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THE EFFECTS OF TEMPERATURE ON THE ABACARD® HEMATRACE® KIT FOR THE IDENTIFICATION OF HUMAN BLOOD

By

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A thesis submitted in fulfilment of the requirements for the degree of

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in

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Murdoch University

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Semester 2, 2017

Declaration

I declare that this thesis does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due reference has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Acknowledgements

The production of this thesis would not have been possible were it not for the efforts of a number of individuals.

Firstly, to my supervisor Associate Professor James Speers, thank you for helping me throughout my project especially with my funding issues. To my second supervisor Dr Dave Berryman, I am grateful for the input you gave towards my project and for allowing me to use the machines in the SABC department. To Professor Emeritus Robert Mead thank you very much for becoming my 'surrogate' supervisor. Without your help I think I would have been lost throughout most of this project.

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Lastly, to the reader, I am truly humbled that you have decided to read this thesis. I hope you will forgive any errors or redundancies that may arise but more importantly, I hope that the contents of this thesis will be useful to you in some way.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
BPA	Blood Pattern Analysis
Hb	Haemaglobin
Ig	Immunoglobulin
IgG	Immunoglobulin G
LFIA	Lateral flow immunochromatographic assay
LC-MS	Liquid chromatography-mass spectroscopy
DSC	Differential scanning calorimetry
Fab	Fragment antigen-binding region
Fc	Fragment crystallisable region
T/C ratio	Ratio between Test and Control band intensities

ABSTRACT

Blood is often cited as the most informative form of evidence at a violent crime scene. DNA analysis enables the identification of individuals involved in a crime whilst blood pattern analysis enables the reconstruction of a crime scene. Prior to analysis, potential bloodstains found at a crime scene are processed via observation, documentation, followed by presumptive testing. This screening process eliminates substances that have a similar appearance to human blood therefore ensuring that time and resources are not wasted on samples with little or no forensic value.

The ABACard® HemaTrace® kit is a lateral flow immunochromatographic assay that targets human haemoglobin using antibodies and is used by the Western Australia (WA) Police as a test for the identification of human blood. However, WA Police officers have noted that in the northern regions of Western Australia, HemaTrace® kits have resulted in false negatives. It was postulated that this is due to prolonged exposure of the HemaTrace® kits to elevated storage temperatures resulting in a decrease in the HemaTrace® kit sensitivity and efficacy. However, a temperature stress test on HemaTrace has yet to be reported in the scientific literature and thus this hypothesis remains inconclusive. Thus, this literature review aims to critically assess the current literature pertaining to the HemaTrace® kits with emphasis on the sensitivity, specificity and robustness of the kits. As there are currently no studies that have investigated the effects of temperature on the performance of ABACard® HemaTrace® kit, this literature review will explore temperature studies on antibodies and other lateral flow immunochromatographic assays in order to postulate the effects of temperature on the HemaTrace® kits. Lastly, this literature review will outline parameters for potential

research in order to evaluate whether elevated temperature exposure for prolonged periods is potentially responsible for the HemaTrace® false negatives obtained in northern WA.

1.0 INTRODUCTION

Blood is one of the most common and valuable forms of biological evidence found at a crime scene.[1] DNA analysis of a blood sample could potentially identify individuals involved in the crime.[2] Alternatively, Blood Pattern Analysis enables the sequencing and reconstruction of blood letting events.[3] Thus, it is critical that human blood is correctly identified at crime scenes during a forensic investigations. To do this, potential bloodstains found at a crime scene are selected based on visual and contextual cues. However, many substances share a similar appearances to bloodstains and animal blood samples are potentially irrelevant to the crimes committed.[4] Thus, this step is often shortly followed by presumptive testing of the potential bloodstain.

The ABACard® HemaTrace® kit is an immunological presumptive test that utilises antibodies to target human heamoglobin present in red blood cells.[5] This kit has been shown to be highly sensitive and specific, with minimal cross reactivity and thus is used by the Western Australia (WA) Police as a test for the identification human blood.[4] However, WA Police officers have reported that the ABACard® HemaTrace® kits used in northern WA have a tendency to result in false negatives.[6] This is potentially detrimental to an investigation as this could result in the exclusion of vital forensic evidence, hindering the progress of an investigation

It was postulated that this was potentially due to the higher temperatures experienced in the northern regions of WA, as it is not uncommon for daily temperatures to exceed 40°C during the summer months.[7] Two studies conducted by, Evans[8] and Teaghan[9] attempted to test this hypothesis by evaluating whether exposure of bloodstains to elevated temperatures could be responsible for the HemaTrace® false negative results. This was based on the assumption that elevated temperature exposure could potentially denature haemoglobin beyond the recognition of the HemaTrace® antibodies. However, it was noted that false negatives only began occurring when bloodstains were exposed to temperatures at 80°C.[9] As it is rare that bloodstains at a crime scene would be exposed to such extreme temperatures, this hypothesis was deemed unsuitable to explain the occurrences of the HemaTrace® false negative results.

Another factor that could potentially explain the occurences of the false negative results is that elevated temperature exposure of the HemaTrace® kits themselves, rather than the bloodstains, could be responsible for the false negative results. A study conducted by Dadour *et al* [10], in Perth, WA, noted the temperature inside the cabin of a vehicle was able to exceed the outside ambient temperature by 20-30°C. Thus, HemaTrace® kits stored in police vehicles are potentially exposed to temperatures up to 60°C. This is further exacerbated as the ABACard® HemaTrace® kits can be stored in police vehicles for months prior to use.[6] However, this has yet to be tested and thus remains to be concluded.

The ABACard® HemaTrace® kit operates based on antibody binding mechanisms.[5] Thus, it is postulated that elevated temperature exposure to the HemaTrace® kits could denature

the antibodies in the kits resulting in protein unfolding of the antibody structure.[11] This could affect the ability of the antibodies to bind to haemoglobin molecules in blood samples resulting in the false negative results. However, there have been no reported studies on the effects of temperature on the HemaTrace® kits in the scientific literature. This literature review aims to evaluate this hypothesis by exploring temperature studies on antibodies and other lateral flow immmunochromatographic assays in order to postulate the effects of temperature on the ABACard® HemaTrace® kits. Furthermore, this literature review will aid in the determination of an experimental design flow in order to address this research gap. The results of such a study could provide information on suitable storage conditions for the HemaTrace kits and potentially provide an adequate explanation for the false negative results both in an investigative sense and in a court of law.

2.0 DISCUSSION

This section aims to describe the mechanism in which the ABACard® HemaTrace® kits function. This will be done by evaluating the current literature pertaining to HemaTrace® with particular emphasis on the sensitivity and specificity of the kit. The second aim is to understand the potential effects of temperature on the HemaTrace® kits. This will be done by reviewing previous temperature studies on antibodies and other lateral flow immunochromatographic assays.

2.1 Composition of Blood

Blood is a complex colloid suspension comprising of blood cells, proteins and amino acids suspended in a fluid matrix called plasma. The three major physiological roles of blood are: (i) transportation of gases, nutrients, hormones and waste products; (ii) regulation of temperature, pH and water content; (iii) protects the body against infection via the immune response. Through centrifugation, blood can be separated into four main components, which are plasma, platelets, white blood cells (WBCs) and red blood cells (RBCs).[12] In DNA analysis, WBCs are integral for STR profiling as they are the only mature nucleated blood cells.[2] In contrast, presumptive tests for blood target RBCs due to the abundance of haemoglobin within these cells.[13]

In a healthy individual, RBCs account for 37-52% of the total blood volume.[14] This percentage is known as the haematocrit and can vary depending on race, age, sex, altitude and smoking.[15] Red blood cells are non-nucleated, circular, biconcave cells with a diameter of approximately 7.5µm.[14] The main function of the RBC is for gas transport throughout the body. This is primarily done using the protein haemoglobin, which constitutes 97% of the RBCs' dry content.[16]

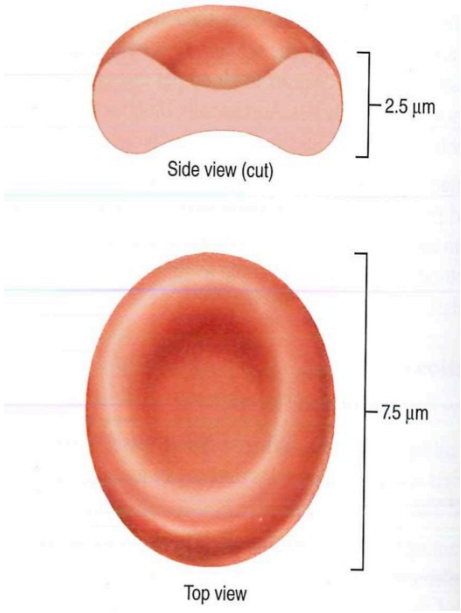

Figure 1: Circular biconcave structure of red blood cells (image from [14])

2.2 Human Haemoglobin

Haemoglobin acts as a carrier to transport oxygen from the lungs to tissue and the removal of carbon dioxide from tissue to be expelled in the lungs.[14] A single haemoglobin molecule is comprised of an aggregate of four globin protein units, each associated with a haem group (Figure 2).

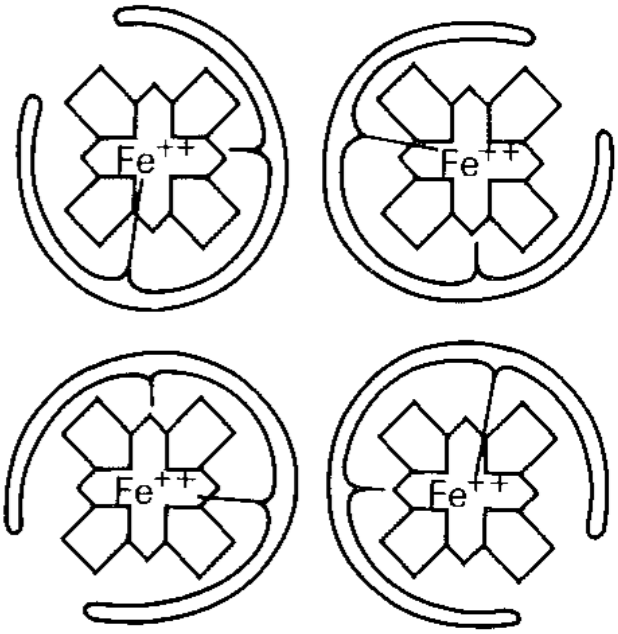

Figure 2: Aggregation of four haemoglobin subunits, forming a tetrameric haemoglobin molecule (Image from [17])

The haem group is responsible for the oxygen carrier capabilities of haemoglobin. Haem is comprised of a ferrous ion (Fe^{2+}) and a protoporphyrin IX ring. [18] Synthesis of the protoporphyrin ring is regulated by an enzyme, δ-aminolaevulinic acid synthase, within the mitochondria.[19] The ferrous ion (Fe^{2+}) is centralised in the protoporphyrin ring by binding to four nitrogen atoms (Figure 3). Two additional bonds are formed with the ferrous ion that are integral to haemoglobin function.[16] The first is reversible binding of an oxygen molecule to the ferrous ion, allowing haemoglobin to act as an oxygen carrier. The second bond formed by the ferrous ion mediates this process. The ferrous ion forms a bond with the associated globin protein on the histidine F8 residue located on residue 87 and residue 92 of the alpha and beta globin subunits respectively.[20] The function of this bond is two-fold. First, the globin protein prevents oxidation of the ferrous ion (Fe^{2+}) into a ferric state (Fe^{3+}) as haem in the ferric state will result in permanent binding of the

oxygen molecule. The second function is evident when oxygen is bound to the ferrous ion in haem, this results in a conformational change in the globin protein resulting in an increase in oxygen binding efficiency to haemoglobin, known as the allosteric effect.[16]

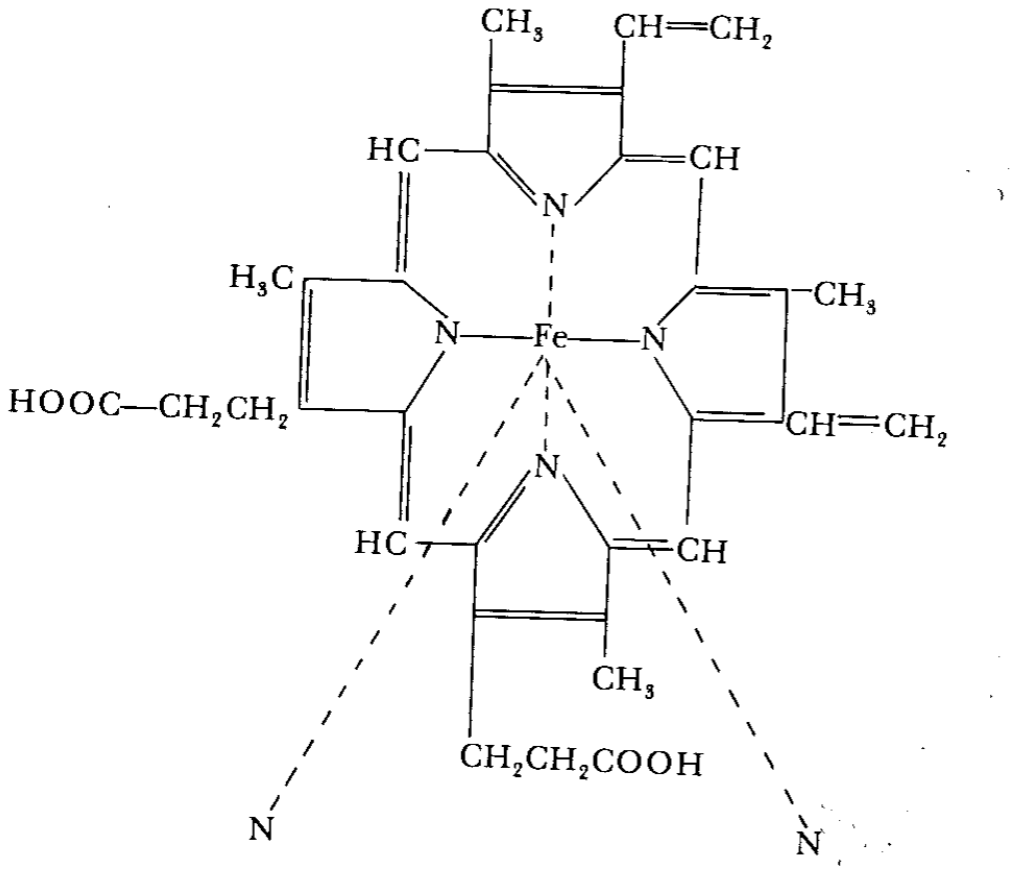

Figure 3: Structure of heam comprising of a centralised ferrous (Fe^{2+}) ion within a protophrpyrin IX ring (Image from [16]).

