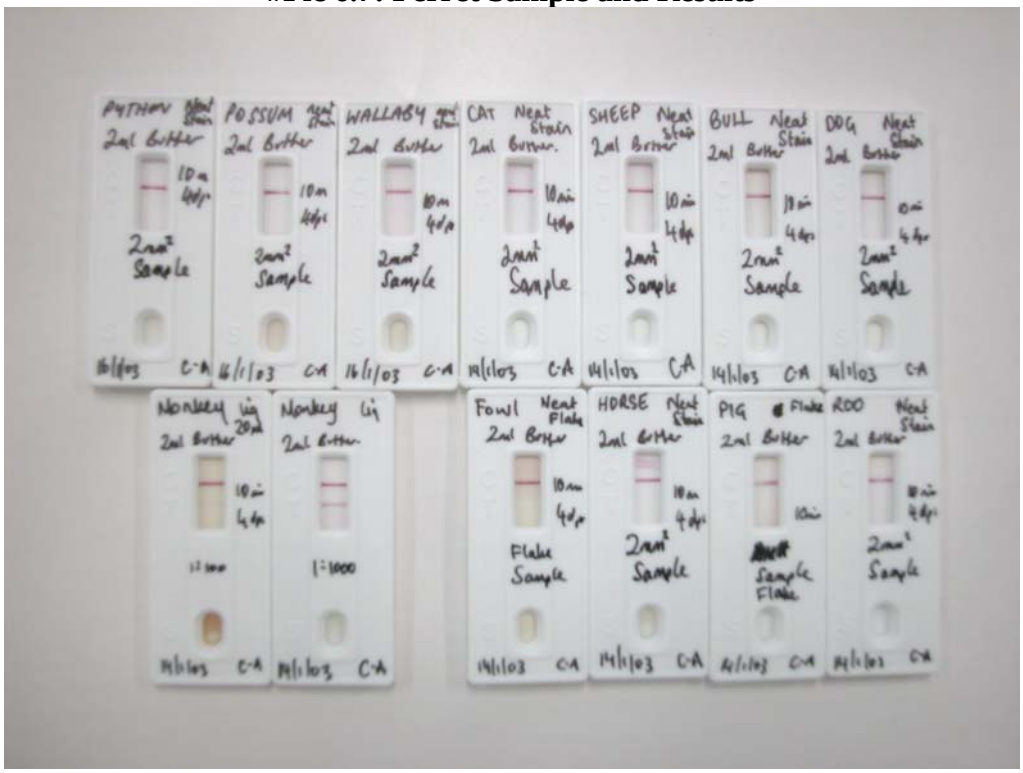
#Pic 6.4: Control contamination



#Pic 6.5: Washed Stain Samples

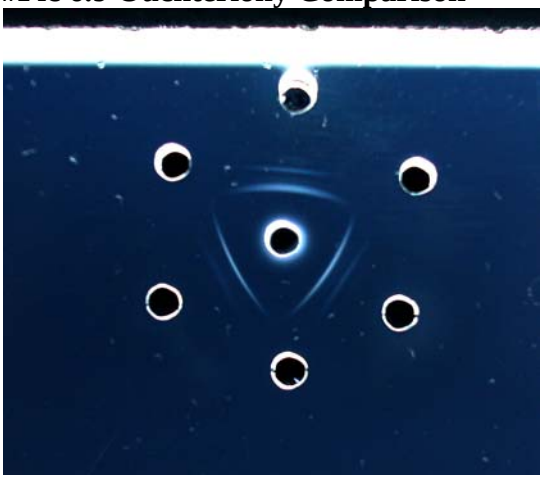
#Pic 6.6: Supernatant Samples and Results

#Pic 6.7: Ferret Sample and Results



#Pic 6.8 Forensic Case Sample: #A

#Pic 6.9 Ouchterlony Comparison



10. Tables .

TABLE #1

SAMPLE SIZES: NEAT BLOOD STAINS

Sample Size	Solution Appearance	Control Window	Test Window			Further Observations
			45 sec	2 min	10min	
Hematrace Buffer Only	Opaque	Positive	Weak +ve	Weak +ve	Weak +ve	Confirmed contamination
Distilled deionised H_20	Clear	Positive	- ve	- ve	- ve	——
Hematrace Buffer Only	Opaque	Positive	- ve	- ve	- ve	———
150µl neat blood	Dk Pink/Red	Positive	Weak +ve	Mod +ve	Strong +ve	**Possible "High Dose Hook Effect"** Colour was visible in sample well and test screen
10mm^2	Dk Orange/red	Positive	Mod +ve	Mod +ve	Strong +ve	Colour was visible in sample well and test screen
5mm^2	Med Straw	Positive	Mod +ve	Mod +ve	Strong +ve	———
2.5mm^2	Straw	Positive	Weak +ve	Mod +ve	Strong +ve	———
1mm^2	No Obvious Change	Positive	Weak +ve	Weak +ve	Strong +ve	———
2mm thread	No Obvious Change	Positive	- ve	Mod +ve	Strong +ve	———
1mm thread	No Obvious Change	Positive	- ve	Weak +ve	Mod +ve	———