The protein components of haemoglobin are represented by the four globin subunits.[16] For adult human haemoglobin, this consist of a pair of alpha globin subunits and a pair of beta globin subunits, known as the α-chain and β-chain respectively. The α-chain spans 142 amino acid residues whilst the β-chain spans 147 amino acid residues.[20] The secondary structure of both of the proteins consists of coiled structures known as α-helices (Figure 4). The α-helix structures fold upon itself and each subunit associates itself

with a haem molecule to form the tertiary structure of each globin subunit. The four globin subunits then bind together via hydrophobic interactions to form the complete tetrameric form of haemoglobin (Figure 4). [21]

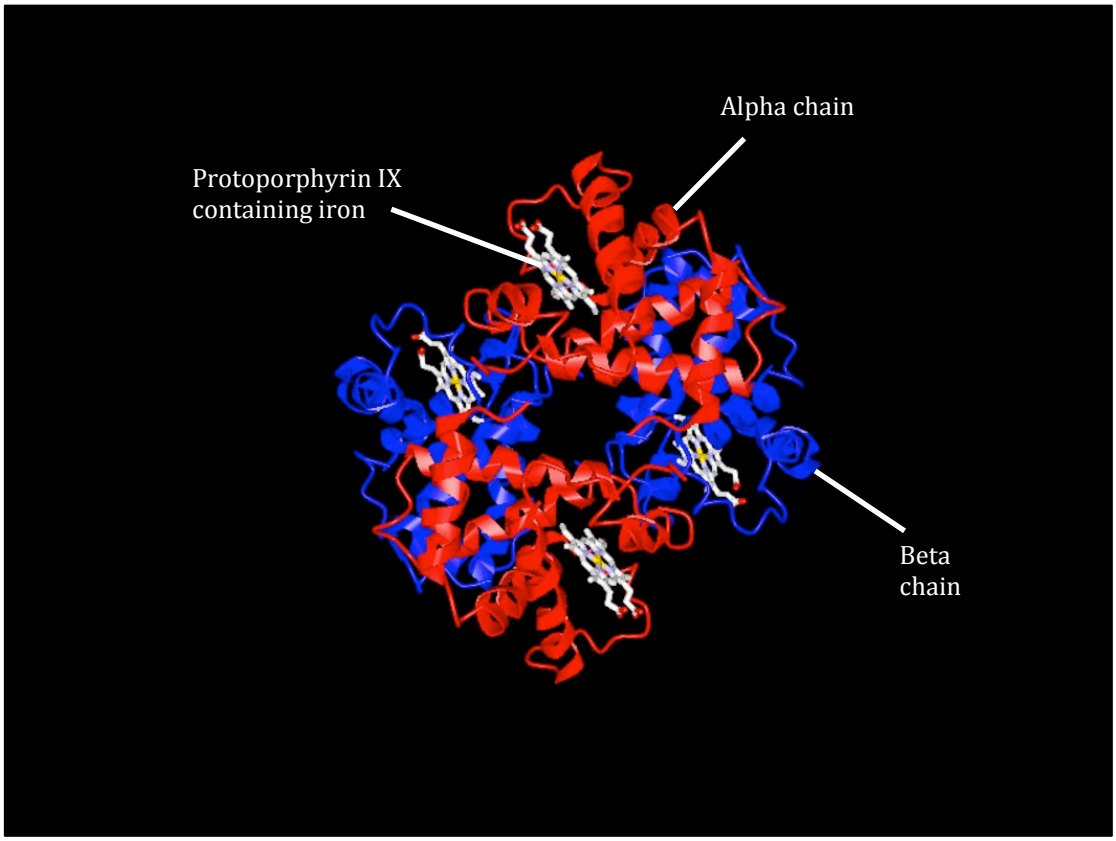

Figure 4: 3-D Haemoglobin structure. Red: Alpha globin subunits; Blue: Beta globin subunits; Grey: Protoporphyrin ring containing ferrous (Fe^{2+}) ion (Image adapted from [21]).

2.2.1 Human Haemoglobin Variants

Variations of the haemoglobin composition are presented throughout different stages of healthy human development.[16] A summary of the multiple haemoglobin variants and its occurrences are summarised in Table 1. Briefly, the tetrameric haemoglobin protein can be comprised of a combination of α, β, γ, δ, ε, or ζ subunit pairs. In an adult, the $\alpha_2\beta_2$ combination constitutes 97.5% of the total haemoglobin whilst the remainder is in the $\alpha_2\delta_2$ isoform. The ABACard® HemaTrace® targets the α-chain of haemoglobin thus, both

isoforms of adult haemoglobin can be detected by the HemaTrace® kit.[5] Furthermore, embryonic and foetal bloodstains are not commonly found at crime scenes. Thus, the ABACard® HemaTrace® is an adequate kit for the forensic detection of bloodstains.

Table 1: Human haemoglobin isoforms present throughout healthy human development (Adapted from [16]).

Developmental stage	Haemoglobin isotype	Globin subunits
Embryonic	HbE_1	$\alpha_2\varepsilon_2$
	HbE_2	$e_2\zeta_2$
	HbE_3	$\zeta_2\gamma_2$
Foetal	HbF	$\alpha_2\gamma_2$
Adult	HbA	$\alpha_2\beta_2$
	HbA_2	$\alpha_2\delta_2$

2.2.2 Haemoglobin Degradation

Haemoglobin can exist in various forms known as haemoglobin derivatives. *In vivo* haemoglobin is mainly present in two forms: (i) Deoxyhaemoglobin: unbound to oxygen; (ii) Oxyhaemoglobin: saturated with oxygen.[14] Small amounts of oxyhaemoglobin in blood can auto-oxidise into methaemoglobin, resulting in the oxidation of the ferrous (Fe2+) ion into a ferric (Fe3+) state. However, *in vivo*, an enzyme, cytochrome b5, reduces methaemoglobin back into deoxyhaemoglobin.[19] In a healthy individual, methaemoglobin only accounts for approximately 1% of the haemoglobin derivitives.[22]

Blood at crime scenes is located *ex vivo* and becomes completely saturated with oxygen resulting in the formation of oxyhaemoglobin.[22] This is due to the high partial pressure of oxygen in atmospheric air (~160mm Hg).[14] As the availability of cytochrome b5 decreases *ex vivo*, oxyhaemoglobin will auto-oxidise into methaemoglobin. Over time, an internal conformational change in the haem group of methaemoglobin results in methaemoglobin

denaturation into hemichrome.[22] Thus, *ex vivo*, haemoglobin follows a sequential degradation from oxyhaemoglobin to methaemoglobin to hemichrome (Figure 5).

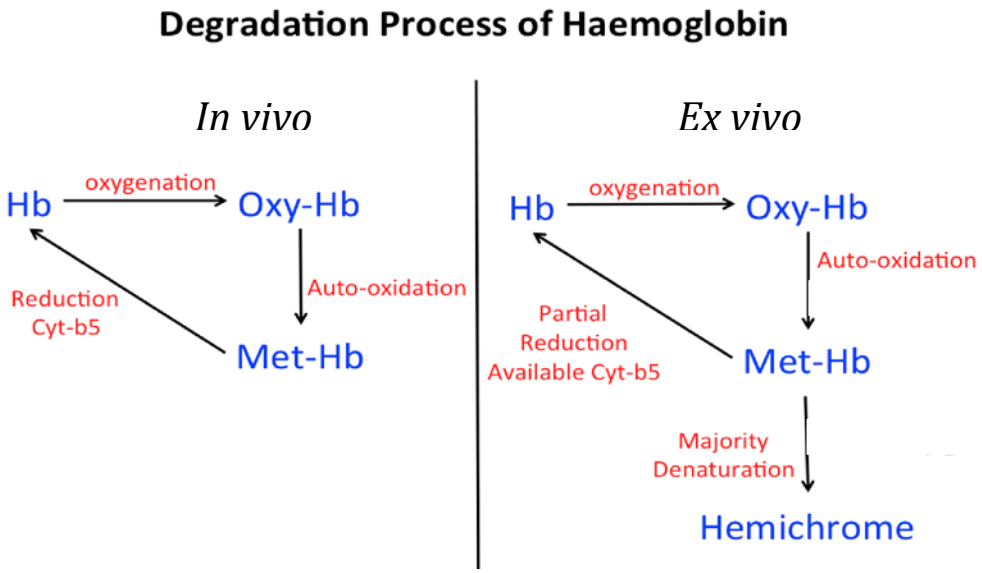

Figure 5: Schematic of oxidation and denaturation of haemoglobin *in vivo* and *ex vivo* (Adapted from [8]).

Marrone and Ballantyne[23] monitored the changes in human haemoglobin derivatives over time using visible spectroscopy. It was noted that for dry haemoglobin samples, the sum of the three haemoglobin derivatives (oxyhaemoglobin, methaemoglobin and hemichrome) decreased with age (Figure 6). This is indicative of a fourth haemoglobin derivative, which was hypothesised to be ferrylhaemoglobin or choleglobin.[23] However, the spectral evidence did not indicate any presence of either ferrylglobin or choleglobin. Thus, it is possible that a fourth unknown haemoglobin derivative is involved in the haemoglobin degradation process, which is undetectable using visible spectroscopy.

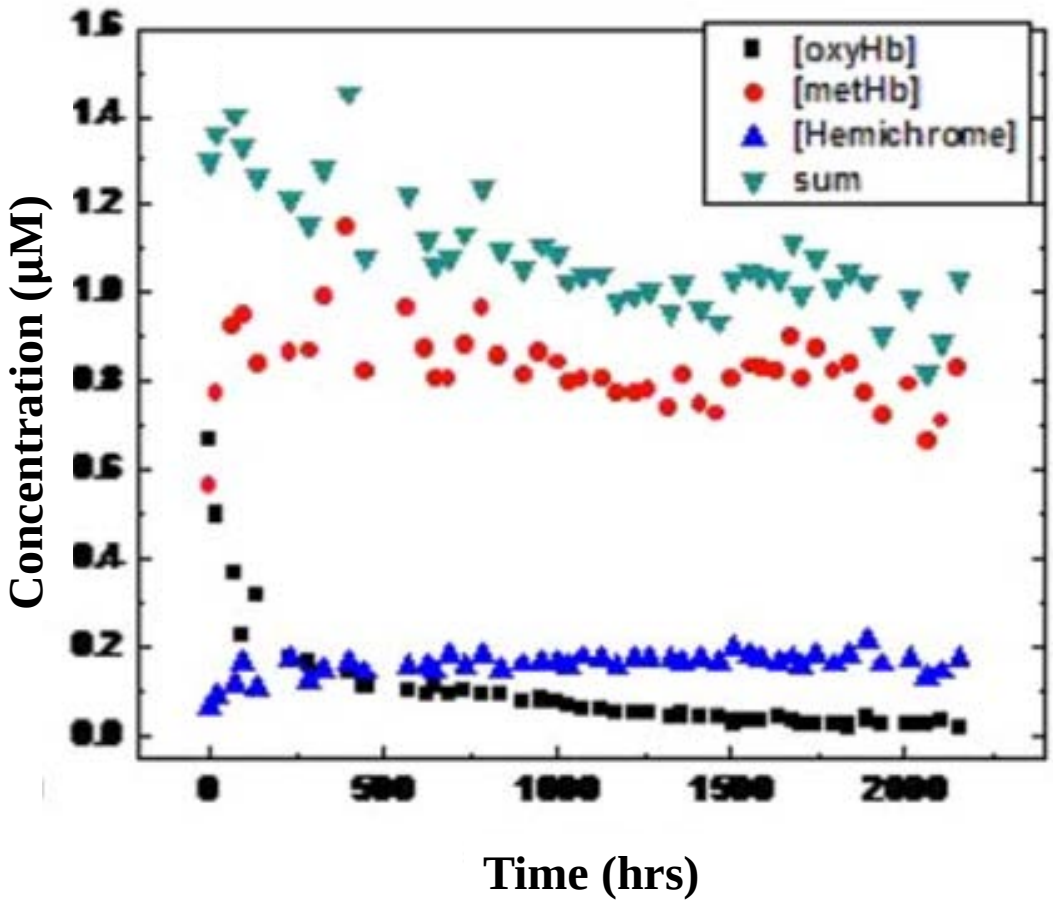

Figure 6: Detection of haemoglobin derivatives, oxyhaemoglobin (□), methaemoglobin(□) and hemichrome (Δ) from dry haemoglobin samples using visible spectroscopy over a period of 2200 hours (Image from [23]).

The ABACard® HemaTrace® kit utilises antibodies to target the α-chain of haemoglobin. However, there has yet to be any evidence in the literature to suggest whether the conformational changes that occur during the degradation process of haemoglobin will affect the efficacy of the HemaTrace® kit. Thus, this is an area that warrants future study.

2.3 ABACard® HemaTrace®

The ABACard® HemaTrace® is a sandwich format lateral flow immunochromatographic assay that utilises antibodies to target the alpha chain of human haemoglobin.[23] Similar to other lateral flow immunochromatographic assays, the HemaTrace® kit consists of four

overlapping strips arranged in a plastic housing.[25] These consist of the sample pad, conjugate pad, nitrocellulose membrane and absorbent pad (Figure 7). Table 2 summarises the components and function of each strip.

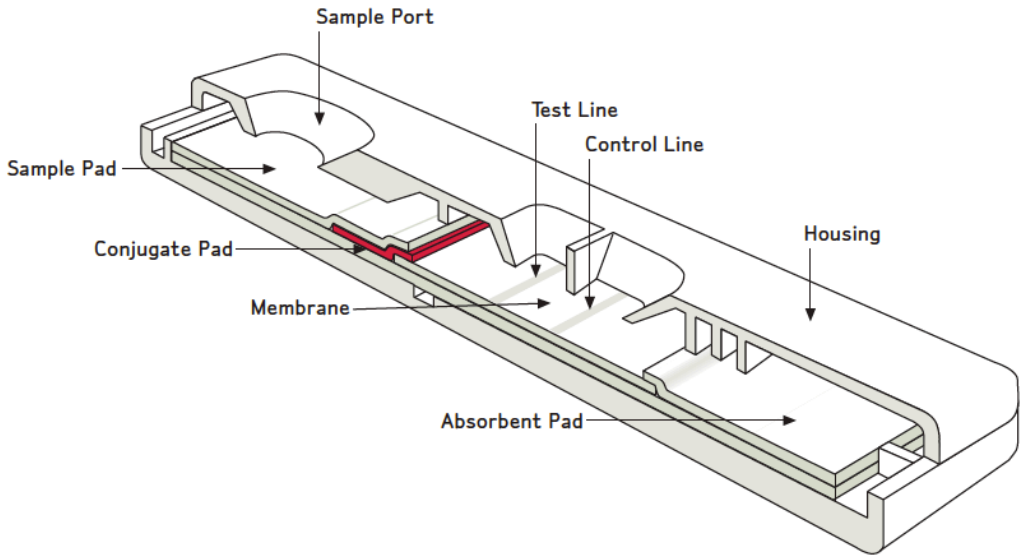

Figure 7: Schematic for HemaTrace® and other lateral flow immunochromatographic assays (Image from [26]).

Table 2: Summary of strip components and function for the ABACard® HemaTrace®. (Information collated from [26],[27]).

Strip name	Materials	Reagents	Function
Sample pad	Cellulose filter or woven mesh[a]	Buffer salts, blocking agents, detergents[a]	To ensure that samples are maintained at optimal conditions for antibody binding
Conjugate pad	Cellulose, glass or plastic non-woven filters[a]	Monoclonal antihuman-Hb antibody-dye conjugates	Contains the biorecognition molecule conjugated to a labelling dye that is responsible for the production of a signal during testing
Nitrocellulose membrane	Nitrocellulose	Immobile polyclonal antihuman-Hb antibodies, Immobile anti-Ig antibodies	Two spatially separated sets of antibodies where one acts as test for the presence of haemoglobin whilst the other acts as an internal control for the HemaTrace® kit
Absorbent pad	Cellulose filter	None	Acts as a reservoir to prevent backflow of the extract therefore reducing background noise resulting from unbound labelling dyes

[a] - As HemaTrace® is a commercial kit, the exact components are not publically known

Prior to HemaTrace® testing, blood samples are diluted in the HemaTrace® extraction buffer. This promotes haemolysis to release haemoglobin from RBCs.[24] To begin the HemaTrace® test, the extract is placed into the Sample Well (Figure 8). Haemoglobin in the extract binds to mobile monoclonal antihuman-Hb antibodies-dye conjugates in the conjugate pad resulting in the formation of mobile antigen-antibody-dye complexes. The mobile complexes diffuse along the nitrocellulose membrane to the Test Area containing immobile polyclonal antihuman-Hb antibodies. The immobilised polyclonal antibodies bind to the mobile complexes to form an antibody-antigen-antibody sandwich, hence the name sandwich format. This results in the accumulation of the conjugated dye at the Test Area resulting in a pink band. Excess mobile monoclonal antihuman-Hb antibodies migrate past the Test Area to the Control Area. Immobilised anti Ig-antibodies at the

Control Area bind to the excess mobile monoclonal antihuman-Hb antibodies resulting in the accumulation of the conjugated dye. Thus, a second pink band is formed that acts as an internal control for the HemaTrace® kit (Figure 8).[24]

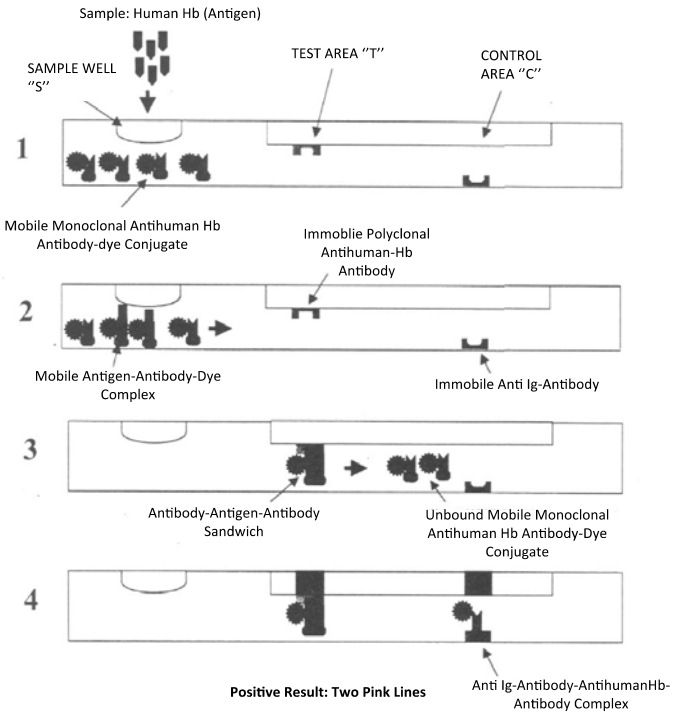

Figure 8: Mechanism of ABACard® HemaTrace® in the presence of human haemoglobin. (Image from [24]).

According to the manufacturer's protocol, HemaTrace® results should be recorded within 10 minutes after application of extract to the sample well. This is to prevent backflow of the extract, which may result in false positives.[5] The results of the HemaTrace® kit is qualitative, where results are recorded as positive, negative or inconclusive. A pink band at both the Test (T) and Control (C) regions indicates a positive result. A negative result is indicated by the absence of a pink band at the 'T' region with the presence of a band at the 'C' region. A result is deemed inconclusive if no band appears at the 'C' region regardless if a band is present or absent at the 'T' region (Figure 9).[5]

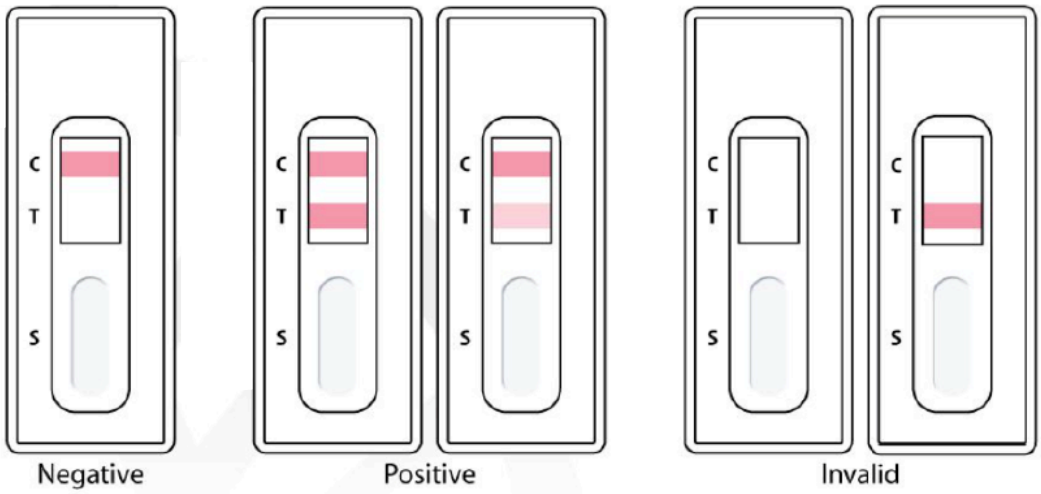

Figure 9: Interpretation of ABACard® HemaTrace® results. (Image from [5]).

As HemaTrace® is a sandwich format immunoassay, the HemaTrace® kit suffers from false negatives at high haemoglobin concentrations due to the High Dose Hook Effect.[5] At these concentrations, the mobile monoclonal antibodies become saturated with haemoglobin, generating mobile antigen-antibody-dye complexes with excess unbound haemoglobin. The excess unbound haemoglobin is smaller than the antigen-antibody-dye complexes and thus will diffuse down the nitrocellulose membrane at a faster rate. This results in unbound haemoglobin binding to the immobilised polyclonal antihuman-Hb antibodies first therefore occupying the binding sites of the antibodies at the Test line.[28, 29] Thus, the mobile antigen-antibody-dye complexes are unable to bind to the Test area, allowing the complexes to migrate to the Control area. In contrast to normal HemaTrace function, the antigen-antibody-dye complexes now bind to the immobilised anti Ig-antibodies at the Control area. This results in a pink band only at the Control area giving the appearance of a negative result.[24] However, according to the manufacturers, the High Dose Hook Effect can be ameliorated by retesting the extract using 1:10 and 1:100 fold dilutions.[5]

2.3.1 ABACard® HemaTrace® Sensitivity

A number of sensitivity studies have been conducted on the ABACard® HemaTrace® kit.[5, 24, 30-33] An overview of all sensitivity studies on HemaTrace® is summarised in Table 3. However, these studies have shown results inconsistent with the Technical Information Sheet provided by the manufacturers, Abacus Diagnostics.[5] The Technical Information Sheet provided by Abacus Diagnostics has noted that the minimum haemoglobin concentration detectable by the HemaTrace® kit is 0.05µg/mL.[5] However, Johnston *et al* [24] and Kristaly *et al* [32] have reported a minimum detection limit that was one magnitude higher than that indicated by Abacus Diagnostics, with a minimum detection limit of 0.26µg/mL and 0.125µg/mL respectively. In contrast, Swander and Stites[33] have reported a minimum detection limit of 0.008µg/mL, which was one magnitude lower than what was reported by Abacus Diagnostics. A detailed review of these studies has revealed three potential areas that could result in the difference in reported sensitivities. These were differences in haemoglobin concentration estimations, differences in HemaTrace® extraction protocols and differences in dilution protocols.

Table 3: Summary of ABACard® HemaTrace® sensitivity studies.

Study	Sample deposition	Extraction technique	Minimum Detection Limit	
			Dilution factor	Estimated Hb concentration (µg/mL)
HemaTrace® Technical Information Sheet[5]	n/a	Recommended to extract using HemaTrace® extraction buffer	n/a	0.05
Horjan *et al*[30]	Blood deposited immediately into HemaTrace® extraction buffer	Extracted using HemaTrace® extraction buffer	1:10 000 000	none
Johnston *et al**[24]	Blood deposited immediately into sterile water	Extracted using sterile water	1:500 000	0.26
	Blood deposited immediately into HemaTrace® extraction buffer	Extracted using HemaTrace® extraction buffer	1:2 000 000	0.07
	Blood diluted with sterile water and deposited on white cotton	Extracted using sterile water	1:256[a]	n/a[b]
		Extracted using HemaTrace® extraction buffer	1:256[a]	n/a[b]
Atkinson *et al*[31]	Blood diluted with sterile water and deposited on white cotton	Extracted using 2mL HemaTrace® extraction buffer	1:8192[a]	none
		Extracted using 300µL HemaTrace® extraction buffer	1:32768[a]	none
	Blood deposited immediately into sterile water	Extracted using sterile water	1:262 144	none
Kristaly *et al*[32]	Blood deposited immediately into sterile water	Extracted using sterile water	1:1 000 000	none
	Blood deposited immediately into HemaTrace® extraction buffer	Extracted using HemaTrace® extraction buffer	1:20 000	0.125
Swander and Stites[33]	Blood deposited immediately into HemaTrace® extraction buffer	Extracted using HemaTrace® extraction buffer	1:16 777 216	0.008
	Blood diluted with sterile water and deposited on white cotton	Extracted using 2mL HemaTrace® extraction buffer	1:32 768[a]	n/a[b]

* - used heparin and EDTA anticoagulated blood.

[a] - dilution factor of diluted bloodstain. Does not include dilution during extraction step.

[b] – unable to estimate Hb concentration as only a portion of the bloodstain on the substrate was extracted.

The first possible explanation for the sensitivity discrepancies is that the sensitivity studies used haemoglobin concentration estimates rather than measured absolute haemoglobin concentrations. Johnston *et al*[24] estimated the haemoglobin concentration of the neat blood sample to be 130g/L by using the average haemoglobin content of a healthy female. However, haemoglobin concentration of an individual can vary depending on race, age, sex, altitude and smoking.[15] Thus, this estimate is unlikely to be accurate. Similarly, Swander and Stites[33] and Kristaly *et al*[32] also estimated the haemoglobin concentration of the neat blood sample to determine the haemoglobin concentrations in the diluted samples. However, both studies failed to outline the methodology used to determine these values. To overcome this issue, future studies attempting to report haemoglobin concentrations as an indicator for HemaTrace® sensitivity should attempt to accurately measure the haemoglobin concentration of the neat blood samples. This can be done using flow cytometry by performing a Complete Blood Count[15] or ideally using high performance liquid chromatography[22].

The second explanation is that some studies used sterile water instead of the HemaTrace® extraction buffer to extract haemoglobin from red blood cells.[24, 31-32] A comparison of the results between the two extraction methods revealed that extraction using the HemaTrace® extraction buffer often resulted in sensitivity readings one magnitude higher than extraction using sterile water (Table 3). As HemaTrace® is a commercial kit, the exact contents of the extraction buffer are not publically known. However, Johnston *et al*[24] has noted that the extraction buffer contains Tris buffer saline. As Tris buffer has been previously shown to increase membrane permeability[34], the Tris buffer potentially increases the sensitivity of the HemaTrace kit by facilitating haemolysis.

Alternatively, the Tris buffer could merely act as a pH buffer to maintain the pH and ionic strength of the blood sample during HemaTrace testing.[26] However, until the full contents of the extraction buffer are known, this cannot be concluded.

Lastly, the differences in HemaTrace® sensitivity could potentially arise from different dilution protocols during extraction. Johnston *et al*[24], Atkinson *et al*[31] and Swander and Stites[33] diluted blood in sterile water which was then deposited on a cotton swatch. This resulted in a threefold magnitude reduction in HemaTrace sensitivity as blood samples were diluted once using sterile water followed by a second dilution during extraction in the HemaTrace® extraction buffer. To illustrate that the reduction in sensitivity was due to the two-step dilution, Atkinson *et al*[31] extracted the diluted blood samples in 300µL of HemaTrace® extraction buffer instead of the conventional 2mL, resulting in a four-fold increase in HemaTrace sensitivity. Thus, it is important that forensic examiners are aware that the HemaTrace® extraction buffer acts as a diluent as extreme dilutions of the extract could result in false negatives.

2.3.2 ABACard® HemaTrace® Specificity

A common trade-off for increased sensitivity is a reduction in specificity. For the HemaTrace® kit, this has been demonstrated in specificity studies on human bodily fluids. Horjan *et al*, Kristaly *et al*, Johnston *et al* and Reynolds[4, 24, 30, 32] tested saliva, semen, faeces, vaginal secretions and urine samples, which resulted in HemaTrace® false positives due to trace haemoglobin levels in the bodily fluids (Table 4). A contrasting study by Swander and Stites[33] noted that saliva and urine samples did not produce false positives. However, Swander and Stites failed to provide an outline of the methodology

employed for saliva and urine testing. As seen in Kristaly et al[32], HemaTrace® kits were only able to detect saliva and urine samples at 1:5 and 1:50 dilutions respectively. Thus, it is possible that the urine and saliva samples in the Swander and Stites[33] study was potentially too dilute. However, without a clear outline of the methodology employed in the Swanders and Stites study, this cannot be concluded. It was hypothesised that the false positive results seen when testing other human bodily fluids is a result of trace amounts of haemoglobin in these fluid.[24,30,32] Thus, although the ABACard® HemaTrace® kit is specific for haemoglobin, due to the high sensitivity of the kit, the ABACard® HemaTrace® kit may not be specific for blood.