TABLE #2

AGED NEAT BLOODSTAINS: EXTRACTION METHODS

Age of Sample	Extraction Method	Hematrace Results		Further Obseravtions
		Control	Test	
2 days old	Hematrace Buffer	Positive	Positive	____
12 y.o. (1991) **Hemastix: Strong <5secs**	2ml deionised distilled H_2O	Positive	Negative	____
	2ml Hematrace buffer	Positive	Moderate Positive	____
	50µl 5%NH_3 Hematrace protocol	Positive	Weak Positive	Initial test @ pH9
	50µl 5%NH_3 Hematrace Protocol	Positive	Weak Positive	After addition of ~5µl glacial acetic acid the test was Repeated @ pH4
26 y.o (1976) **Hemastix: Moderate <15secs**	2ml Hematrace buffer	Positive	Moderate Positive	Sample broken up with wooden applicator

TABLE #3
SERIAL DILUTIONS : STAINED SWATCHES

Sample dilution	Hemastix Results	Hematrace		
		Control window	Test window 2 min	Test window 10 min
1: 262144 (18B)	No reaction	Positive	Negative	Negative
1: 131072 (17B)	No reaction	Positive	Negative	Negative
1: 65536 (16B)	No reaction	Positive	Negative	Negative
1: 32768 (15B)	No reaction	Positive	Negative	Negative
1: 16384 (14B)	< 60secs Trace	Positive	Negative	Negative
1: 8192 (13B)	< 60secs Trace	Positive	Negative	Weak Positive
1: 4096 (12B)	< 60secs Trace	Positive	Positive	Moderate Positive
1: 2048 (11B)	< 60secs Trace	Positive	Positive	Strong Positive
1: 1024 (10B)	< 5Secs Moderate	Positive	Positive	Strong Positive
1: 512 (9B)	< 5secs Strong	Positive	Positive	Strong Positive

RE-TEST SERIAL DILUTION: STAIN SAMPLES

Retest Hematrace using 300µl Buffer for extraction.			
Dilution	**Control window**	**Test Window**	
		2min	**10min**
1: 65536	Positive	Negative	Negative
1: 32768	Positive	Negative	Not Reportable Positive
1: 16384	Positive	Negative	Weak Positive
1: 8192	Positive	Weak Positive	Strong Positive

TABLE #4

SERIAL DILUTUONS: LIQUID SAMPLES

Dilution	Hemastix	Hematrace		
		Control window	Test window	
			2min	10min
1: 1, 048576	Partial <20secs	Positive	Negative	Not reportable Positive
1: 262144	Weak <10sec	Positive	Negative	Weak Positive
1: 32768	Moderate <10secs	Positive	Moderate Positive	Moderate Positive
1: 1024	Strong <5secs	Positive	Strong Positive	Strong Positive

TABLE #5

WASHED SAMPLES

Sample	Hemastix of solution	Hematrace		
		Control Window	Test Window	
			2 min	10min
1 year old Hot water wash	<30secs Weak	Positive	Weak Positive	Moderate Positive
2 day old Hot water wash	<30secs Weak	Positive	Weak Positive	Moderate Positive
1 year old 1/2 hr Detergent soak	<20secs Moderate	Positive	Negative	Very weak Positive
2 day old 1/2 hr Detergent soak	<10secs Moderate	Positive	Negative	Weak Positive

TABLE #6

SUPERNATANT SENSITIVITY

Supernatant appearance	Hemastix	Hematrace			Further Observations
		Control Window	Test Window		
			2 min	10min	
#1 Colourless	<5secs Moderate	Positive	Negative	Not reportable Positive	supernatant was diluted more than the standard wash volume
#2 Colourless	<5secs Strong	Positive	Weak Positive	Strong Positive	___
#3 Light orange	<5secs Strong	Positive	Weak Positive	Strong Positive	pH6
#4 Straw	<5secs Strong	Positive	Weak Positive	Strong Positive	____
#5 Opaque	<5secs Moderate	Positive	Weak Positive	Strong Positive	____
#6 Colourless	Nil	Positive	Negative	Negative	____
#7 Colourless	<10secs Weak	Positive	Positive	Positive	____
#8 Colourless	<40secs Trace	Positive	Positive	Positive	pH5