Table 4: Summary of ABACard® HemaTrace® bodily fluid specificity studies.

Study	Bodily fluid specificity	
	Human bodily fluids tested	**Results**
HemaTrace® Technical Information Sheet[5]	n/a	Trace amounts of Hb in urine, semen, faeces, saliva, vaginal secretions and perspiration can result in false positives
Horjan *et al*[30]	Saliva	Saliva resulted in HemaTrace® false positive
Reynolds[4]	Saliva, urine	Saliva and urine resulted in HemaTrace® false positive
Johnston *et al*[24]	Vaginal secretions, semen, faeces, menstrual blood, nasal secretions, saliva, urine	Oral, vaginal, anal and rectal swabs resulted in HemaTrace® false positives
Kristaly *et al*[32]	Semen, urine, saliva	All three fluids resulted in false positives
Swander and Stites[33]	Saliva, urine	No false positives detected in saliva and urine samples

Species specificity studies on HemaTrace® have demonstrated that HemaTrace produces false positives with higher primates and ferret blood samples[2-5,24, 31-33]. An overview of species specificity studies is illustrated in Table 5. A contrasting study by Kristaly *et al*[32] noted that ferret blood did not result in a false positive result. In the study conducted by

Atkinson *et al*[31], it was noted that four drops of ferret blood placed into the sample well followed by two drops of extraction buffer, only produced a weak positive result. In order to show that this was not due to the high dose hook effect, one drop of ferret blood was diluted into 2mL of the HemaTrace® extraction buffer, which also produced a weak positive result. Thus, this is suggestive that the sensitivity of the HemaTrace® kit against ferret blood is potentially low. This may potentially provide a possible explanation for the negative result seen in Kristaly *et al*[32]. However, this cannot be concluded, as neither Kristaly *et al*[32] nor Atkinson *et al*[31] noted the dilution factors used to test the ferret blood samples.

Table 5: Summary of ABACard® HemaTrace® species specificity studies.

Study	Species specificity	
	Animals tested	**Results**
HemaTrace® Technical Information Sheet[5]	n/a	Primates (anthropoideae) and ferret blood will result in a positive HemaTrace® result
Reynolds[4]	Canine (dog), Equine (horse), Porcine (pig), Feline (cat)	All animals tested negative. However, when animal blood was mixed with human blood, mixtures tested positive
Johnston *et al*[24]	Dog, Cat, Horse, Pig, Goat, Cow, Domestic ferret, cockatiel, Dall's sheep, European reindeer, Barbary ape, Mara, Wapiti (elk), Black footed ferret, Tamandua, Ring-tailed lemur, Snow leopard, Sable antelope, Boa constrictor, Trumpeter swan, Japanese macaque, Sumatran tiger, Chamois, African cheetah, African elephant, Grevy's zebra, Olive baboon, Canadian lynx, Harbour seal, Striped skunk, Racoon, Red fox, Moose, San Clemente Loggerhead Shrike, Gray Jay, Eastern wolf, Black bear	Only higher primates and ferrets blood samples resulted in false positives
Atkinson *et al*[31]	Cat, Kangaroo, Horse, Dog, Bull, Sheep, Pig, Fowl, Python, Possum, Wallaby, Monkey, Ferret	Only the monkey and ferret blood samples resulted in false positives
Kristaly *et al*[32]	Skunk, Rabbit, Chicken, Porcupine, Snake, Lion, Waterbuck, Fox, Tortoise, Elephant, Pig, Flamingo, Camel, Horse, Spoonbill, Dog, Cat, Ferret	All animal blood samples tested negative including ferret blood samples
Swander and Stites[33]	Deer, Cow, Pig, Horse, Dog, Cat,	All animal blood samples tested negative

A possible explanation for the false positive results for higher primates and ferret blood was proposed by Johnston *et al*.[24] It was stated that the amino acid sequence TNAVAHV that is located on residues 67-73 on the α-chain of the human haemoglobin protein could potentially serve as an epitope for monoclonal antibody recognition. This region was noted to show variability between human and commonly encountered animal haemoglobins. Also, this sequence was noted to be sufficiently different from the corresponding amino acid sequence in rabbits and mice to enable an immunogenic reaction, which is required for the production of monoclonal and polyclonal antibodies.[35] However, no evidence or references were provided to support this statements in the study. Thus to reappraise this statement, the author of this literature review conducted a GenBank search to compare the alpha chain haemoglobin amino acid sequences between humans, higher primates, ferrets and other commonly found household animals.[20] The gorilla and chimpanzee were used as a representative for higher primates. The sequence for cats, dogs and rats were used, as these are common household animals. Lastly, chicken cow and pig sequences were analysed, as these are common meat products found in kitchens. A ClustalW alignment, using the Unipro UGENE 1.27 software, revealed that the TNAVAHV sequence is shared between humans, gorillas, chimpanzees and ferrets whilst dogs, cats, rats, chickens, cows and pigs showed variability in the amino acid sequence at this region (Figure 10). Thus, this supports the hypothesis proposed by Johnston *et al.*[24] However, as the antibodies in the HemaTrace® kit are not publically known, this is a mere postulation.

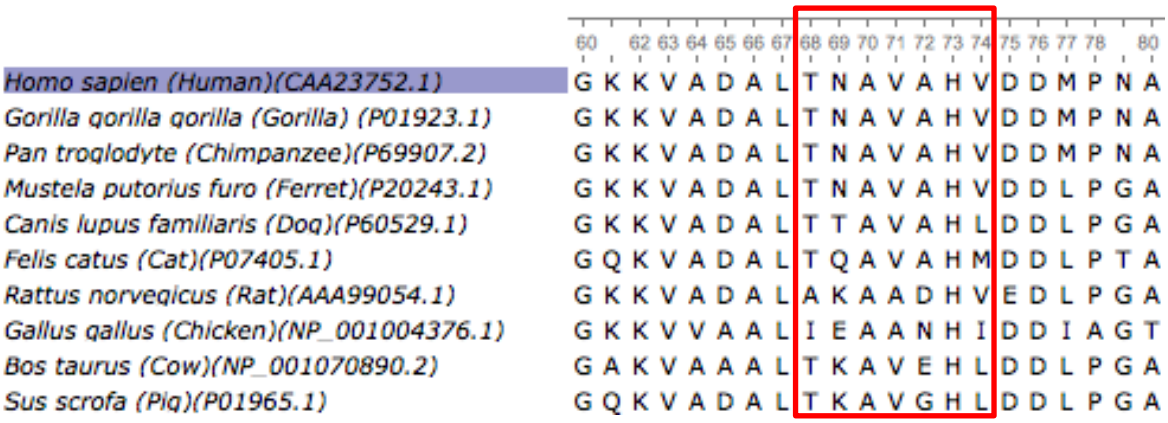

Figure 10: ClustalW alignment of the alpha chain amino acid sequence of haemoglobin for multiple animal species using Unipro UGENE 1.27 software.

In conclusion, the ABACard® HemaTrace® kit is neither specific to human samples nor blood samples. Thus, there has been some debate in the literature as to whether HemaTrace® is a confirmatory or presumptive test for the identification of human blood. One justification for the confirmatory test argument is that ferret and higher primate blood samples are unlikely to be found at the majority of crime scenes. Furthermore, bloodstains at a crime scene are selected based on visual cues and contextual cues whilst a Kastle-Meyer test is conducted prior to HemaTrace testing to eliminate the inclusion of other human bodily fluids in the HemaTrace results.[31] Thus, the consideration of the HemaTrace® kit as a confirmatory test for human blood at crime scenes is somewhat practical. This was shown in case studies where the specificity of the ABACard® HemaTrace® kit was sufficient to be able to differentiate human blood from animal blood at crime scenes. Furthermore, HemaTrace® kits were able to identify human blood samples in human and animal blood mixtures.[4]

2.4 Antibody Structure and Function

The ABACard® HemaTrace® utilises antibodies, also known as immunoglobulins, to target the α-chain of human haemoglobin.[24] *In* vivo, there are four main immunoglobin isotypes known as, IgG, IgM, IgE, IgD and IgA.[35] However, for commercial immunoassays such as the ABACard® HemaTrace®, the IgG isotype is preferentially used as IgG antibodies have been shown to have long resident half-lives, thus resulting in longer shelf lives.[36]

A typical IgG antibody molecule is composed of four polypeptide chains. This consists of two identical Heavy chains and two identical Light chains bound together by disulphide bridges (Figure 11).[35] These four polypeptides can be functionally broken down and grouped together into different segments consisting of two identical Fab segments and a single Fc segment. This results in a Y-shaped conformation where the antigen binding sites are located at the far end of the Fab segments (Figure 11).[37]

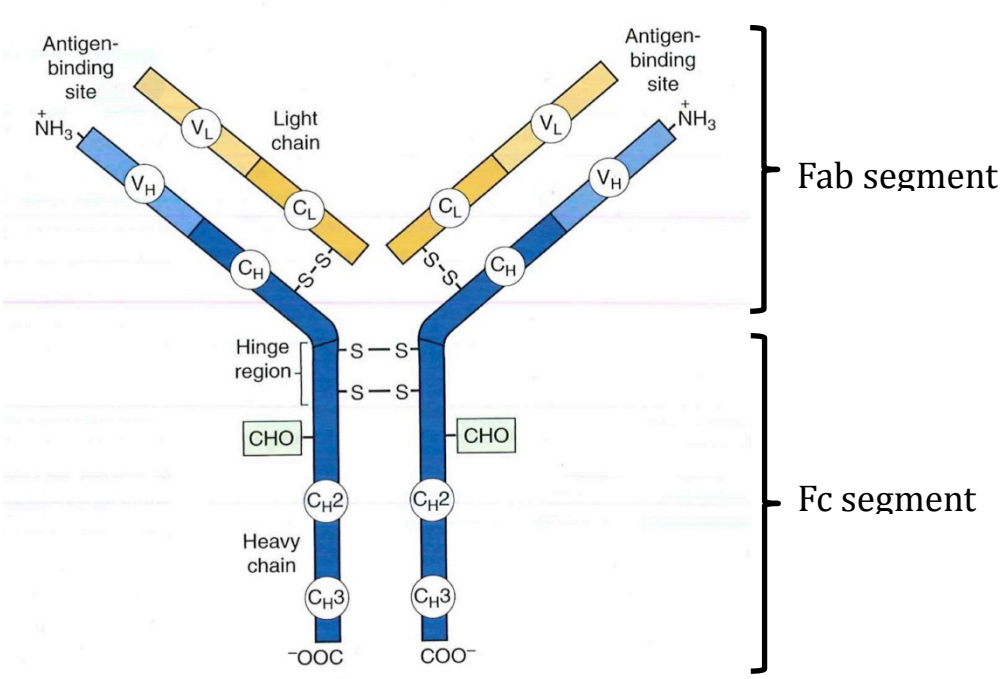

Figure 11: Schematic of Immunoglobulin G (IgG) structure (Image from [19])

The secondary structure of the Fab segment is comprised of β-strands that flank three hyper variable regions in the Light chain and Heavy chain each.[35] The β-strand structure, folds onto itself to form the tertiary and quaternary structure of the Fab segment. A result of this folding is that the six hypervariable domains from the Heavy and Light chains are concentrated at the outer edge of the antigen binding site (Figure 12). This region is responsible for the specificity and binding capabilities of the IgG antibody and is called the Complementarity-determining region (CDR).[35]

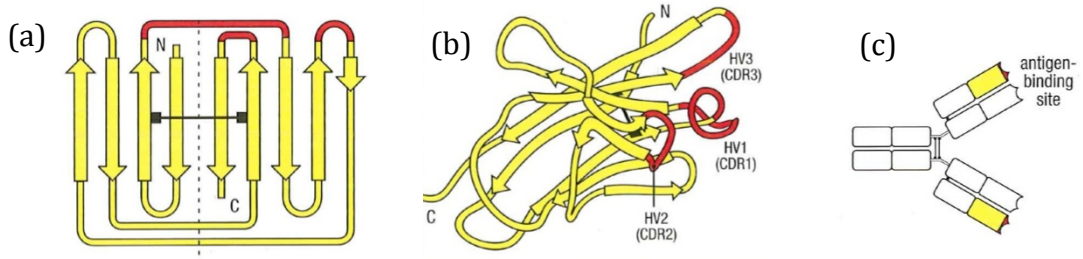

Figure 12: Formation of the CDR of the Light Chain at the antigen-binding site. (a) Secondary structure, (b) Tertiary structure and (c) Quaternary structure of the Light Chain CDR (Image adapted from [35])

Antibody binding to an antigen is reliant on shape and chemical complementarity of the antigens and the CDR of the antibody.[35] Chemical complementarity is responsible for the actual binding of the antibody to the antigen where amino acid side chains form reversible non-covalent interactions between the antigen binding site and the antigen. Shape complementarity ensures that the amino acid side chains in the antibody and the antigen are in the appropriate orientation for binding.[38] Degradative agents such as temperature have been known to denature proteins resulting in a change in protein folding conformation.[35] Thus, for the ABACard® HemaTrace®, exposure to elevated temperatures could potentially result in a conformational change in the CDRs of the HemaTrace® antibodies resulting in a loss of antibody binding function.

2.5 Elevated Temperature Exposure

The effects of temperature on the ABACard® HemaTrace® has yet to be studied. Thus, this section will aim to postulate the effects of temperature on HemaTrace® by evaluating temperature studies on antibodies and other lateral flow immunochromatographic assays.

2.5.1 Temperature and antibodies

ABACard® HemaTrace® kits stored in police vehicles are potentially exposed to elevated temperatures[6], which could potentially lead to antibody denaturation. A study by Vemeer and Norde[39] used differential scanning calorimetry (DSC) to monitor the changes in IgG antibodies in response to temperature. They found that at 61°C, the Fab segment denatures irreversibly followed by Fc segment denaturation at 71°C. In order to measure the rate of Fab segment denaturation, Vemeer and Norde[39] incubated IgG antibodies at temperatures ranging from 41°C to 70°C and measured heat flow using isothermal calorimetry. Similar to the DSC results, antibodies denatured at approximately 60°C. However, it was also noted that at 55°C, IgG antibodies denatured at the Fab segment but at a much slower rate, taking up to 25 hours for full denaturation. Thus, this showed that the level of antibody denaturation is dependant on both temperature and exposure duration. As antibody binding is dependant on shape complementarity of the Fab segment with the antigen, denaturation of the antibody at 55°C could potentially result in a change in antibody binding activity.[35] However, Vemeer and Norde[39] did not test the antibody reactivity after denaturation thus this remains to be concluded.

Johnstone *et al*[40] evaluated the effects of temperature on the binding kinetics of four monoclonal antibodies using flow cytometry at temperatures ranging from 1°C to 37°C. It was found that for the majority of antibodies, an increase in temperature resulted in decrease in the antibody binding equilibrium constant. This indicated that at higher temperatures, antibodies were more likely to dissociate from their respective antigens. However, Johnstone *et al*[40] noted that the decrease in antibody binding kinetics for the four monoclonal antibodies did not show a predictive pattern, indicating that different antibodies are affected differently by elevated temperature exposure.

Labrousse *et al*[41] and Imagawa *et al*[42] studied the effects of temperature on IgG antibodies in regards to antibody reactivity and specificity. Both studies showed a similar trend where antibody reactivity increased with temperature. This is somewhat contrasting to the study by Johnstone *et al*[40] that indicated that antibodies were more likely to dissociate from antigens at higher temperatures. However, Labrousse *et al*[41] and Imagawa *et al*[42] noted that the increase in antibody reactivity was also associated with a decrease in antibody specificity. Thus, this could potentially explain the difference seen between the Imagawa and Labrousse studies and the Johnstone *et al* study.[40-42] Additionally, Labrousse *et al*[41] noted that at 56°C, antibody reactivity began to decrease. As noted by Vemeer and Norde[39], this temperature coincides with the denaturation temperature of the Fab segment. Thus, it is possible that the Fab segment denaturation could contribute to the reduction in antibody reactivity. However, as both antibodies and antigens were incubated simultaneously at the experimental temperatures, Labrousse *et al*[41] could not conclude whether the reduction in antibody reactivity was a result of antibody denaturation or antigen denaturation.

The literature thus far suggests that at temperatures above 55°C, antibody reactivity will reduce due to Fab denaturation. However, these studies evaluated antibodies in solution. In contrast, the ABACard® HemaTrace® antibodies are immobilised on a nitrocellulose membrane. Wang *et al*[36] and Wu *et al*[43] studied the effects of temperature on antibodies immobilised on paper by incubating the antibodies at temperatures ranging from 40°C to 140°C. It was found that antibodies remained relatively stable at temperatures below 80°C, showing only a slight reduction in antibody reactivity with exposure times up to 24 hours (Figure 13). The stable temperature ranges noted in Wang *et al*[36] and Wu *et al*[43] are highly contrasting with what was reported by Vemeer and Norde.[11] Thus, this suggests that the immobilisation of antibodies to a surface could potentially increase antibody resistance to denaturation at elevated temperatures.

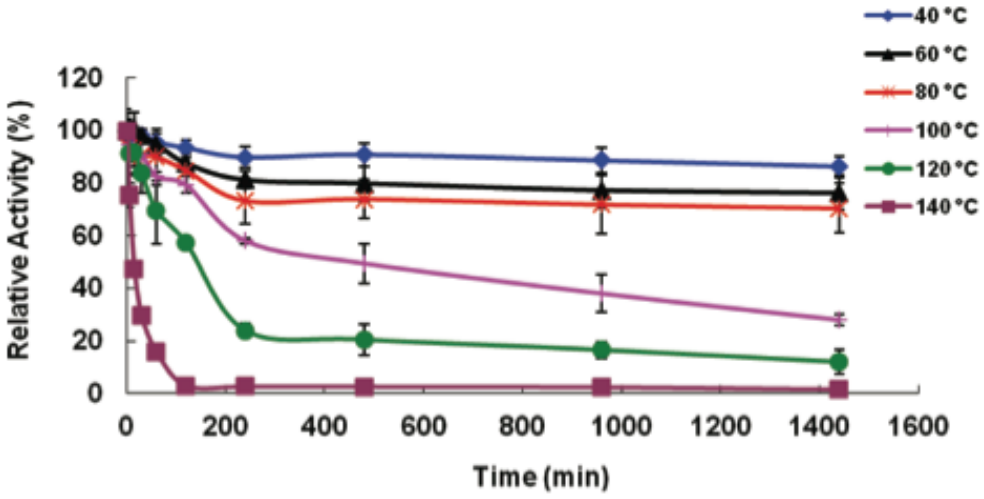

Figure 13: Influence of temperature and exposure time on antibodies immobilised on paper. Error bars give the standard deviation of the triplicates (Image from [36])

In conclusion, the current evidence indicates that elevated temperature exposure results in antibody denaturation, resulting in a reduction in antibody binding efficacy. Therefore the exposure of the HemaTrace® kits to elevated temperatures may result in a decrease

in antibody binding to haemoglobin resulting in a reduction in kit efficacy. However, different antibodies appear to respond differently to elevated temperature exposure. Furthermore, antibodies immobilised on paper have been shown to increase the stability of antibodies at higher temperatures. Thus as the antibodies used in the ABACard® HemaTrace® kit are not publically known, it is difficult to infer the exact effects of temperature on the HemaTrace® antibodies. Thus, this is a potential area for future research.

2.5.2 Temperature and Lateral Flow Immunochromatographic Assays

The ABACard® HemaTrace® kit is a sandwich format lateral flow immunochromatographic assay (LFIA).[5] Thus, temperature studies done on other LFIAs could potentially provide insights on the possible effects of temperature on the HemaTrace® kits.

A common approach to monitor the temperature stability of LFIAs is by quantitative measurement of the Test and Control band intensities (Figure 14).[44-51] Peng *et al*[44] noted that the Test line intensity correlates well with sample concentration. However, Li *et al*[45] indicated that pH, ionic strength and temperature variations could lead to variability in the Test line intensity. To overcome this, the majority of studies use the Control line as a normalising factor, thus using he ratio of the Test band intensity against the Control band intensity (T/C ratio) as a representation of target concentration.[44-51] Furthermore, Liu *et al*[46] and Alfonssi *et al*[47] found that T/C ratio concentration measurements were comparable to liquid chromatography-mass spectrometry (LC-MS) concentration measurements (Figure 15).

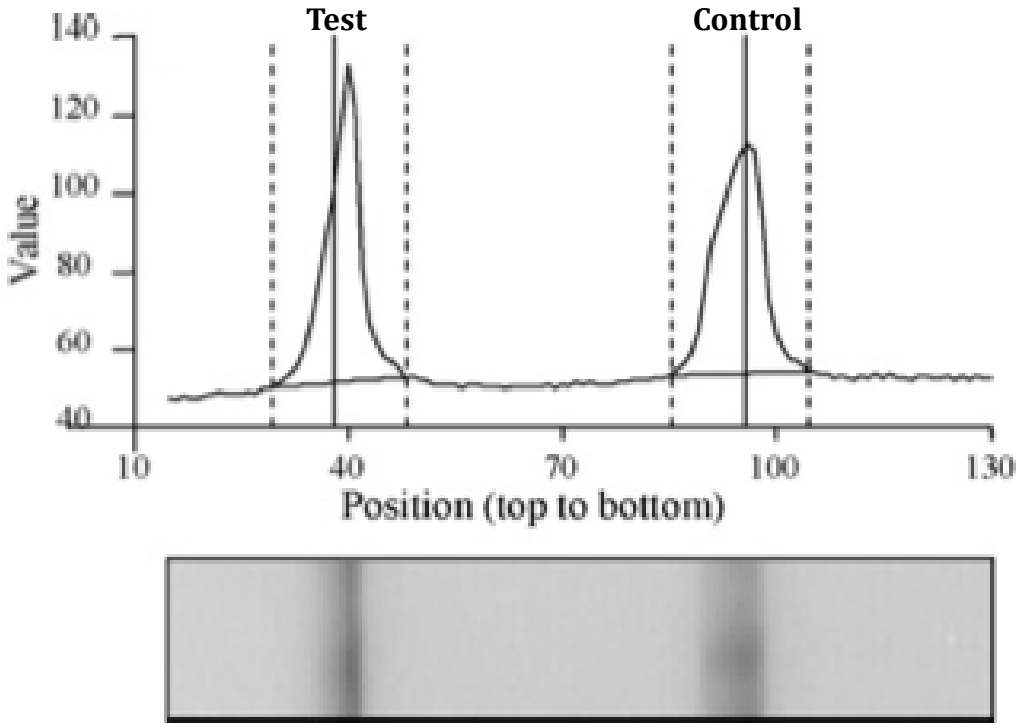

Figure 14: Measurement of Test and Control band intensities of a lateral flow immunochromatographic assay (LFIA)(Image from [47]).

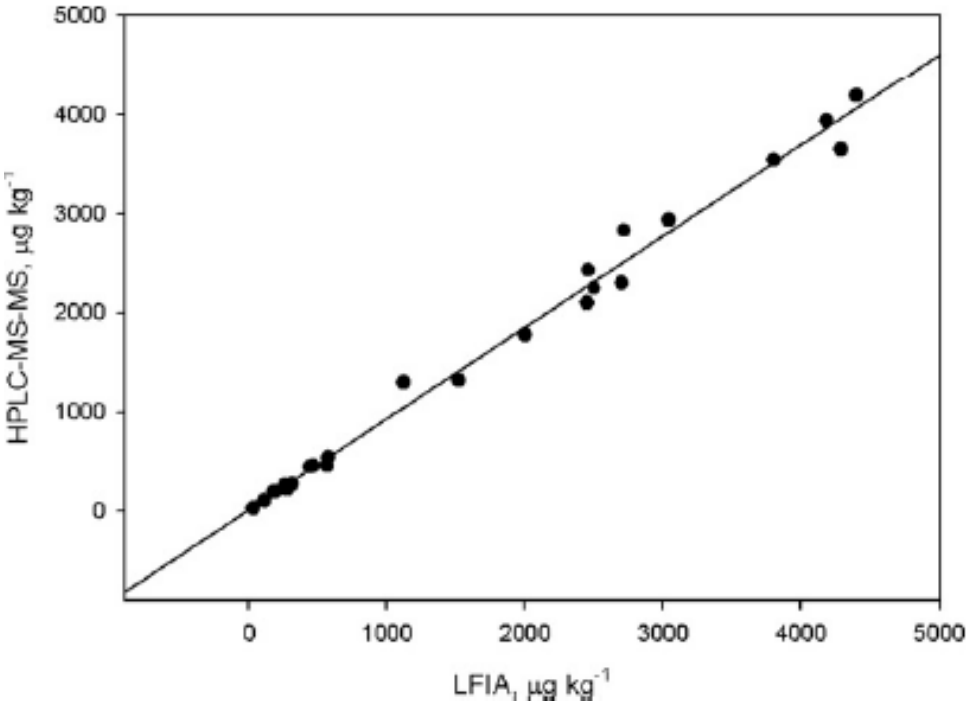

Figure 15: Correlation of results between C/T ratio concentration measurements and liquid chromatography-mass spectrometry (LC-MS) concentration measurements. Linear regression analysis showed good correlation between the methods(y = 1.07x – 0.2, R^2 = 0.0990, n = 24)(Image from [47]).

Peng *et al*[44] conducted a study to evaluate the temperature stability of an LFIA used to detect clenbuterol in swine urine. It was found that when tested against a positive control sample of 5.0μg/L incubation of the LFIA at 60°C resulted in a steady decline of T/C ratio with the duration of temperature exposure. A similar study by Di Nardo *et al*[48] showed that at room temperature and 4°C, the LFIA tested took 180 days to degrade to unreliable levels. Incubation of the LFIA at 37°C took a week to reach similar levels. Thus this showed that temperature exposure reduced the efficacy of the LFIA kits by accelerating the rate of degradation of the LFIA kits.

Incidentally, exposure of LFIAs to elevated temperatures is routinely used to determine kit shelf life.[52] This is known as an accelerated aging test. A typical accelerated aging test subjects LFIAs to temperatures 15°C or more above the recommended storage temperature.[52] By monitoring the change in T/C ratio of the LFIA kit with time at elevated temperatures, the LFIA shelf life can be calculated using the Arrhenius equation. The equation gives the rate constant of LFIA degradation, *k*, which is influenced by the temperature, *T*, and the activation energy E_a. The Arrhenius equation is shown below where *A* is the pre-exponential factor, and *R* is the universal gas constant.

$$k = Ae^{-Ea/RT}$$

The rate constant, *k*, can then be used to calculate the duration an LFIA kit remains viable at a given temperature.

Thus, for the ABACard® HemaTrace®, exposure to elevated temperatures is expected to result in a decrease in kit efficacy. However, without experimental evidence, this cannot be concluded. Furthermore, a study by Peng *et al*[44] demonstrated that addition of stabilising compounds such as WellChampion™ (Kem-En-Tec Diagnostics) can increase the thermal stability of LFIAs. Thus, as the components of the HemaTrace® kits are not publically known, the effects of temperature on HemaTrace® remains to be elucidated.

3.0 EXPERIMENTAL DESIGN ELEMENTS

3.1 Temperature Conditions in Parked Vehicles

It has been reported that the high temperatures experienced in the northern regions of WA may be responsible for the HemaTrace® false negatives reported by WA Police officers.[6] A report by the Bureau of Meteorology[7] in 2017 showed that a northern town, Broome, had ambient temperatures consistently higher than in Perth (Table 6). Furthermore, during the summer months, it was not uncommon for temperatures to exceed 40°C.

Table 6: A summary of monthly temperatures recorded for Perth (International Airport Station) and Broome (Airport Station) during 2016 as reported by the Bureau of Meteorology. (Information collated from [7])

Month	Perth		Broome	
	Monthly mean temperature (°C)	Maximum recorded temperature (°C)	Monthly mean temperature (°C)	Maximum recorded temperature (°C)
January	32.4	43.0	34.4	40.5
February	32.8	42.7	34.2	39.6
March	30.0	40.1	35.0	40.8
April	25.0	33.9	36.8	41.0
May	20.8	26.2	32.0	38.2
June	18.5	24.7	31.1	34.0
July	17.7	22.6	30.9	35.1
August	17.5	22.3	30.7	34.8
September	18.5	23.4	32.3	37.1
October	22.0	32.6	34.1	40.1
November	28.3	38.2	34.8	42.6
December	29.3	42.8	33.8	41.9

Dadour *et al*[10] conducted a study looking at the temperatures experienced inside parked vehicles in Perth, WA. It was found that the temperature inside the cabin of a vehicle was able to exceed the outside ambient temperature by 20-30°C. Thus an appropriate temperature range for HemaTrace® testing would be temperatures ranging from approximately 40-70°C.