TABLE #7

SPECIES CROSS REACTIVITY

Species	Hemastix	Solution appearance	Hematrace		Further Observations
			Control Window	Test Window 10min	
Cat	<5secs Strong	Straw	Positive	Negative	____
Kangaroo	<5secs Strong	Straw	Positive	Negative	____
Horse	<5secs Strong	Straw	Positive	Negative	____
Dog	<5secs Strong	Straw	Positive	Negative	____
Bull	<5secs Strong	Dark Straw	Positive	Negative	____
Sheep	<5secs Strong	Dark Straw	Positive	Negative	____
Pig	<5secs Strong	Orange	Positive	Negative	____
Fowl	<5secs Strong	Dark Orange	Positive	Negative	____
Python	<5secs Strong	Dark Straw	Positive	Negative	____
Possum	<5secs Strong	Dark Orange	Positive	Negative	____
Wallaby	<5secs Strong	Dark Orange	Positive	Negative	____
Monkey	<5secs Strong	Dark Red	Positive	Not reportable Positive	**Appeared to give a slight "High Dose Hook effect**"
	<5secs Strong	Straw	Positive	Weak Positive	Original sample further diluted and retested
Ferret	<5secs Strong	Dark Red	Positive	Weak Positive	4dps of neat blood into test well followed by 2dps of buffer
	<5secs Strong	Dark Pink	Positive	Weak Positive	1dp of neat blood into 2ml buffer

TABLE #8

FORENSIC CASES

Evidence item Description		Sample Size	Luminol	Hemastix	Hematrace Buffer volume	Extraction time	Hematrace		
							Control Window	Test Window	
								2 min	10min
#A **Blue denim Shirt**	**#1**	8mm^2 cutting	Positive	V.Weak +ve @ 60secs	500µl	30 min	Positive	-ve	-ve
	#2	15mm^2 cutting	Negative	Weak +ve @ 60secs	500µl	30 min	Positive	-ve	-ve
	#3	25mm^2 cutting	Positive	V.Weak +ve @ 90secs	1000µl	30 min	Positive	-ve	-ve
#B **Crime scene Bath Water**		5drops	n/a	Negative	Nil	n/a	Positive	-ve	-ve
#C **Crime Scene Floor linoleum**		Micro Swab from 20mmϕ stain	n/a	Strong positive @ <5secs	2ml	10min	Positive	-ve	-ve
		Micro Swab from 10×20mm stain	n/a	Strong positive @ <5secs	500µl	10min	Positive	-ve	-ve
#D **T-Shirt found on a decomposed body at scene for 2-3wks**		5mm^2	n/a	Strong positive @ <5secs	2ml	10min	Positive	mod +ve	strong +ve

TABLE #9

OUCHTERLONY GEL SENSITIVITY

Dilution	Ouchterlony		Dilution	Ouchterlony	
	Anti Human Serum	**Anti Human Hemoglobin**		**Human Serum**	**Anti Human Hemoglobin**
1:2	Positive	Positive	**1:128**	Positive	Positive
1:4	Positive	Positive	**1:256**	Positive	Positive
1:8	Positive	Positive	**1:512**	Positive	Positive
1:16	Positive	Positive	**1:1024**	Positive	Positive
1:32	Positive	Positive	**1:2048**	Negative	Negative
1:64	Positive	Positive	**1:4096**	Negative	Negative

8. References .

ABAcard Hematrace Onestep Test Kit – Technical Information Sheet. ABACUC Diagnostics Inc USA

Swander, C.J and Stites, J.G., Michigan State Police, Forensic Laboratory. The Evaluation of the ABAcard HemaTrace for the Forensic Identification of Human Blood.

Reynolds, M. The Abacard HemaTrace: Confirmatory Identification of Human Blood located at Crime Scenes. The Forensic Bulletin, December 2002.

Kristaly, A and Smith, D.A.S, Forensic Biology Section, Crime Laboratory Bureau, Miami-Dade Police Department, Miami, Florida, USA. Validation of the *OneStep* ABAcard HemaTrace for the rapid forensic detection of human blood.

9. Appendix 1 .