Interestingly, Dadour *et al*[10] also demonstrated that the internal temperature of a vehicle was influenced by the amount of solar radiation that was able to penetrate into the cabin. The orientation and level of shading of the vehicle also influenced the internal temperature, as both would determine the level of solar radiation exposure. Car colour and ventilation also had an effect on the internal cabin temperature where white cars were found to have lower average internal temperatures than black cars. Furthermore, it was noted that the trunk of the vehicle had a temperature that was significantly lower

than the cabin temperature but higher than the ambient temperature (Figure 16). As multiple variables appear to be affecting the internal temperature of a car, temperature data loggers could be used to record temperatures experienced by the ABACard® HemaTrace® kits stored in police vehicles. This would enable the determination of a more accurate temperature range for testing, in order to simulate the storage conditions experienced by the HemaTrace® kits in WA Police vehicles.

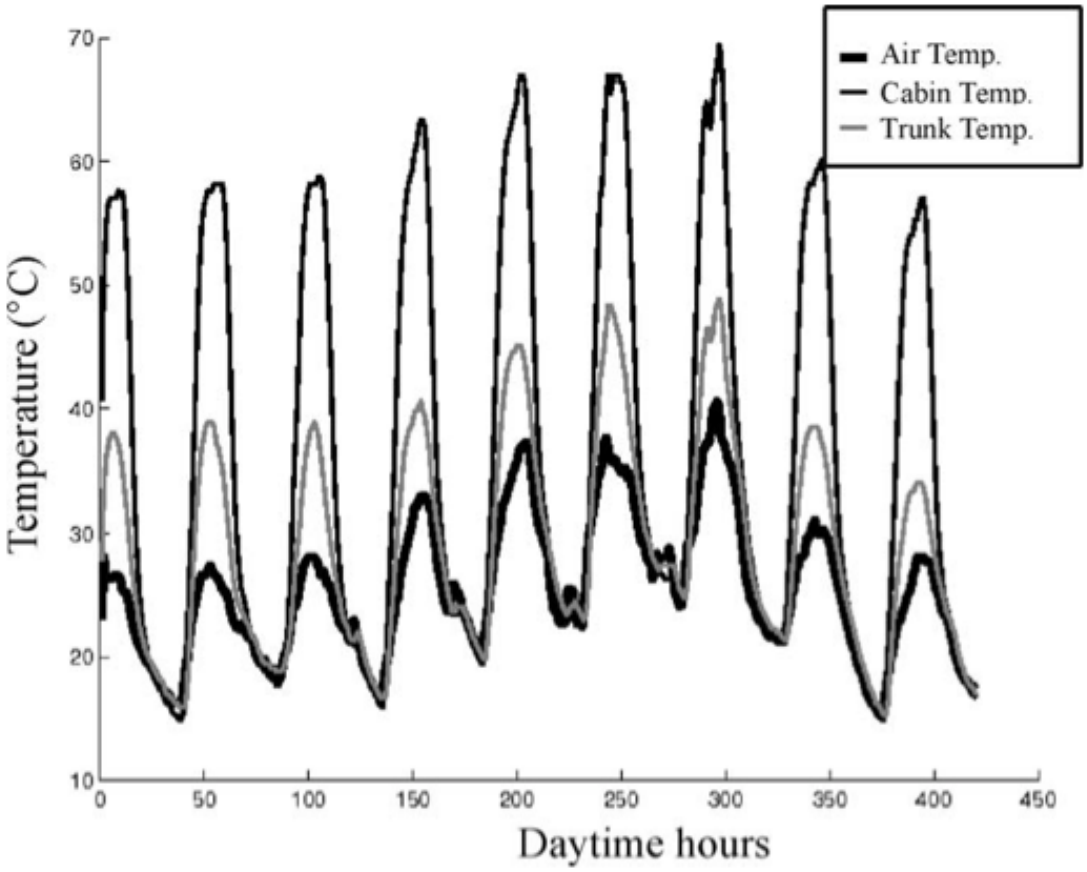

Figure 16: Ambient outside air temperature (the lowest curve), temperature measured in the trunk (middle curve) and temperature measure in the cabin (upper curve) of a parked unshaded vehicle in Perth, WA (summer 2000)(Image from [10])

3.2 Exposure Time

The degradative impact of temperature on LFIAs is dependent on the level and exposure time. King *et al*[53] provided a recommended outline for stability testing of ligand binding assays. It was noted that an ideal study would employ a real-time stability test by incubating the kits at the desired storage temperature until the kits failed to produce the expected results. According to the HemaTrace® Technical Sheet provided by Abacus Diagnostic, the HemaTrace® kits, upon receipt, should have a shelf life of approximately one year, with a recommended storage temperature of 28°C and below.[5] Therefore, an ideal study looking at the effects of temperature on the HemaTrace® kits should monitor the sensitivity of the kits throughout its shelf life (i.e. one year).

However, due to practical reasons, an accelerated stability test could be employed. Previous studies looking at accelerated stability tests of LFIAs often incubate LFIA kits at 37°C or 60°C for 1-4 weeks. This has been shown to demonstrate similar results to the real-time stability tests where real-time stability can be estimated using the Arrhenius equation. Thus, an appropriate incubation period for the HemaTrace® kits should be 1-4 weeks at experimental temperature conditions.

3.3 Qualitative Assessment of HemaTrace with Temperature

The sensitivity of the HemaTrace® kits can be monitored using similar qualitative protocols used in previous HemaTrace® studies. This can be done by testing the HemaTrace® kits against a dilution series of blood samples.[24, 30-33] The minimum detection limit can be estimated by recording the most dilute sample with a positive HemaTrace®

result. The effects of temperature on the HemaTrace® kit can be monitored by observing if a change in the minimum detection limit is seen after incubation of the kits at experimental temperatures.

However, one disadvantage of this qualitative method is that a weak positive band can be classified as positive or negative, depending on the interpretation of the observer. Secondly, for a qualitative study, the minimum detection limit is more accurately represented as a range rather than a specific value. For example, the minimum detectable limit for HemaTrace®, as reported by Swander and Stites[33], was a 1:16 777 216 dilution factor. The subsequent dilution factor of 1:33 554 432 produced a negative result. Therefore, the minimum detection limit lies between the two dilution factors. Thus it would be beneficial to be able to quantify the HemaTrace® results to objectively measure the minimum detection limit, which is stated in the Technical Information Sheet to be 0.05µg/mL.[5]

3.4 Quantitative Assessment of HemaTrace with Temperature

Quantitative measurements of the ABACard® HemaTrace® kits can be done similar to previous studies, using T/C ratio measurements. For the HemaTrace® kits, this could potentially be done by measuring the Test and Control band intensities using reflectance spectroscopy (Figure 17). However, the labelling molecule responsible for the appearance of the pink band is not publically known. Hence, the optical properties of the labelling molecule are not publically known. Furthermore, haemoglobin is a chromophore that when mixed in the HemaTrace® extraction buffer can result in various solution colours ranging from straw coloured to orange to red.[31, 33] Thus, this could potentially lead to

background interference resulting in inaccurate spectroscopic measurements. Thus this method will have to be validated before a stability test can be performed on the HemaTrace® kits.

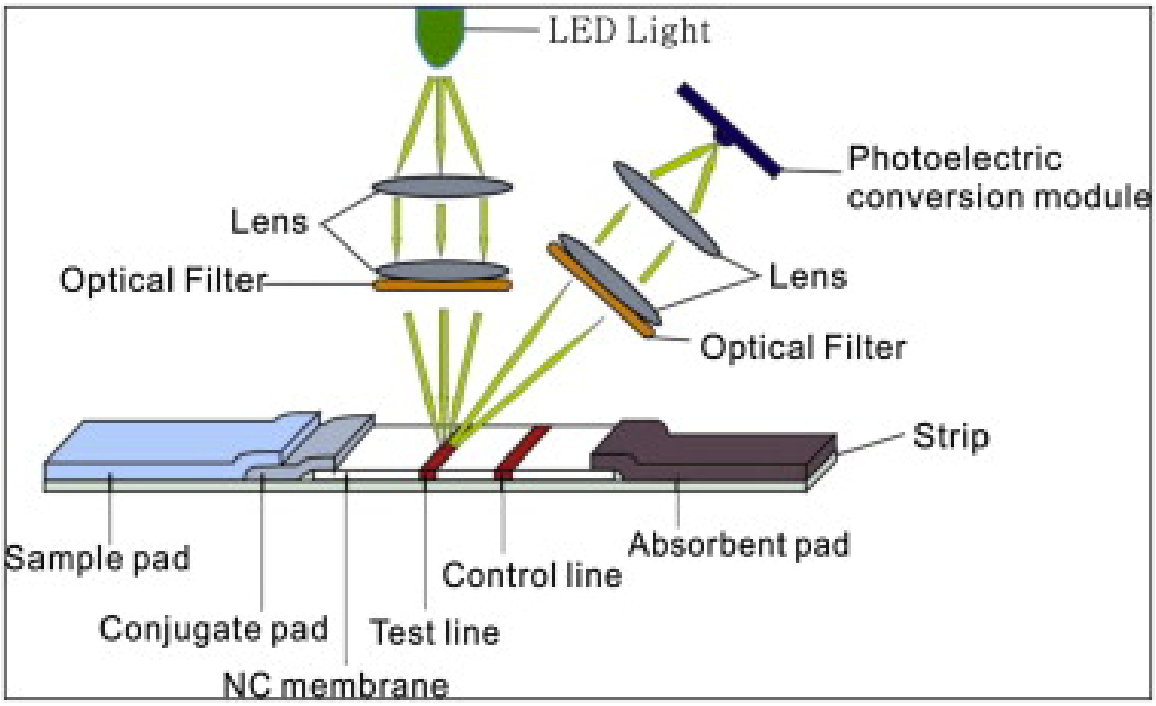

Figure 17: Schematic of band intensity measurement using reflectance spectroscopy (Image from [45]).

However, if this method is successful, this will allow the calculation of the Arrhenius equation to determine the rate of HemaTrace® degradation at a given temperature. This would be beneficial, as this will provide an indication toward the shelf life of the HemaTrace® kits stored in the WA Police vehicles

3.5 Miscellaneous Factors

The experimental design aims to evaluate the effects of temperature on the HemaTrace® kits and any potential factors that may effect HemaTrace® efficacy will be controlled. Humidity has been shown to increase the rate of antibody degradation on antibodies stored on paper.[43] Furthermore, pH and ionic strength variations can disrupt protein interactions due protein unfolding.[54] Thus, these factors should be controlled to ensure that these factors do not complicate the interpretation of the HemaTrace® thermal stability results.

Lastly, Atkinson *et al*, Johnston *et al* and Horjan *et al*[24, 30-31] noted that aged bloodstains could produce false negatives. It was hypothesised that this is due to a reduction of protein solubility as the bloodstain ages. Thus, a HemaTrace® thermal stability study should standardise bloodstain age by using fresh blood samples.

4.0 EXPERIMENTAL AIMS AND HYPOTHESIS

From the research presented in this literature review, it has been demonstrated that elevated temperatures and prolonged exposure durations may result in a reduction in HemaTrace® kit efficacy. This is potentially due to antibody denaturation resulting in the inability of antibodies to bind to haemoglobin molecules in human blood samples. The occurrence of false negative HemaTrace® results could potentially lead to the exclusion of relevant forensic evidence. Thus, the experiment dictated by this literature review aims to

assess whether the elevated storage conditions experienced by the HemaTrace® kits could potentially result in false negatives. This will be done by testing two hypotheses:

Hypothesis 1

H_0: Exposure of the HemaTrace® kits to elevated temperatures will not affect the sensitivity of the kits.

H_1: Exposure of the HemaTrace® kits to elevated temperature will result in an increase or decrease in the sensitivity of the kit.

Hypothesis 2

H_0: The duration of elevated temperature exposure does not effect the sensitivity of the HemaTrace® kits.

H_1: The duration of elevated temperature exposure will result in an increase or decrease in the sensitivity of the kits.

5.0 Conclusion

In conclusion, the ABACard® HemaTrace® kit is a sensitive kit that shows some cross reactivity with ferret and higher primate blood samples. Assessment of temperature studies on antibodies, showed that elevated temperature exposure of the HemaTrace® kits could result in antibody denaturation resulting in a decrease in antibody binding efficacy. This in turn would result in a decrease in overall kit efficacy which could potentially provide a suitable explanation for the HemaTrace® false negative results seen

in the northern regions of Western Australia. In order to test this, an accelerated stability test could be conducted to monitor the changes in HemaTrace® kit efficacy in response to prolonged temperature exposure. The literature pertaining to accelerated stability tests of other lateral flow immunochormatographic assays suggest that an increase in temperature and exposure time will result in a decrease in HemaTrace® kit efficacy. However, as the components of the HemaTrace® kit is not publically known, future research is required to test this hypothesis. The findings of such a study would inform forensic practitioners on recommended storage conditions for the HemaTrace® kits and to ensure that negative results found at a crime scene are true negatives and not due to the failure of the HemaTrace® kits.

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Part Two

Manuscript

THE EFFECTS OF TEMPERATURE ON THE ABACARD® HEMATRACE® KIT FOR THE IDENTIFICATION OF HUMAN BLOOD

The Effects of Temperature on the ABACard® HemaTrace® Kit for the Forensic Identification of Human Blood

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ABSTRACT

The examination of bloodstains at a crime scene is a valuable tool for forensic investigations. One of the early steps in analysing a bloodstain is to first identify whether the stain is indeed blood and if it is of human origin. The ABAcard® HemaTrace® kit is a lateral flow immunochromatographic assay used by the Western Australia (WA) Police as a tool to identify human blood at crime scenes. However, WA Police officers have noted that ABAcard® HemaTrace® kits in the northern regions of WA have been producing false negative results. The current hypothesis for these occurrences is that the false negatives are due to the increased temperatures experienced in northern WA. Thus, this study aimed to evaluate the effects of elevated storage temperature on the efficacy of the ABAcard® HemaTrace® kits. This was done by testing ABAcard® HemaTrace® kits against blood samples diluted as a dilution series ranging from 1:10 to 1:10 000 000 after incubation at 40°C, 50°C and 60°C for a period of 4 weeks. Incubation of the ABAcard® HemaTrace® kits at 60°C for 2 weeks was enough to produce false negative results across the entire dilution series tested. A secondary aim of this study was to determine the duration the ABAcard® HemaTrace® would remain valid for at elevated temperatures. This study estimated that at 40°C, ABAcard® HemaTrace® kits would remain valid for only 70

days. Overall, this study demonstrated that temperature does adversely affect the ABAcard® HemaTrace® kits and could provide a valid explanation for the false negative results obtained in northern WA.

Keywords: Forensic science, ABAcard® HemaTrace®, Temperature, Stability test, Blood tests

INTRODUCTION

Analysis of blood stains found at a violent crime scene can be used as an important investigative tool.[1] DNA analysis[2] can potentially identify individuals involved in the crime and Blood Pattern Analysis[3] can be used for the reconstruction of a bloodshed event. As many substances share a similar appearance to blood, it is important that bloodstains found at a crime scene are correctly identified before further analysis is conducted.[1] This is to prevent the loss of valuable time and resources on samples that may not have much evidentiary value.

The ABAcard® HemaTrace® kit is a lateral flow immunochromatographic assay that targets human haemoglobin and is currently used by the Western Australia (WA) Police as a tool to identify human blood at crime scenes.[4] The test procedure of a potential bloodstain involves the addition of the stain to an extraction buffer that facilitates haemolysis, followed by addition of the extract to the ABAcard® HemaTrace® kit. If human haemoglobin is present, the human haemoglobin will bind to mobile monoclonal antibodies-dye conjugates located in the absorbent test strip. This results in the formation of mobile antigen-antibody-dye complexes. The mobile complexes migrate along the test strip to a Test Area containing immobile polyclonal antihuman-Hb antibodies. The immobilised polyclonal antibodies bind to the mobile complexes to form an antibody-antigen-antibody sandwich. This results in the accumulation of the conjugated dye at the Test Area resulting in a pink band. Excess mobile monoclonal antibody-dye conjugates migrate past the Test Area to a Control Area, where a second set of immobilised antibodies are present that bind to the excess mobile monoclonal antibodies. This results in the

accumulation of the conjugated dye at the Control Area. Thus, a second pink band is formed, which acts as an internal control for the ABAcard® HemaTrace® kit.[5]

The results of ABAcard® HemaTrace® testing are interpreted qualitatively. A positive result is indicated by a band at the Test and Control area whilst a negative result is indicated by the absence of a band at the Test area and the presence of a band at the Control area.[6] However, a common feature noted for the ABAcard® HemaTrace® kit is that the kit suffers from the 'high dose hook effect', where high concentrations of haemoglobin result in ABAcard® HemaTrace® false negatives.[7]

Reports from WA Police officers have noted that ABAcard® HemaTrace® kits in the northern regions of WA have been producing false negatives.[8] This can be detrimental to an investigation as this may result in the exclusion of relevant forensic evidence. Blood deposited at a crime scene is potentially exposed to degradative conditions. Thus, it was hypothesised that the false negatives may be due to the degradation of the blood proteins as a result of the elevated temperatures experienced in northern WA.[9] However, previous studies evaluating the effects of high temperature exposure to blood samples on ABAcard® HemaTrace® testing have reported that false negatives were only seen when blood samples were exposed to extreme temperatures of approximately 80°C.[10] It is unlikely that this would be responsible for the false negative results obtained in northern WA as even surfaces such as asphalt rarely exceed 70°C.[11] Thus, an alternative explanation is needed to account for the occurrences of these false negative results.

Another factor that could potentially contribute to the appearance of the ABAcard®

HemaTrace® false negatives is that the ABAcard® HemaTrace® kits themselves are exposed to elevated temperatures. A study conducted by Dadour *et al*[12] noted that the internal temperature of parked vehicles can potentially exceed the external ambient temperature by 20-30°C. Thus, ABAcard® HemaTrace® kits stored in police vehicles[8] are potentially exposed to temperatures up to 60°C. This may potentially adversely affect the ABAcard® HemaTrace® kits, however, this has yet to be tested.

As the ABAcard® HemaTrace® kit is a qualitative test, the thermal stability of the kit can be assessed by incubating the kits at various temperatures and testing the kits against a series of diluted blood samples. This would enable the examiner to monitor the sensitivity of the kits in response to elevated temperature exposure. However, in order to calculate the shelf life of the kits, quantitative data is needed.[13] Previous studies looking at quantifying lateral flow immunochromatographic assays utilise the Test and Control band intensities to measure the concentration of a target molecule.[14-17] These studies noted that the Test band intensity is proportional to the concentration of the target molecule.[17] However, it was found that the Test band intensity alone displayed a large amount of variation as immunoreaction time, batch variability and pH and ionic strength of the sample can affect the Test band intensity.[17,18] Thus, these studies used the Control band as a normalising factor, using the Test band intensity divided by the Control band intensity (T/C ratio) as a quantitative measurement of the immunoassay signal.[14-20] Using the T/C ratio measurements, two previous studies have been able to calculate the shelf life of lateral flow immunochromatographic assays by assuming that the degradation of the kits follow a zero order or first order reaction.[21,22]

Thus, this study aimed to evaluate the effects of temperature on the ABAcard® HemaTrace® kit by monitoring the sensitivity of the kits after exposure to temperatures of 40°C, 50°C and 60°C for a period of 4 weeks. A secondary aim was to determine the duration the ABAcard® HemaTrace® kits would remain valid under elevated temperature conditions. This was done by performing a stability test on the ABAcard® HemaTrace® kits where shelf life was calculated using T/C ratio measurements and assuming the degradation of the kits followed first order reaction kinetics.

MATERIALS AND METHODS

A total of 147 commercially available ABAcard® HemaTrace® kits (Abacus Diagnostics Inc., West Hills, CA, USA) were evaluated in this study. Fresh blood samples were obtained via venepuncture from a healthy, non-smoking, 25 year old male. To simulate blood found at a crime scene, no anticoagulants were used. Immediately after incubation, blood samples were diluted in the ABAcard® HemaTrace® extraction buffer in a 10-fold serial dilution, resulting in dilution factors ranging from 1:10 to 1:10 000 000. Haemoglobin concentration of the diluted blood samples were estimated by measuring the haemoglobin content of the neat blood sample using a complete blood count (CBC), which was found to be 152.5g/L.

Qualitative Assessment of ABAcard® HemaTrace®

A total of 126 ABAcard® HemaTrace® kits were used for the qualitative assessment of the kits. The ABAcard® HemaTrace® kits were incubated at 40°C, 50°C and 60°C in Hybridiser HB-1D (Techne®) incubator ovens. A control set of ABAcard® HemaTrace® kits were

incubated at room temperature (~22°C). Temperatures were monitored using Rowe Scientific Pty. Ltd Digital Thermometers and ABAcard® HemaTrace® kits were tested in duplicate at 0, 2 and 4 weeks against a blood sample dilution series ranging from 1:10 to 1:10 000 000.

Qualitative interpretation

Prior to testing, ABAcard® HemaTrace® kits were left to cool at room temperature. The ABAcard® HemaTrace® kits were tested according to the manufacturer's protocol.[6] Briefly, 150µL of extracted blood sample was pipetted into the sample well and results were recorded after 10 minutes. A pink band at both the Test (T) and Control (C) region indicated a positive result. In order to minimise subjective interpretation of the ABAcard® HemaTrace® results, weak 'T' band intensities that were too faint to conclusively score and too faint to photograph, were categorised as 'partial negatives'. A negative result was indicated by the absence of a pink band at the 'T' region with the presence of a band at the 'C' region. A result was deemed inconclusive if no band appeared at the 'C' region regardless if a band was present or absent at the 'T' region.

Measurement of ABAcard® HemaTrace® band intensities

ABAcard® HemaTrace® 'T' and 'C' bands were imaged using a GS-900™ Calibrated Densitometer (BioRad). The ABAcard® HemaTrace® kits were scanned using the Reflective, Green Filter, Colloidal Gold setting with a resolution of 63.5 microns. The 'T' and 'C' bands were viewed using the Image Lab™ software (Version 6.0), where band intensities were measured by obtaining the area under the curve (Figure 1). The 'T' band intensity divided

by the 'C' band intensity (T/C ratio) was used to quantitatively assess the performance of the ABAcard® HemaTrace® kit.

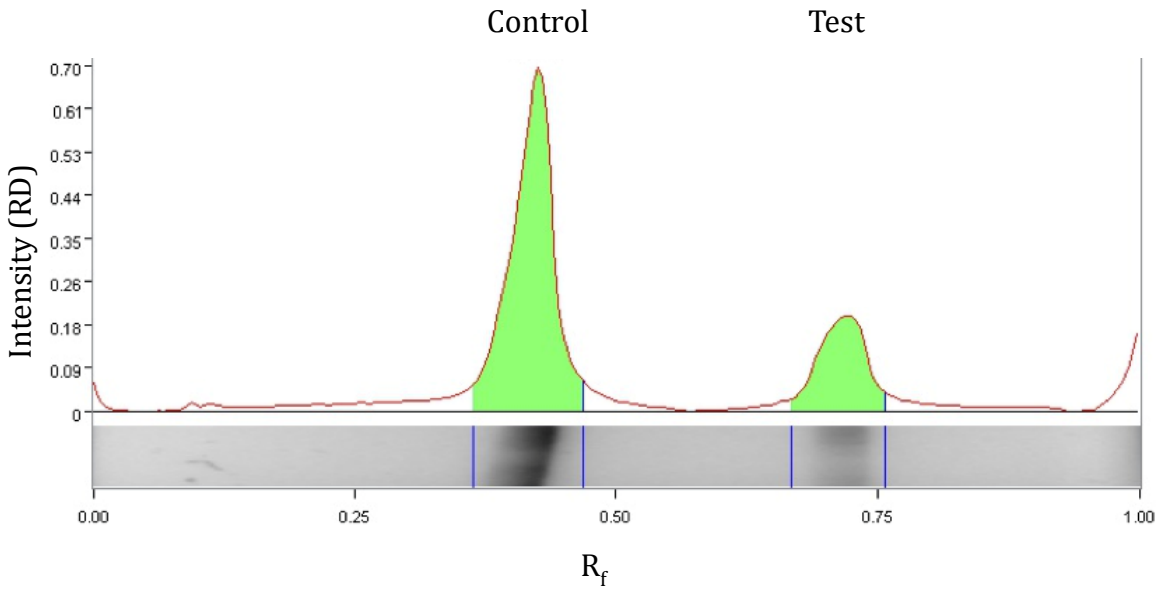

Figure 1: Measurement of Test and control band intensities to calculate T/C ratio.

Stability test

ABAcard® HemaTrace® kits were incubated at 40°C for 0, 1, 2, 5, 7 and 14 days. The ABAcard® HemaTrce® kits were tested in triplicate against a positive control at 1:10 000 dilution. Test and Control band intensities were obtained and shelf life of the kits stored at 40°C was estimated using a first order reaction kinetic equation shown below (Equation 1).

$$\mathrm{Ln(T/C)} = -kt + \mathrm{Ln(T/C)_0} \qquad (1)$$

In this equation, T/C is the Test band divided by Control band intensity (T/C ratio) at time *t*, *k* is the rate constant of the reaction, and $(T/C)_0$ is the initial T/C ratio, prior to incubation.

The T/C ratio value of a negative control was used in Equation 1 to determine the shelf life, *t*.

RESULTS

Temperature and ABAcard® HemaTrace® sensitivity

To assess the effects of temperature on the ABAcard® HemaTrace® kits, the sensitivity of the kits were evaluated after incubation at 40°C, 50°C and 60°C over a 4 week period.

The control group, at room temperature, showed that the ABAcard® HemaTrace® kits produced consistent positive results within a dilution range of 1:100 to 1:100 000. At dilution factors of 1:10, the 'high dose hook effect' was seen, producing false negative results. Similarly, ABAcard® HemaTrace® kits tested against dilution factors of 1:1 000 000 and 1:10 000 000 also produced false negative results. The effective working range of the control group remained consistent throughout the 4 week testing period (Table 1).

Kits incubated at 40°C showed a similar effective range to the control group over the 4 week period (Table 1). Consistent positive results were obtained from samples diluted from 1:100 to 1:100 000 at both 2 weeks and 4 weeks.

Kits incubated at 50°C showed a gradual decrease in the effective range of the kits with time. At 2 weeks after incubation, the kits began producing unreliable results at dilution factors of 1:100. At 4 weeks after incubation, unreliable results were obtained at dilution factors of 1:100 and 1:100 000, thus effectively shrinking the effective range of the kits by two dilution factors (Table1).

Lastly, kits incubated at 60°C failed to produce any reliable positive results across the entire dilution series tested. A single weak positive result was seen after incubation for 2 weeks when tested against a blood sample diluted to 1: 10 000. However, at 4 weeks post incubation, none of the kits produced a positive result (Table 1).

In summary, within the 4 week test period, at temperatures of 50°C and above, an increase in temperature and exposure duration resulted in a decrease in the effective range of the ABAcard® HemaTrace® kits.

Table 1: ABAcard® HemaTrace® results after incubation at room temperature, 40°C, 50°C and 60°C for a total of 4 weeks.

Temperature	Dilution factor	Exposure Time					
		0		2 weeks		4 weeks	
		Replicate		Replicate		Replicate	
		1	2	1	2	1	2
~22°C (Room Temperature)	1:10	-	-	-	-	+*	-**
	1:100	+*	+*	+	+	+	+
	1:1 000	+	+	+	+	+	+
	1:10 000	+	+	+	+	+	+
	1:100 000	+	+	+	+	+	+*
	1:1 000 000	-**	-**	-	-**	+*	-
	1:10 000 000	-	-	-	-	-	-
40°C	1:10	n/a	n/a	-	-	-	-
	1:100	n/a	n/a	+	+*	+	+
	1:1 000	n/a	n/a	+	+	+*	+
	1:10 000	n/a	n/a	+	+	+	+
	1:100 000	n/a	n/a	+	+	+	+
	1:1 000 000	n/a	n/a	-	-	-	-
	1:10 000 000	n/a	n/a	-	-	-	-
50°C	1:10	n/a	n/a	-	-	-	-**
	1:100	n/a	n/a	+*	-**	+	-**
	1:1 000	n/a	n/a	+	+	+	+
	1:10 000	n/a	n/a	+	+	+*	+
	1:100 000	n/a	n/a	+*	+*	-**	+*
	1:1 000 000	n/a	n/a	-	-	-	-
	1:10 000 000	n/a	n/a	-	-	-	-
60°C	1:10	n/a	n/a	-	-	-	-
	1:100	n/a	n/a	-	-	-**	-
	1:1 000	n/a	n/a	-	-**	-	-
	1:10 000	n/a	n/a	+*	-**	-	-
	1:100 000	n/a	n/a	-	-	-	-
	1:1 000 000	n/a	n/a	-	-	-	-
	1:10 000 000	n/a	n/a	-	-	-	-

\+ Positive ABAcard® HemaTrace® result

\- Negative ABAcard® HemaTrace® result

+* Weak positive: Very faint band at Test Area

-** Partial negative: Band present at the Test Area but too faint to confidently classify as positive

n/a Not applicable

ABAcard® HemaTrace® Dose Response Curve

In order to assess the applicability of using band intensities to quantitatively measure the ABAcard® HemaTrace® signal, Test and Control band intensities were obtained from ABAcard® HemaTrace® kits that were tested against a dilution series ranging from 1:10 to 1:10 000 000. The T/C ratio of the kits showed an increase with haemoglobin concentration, reaching a peak at 15.25µg/mL of haemoglobin. At haemoglobin concentrations above 15.25µg/mL a decrease in the T/C ratio was observed, demonstrating the 'high dose hook effect' (Figure 2).

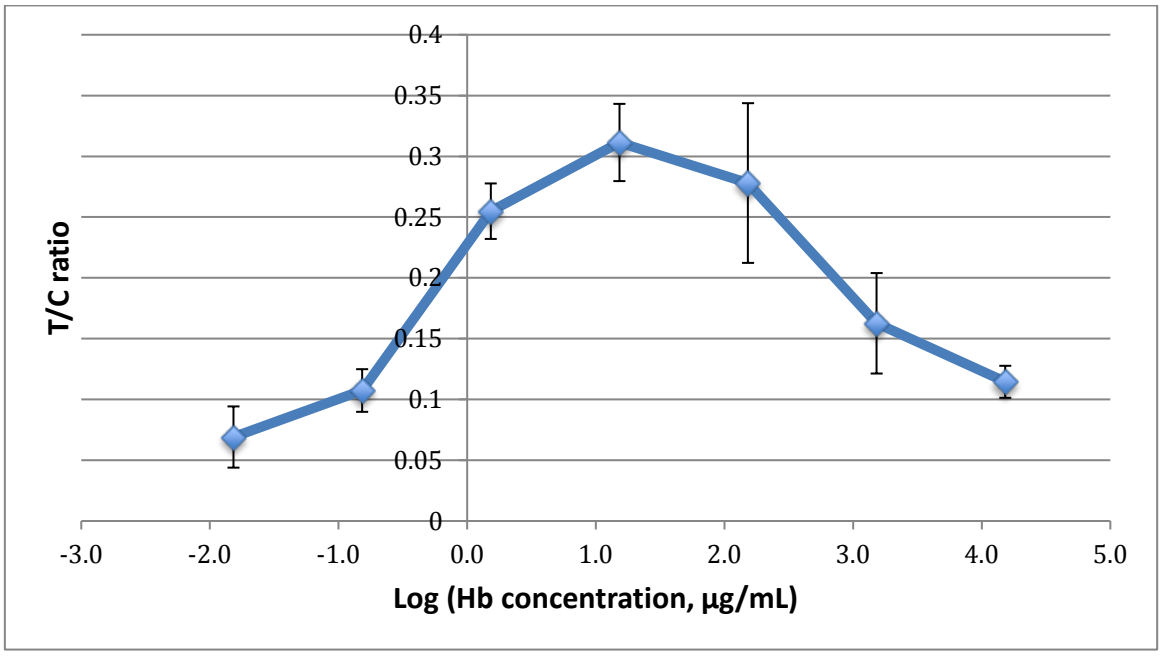

Figure 2: Dose response cure of ABAcard® HemaTrace® kits tested against a dilution series ranging from 1:10 dilution to 1:10 000 000 dilutions (Error bars pertain to the standard error of the mean).

Stability test

The thermal stability of the ABAcard® HemaTrace® kit was tested at 40°C for a period of 14 days. It was noted that an increase in exposure time resulted in a decrease in the T/C ratio. To calculate the duration the kits would remain valid for at 40°C, the logarithm of the T/C

ratio was plotted against time according to a first order reaction (Equation 1) (Figure 3). The gradient of the curve was taken as the rate constant (*k*), which was found to be 0.0132day^{-1}. Using Equation 1, it was estimated that the ABAcard® HemaTrace® kits would be valid for 70 days if stored at 40°C. However, it was noted that linear regression analysis of the curve had a low R-squared value (R^2= 0.5559) (Figure 3).

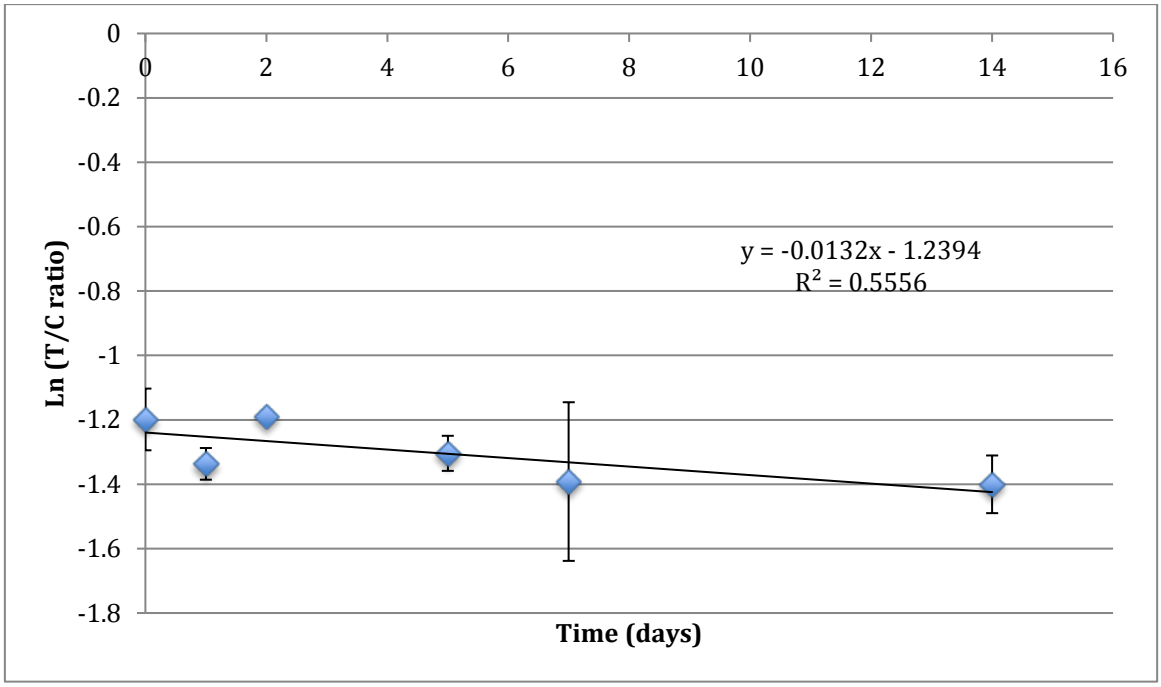

Figure 3: Shelf life estimation of the ABAcard® HemaTrace® kits stored at 40°C using a first order reaction equation (Equation 1: $Ln(T/C) = -kt + Ln(T/C)_0$) (Error bars pertain to the standard error of the mean).

DISCUSSION

The main objective of this study was to evaluate effects of elevated temperature exposure on the ABAcard® HemaTrace® kit. From the results, this study successfully demonstrated that elevated storage temperatures above 50°C and prolonged exposure durations adversely effected the ABAcard® HemaTrace® kits by reducing the effective working range

of the kits, resulting in the occurrences of false negatives. Furthermore, it was found that storing the ABAcard® HemaTrace® kits at 40°C reduced the kit shelf life to only 70 days.

Thermal degradation of the ABAcard® HemaTrace® kit

This study showed that elevated temperature has a degradative effect on the ABAcard® HemaTrace® kits. However, this study does not elucidate the mechanism behind the degradation. One possible explanation is that at these elevated temperatures, antibodies within the kits denature resulting in the inability of the antibodies to recognise their respective targets. This hypothesis is supported by antibody denaturation studies performed by Vemeer and Norde[23] that showed that the segment that contains the antigen binding site, called the Fab segment, denatures at temperatures of approximately 60°C. This is similar to what was seen in the quantitative test where at 60°C, ABAcard® HemaTrace® kits produced false negatives throughout the dilution series tested. Vemeer and Norde[23] also noted that at 55°C, the Fab segment still denatures but at a much slower rate. Thus, this suggests that the level of antibody denaturation is dependent on both temperature and exposure time. This is similar to what was found for the kits incubated at 50°C, where the effective working range of the kits diminished gradually with time (Table 1).

Multiple other factors could also be responsible for the thermal degradation of the ABAcard® HemaTrace® kit. A study by Wang *et al*[21] noted that an increase in storage temperature could result in displacement of the labelled molecule conjugated to the mobile monoclonal antibodies. This too could result in a decrease in the ABAcard® HemaTrace® band intensities resulting in the occurrences of false negative results.[24] Thus,

the mechanism responsible for ABAcard® HemaTrace® thermal degradation remains to be concluded.

ABAcard® HemaTrace® shelf life calculation

The stability test performed in this study estimated that ABAcard® HemaTrace® kits would only be valid for 70 days if stored at 40°C. However, it was noted that the linear regression analysis of the results showed a very low R-squared value (R^2=0.5559) (Figure3). Thus, the shelf life estimation performed in this study may not be accurate. A possible explanation for this inaccuracy was that the incubation time of the blood samples in the ABAcard® HemaTrace® extraction buffer was not controlled according to the manufacturer's guidelines. Blood samples were incubated in the extraction buffer for over 30 minutes in contrast to the 5 minute recommended incubation time noted by the manufacturers.[6] An increase in the incubation time would provide more time for haemolysis and allow more time for the haemoglobin proteins to solubilise, which is required for antibody binding within the ABAcard® HemaTrace® kits. However, this may have instead contributed to the variability seen in the T/C ratio measurements.

Another possible explanation for this inaccuracy is that the degradation of the ABAcard® HemaTrace® kit was assumed to follow first order reaction kinetics. Previous studies assessing the shelf life of lateral flow immunochromatogrphic assays estimate the shelf life using the Arrhenius equation thus suggesting that the degradation of the kit follows either zero order or first order reaction kinetics.[25] However, the low R-squared value obtained in this study potentially indicates that the degradation of the ABAcard® HemaTrace® kit does not follow first order reaction kinetic (Figure 3). Thus, further study is required to

determine the degradative trend of the ABAcard® HemaTrace® kits to enable better shelf life estimations.

ABAcard® HemaTrace® sensitivity

The sensitivity of the ABAcard® HemaTrace® kits in the control group showed a similar limit of detection as described by the manufacturers, where at haemoglobin concentrations below 0.05µg/mL, ABAcard® HemaTrace® results became unreliable. However, this is in contrast to a study conducted by Horjan *et al*[26], where positive results were obtained at dilutions of 1:10 000 000, corresponding to a haemoglobin concentration estimate of 0.013µg/mL. A potential explanation for this slight discrepancy could be that this study restricted the immunoreaction time of the kits to 10 minutes. This is in concordance with the Technical Information Sheet[6] provided by the manufacturers that states that the maximum time for reading the ABAcard® HemaTrace® result is 10 minutes, after which non-specific binding could potentially results in false positive results. This was demonstrated in this study as negative results for kits tested at concentrations below 0.05µg/mL did produce a positive result after the 10 minute immunoreaction time (results not shown).

The dose response curve of the ABAcard® HemaTrace® kits indicates that T/C ratio measurements could potentially be used to quantify haemoglobin concentration (Figure 2). This method has been used by multiple lateral flow immunochromatographic assays to quantify the concentration of a target molecule.[14-16] Obtaining quantitative results in this manner could potentially increase the sensitivity of the kit and would eliminate the ambiguity associated with a qualitative test, where a weak positive can be classified as a

positive or negative depending on the interpretation of the observer.[14-16] However, this method requires further validation and thus warrants further research.

Forensic Implications and recommended storage conditions

Temperature studies conducted on parked vehicles have reported that the internal cabin temperature of a vehicle can exceed 60°C.[12] The results of this study makes this finding particularly concerning as incubation of the ABAcard® HemaTrace® kits at 60°C caused the kits to become completely invalid at as early as 2 weeks. Furthermore, ABAcard® HemaTrace® kits can be stored for months prior to use.[8] However, it should be noted that this study incubated the kits at a constant temperature throughout the testing period. In reality, temperatures experienced by the kits will be dependent on the diurnal changes in ambient temperature. Furthermore, Dadour *et al*[12] noted that the internal temperature of a vehicle is influenced by the level of solar radiation exposure, car colour and level of ventilation. Thus, as multiple factors can affect the temperatures experienced within a vehicle, future studies evaluating the temperatures experienced in police vehicles would be beneficial to conclusively determine whether ABAcard® HemaTrace® kits stored in these vehicles are actually exposed to detrimental temperatures.

The stability test performed in this study estimated that the ABAcard® HemaTrace® kits would only be valid for 70 days if stored at 40°C. In contrast, according to the manufacturers, the ABAcard® HemaTrace® kits should have a shelf life of at least a year if stored below 28°C.[6] Thus, this demonstrates that even mild heating temperatures can affect the ABAcard® HemaTrace® kit by greatly reducing the shelf life of the kits. This too is

concerning as it is not uncommon for ambient temperatures to reach 40°C during the Australian summer months.[9]

In summary, the main findings of this study demonstrate that forensic practitioners should adhere to the recommended storage conditions for the ABAcard® HemaTrace® kits more strictly. Thus, it is recommended that WA Police officers should refrain from storing the ABAcard® HemaTrace® kits in police vehicles. It is suggested that the kits should be stored indoors at room temperature away from sunlight exposure. When using the kits in the field, special care should be taken to protect the kits from elevated temperatures. Thus, kits used in the field should be placed in a thermally insulated container, such as a Styrofoam box; to protect the kits from elevated ambient temperatures especially during the summer months.[9] The occurrences of false negatives can have serious implications for a forensic investigation and also in a court of law. Thus, the authors advise that forensic practitioners adhere to these guidelines to prevent future occurrences of ABAcard® HemaTrace® false negative results.

Lastly, multiple other factors could contribute to the occurrence of an ABAcard® HemaTrace® false negative result.[10,27] Previous studies have shown that humidity plays a role in antibody degradation[28,29] and thus could also potentially effect ABAcard® HemaTrace® testing. Furthermore, the conditions at a crime scene are not controlled, thus, bloodstains at a crime scene could be exposed to degradative agents especially if a crime scene clean up has occurred. Although a previous study has noted the effect of sodium hypochlorite (bleach) as an oxidising agent on ABAcard® HemaTrace® testing[27], the study did not look at how sodium hypochlorite affected the pH of the blood samples.

Factors such as this require further study as pH changes can disrupt antibody binding[30], which could potentially effect ABAcard® HemaTrace® testing.

CONCLUSION

The purpose of this study was to evaluate the effects of temperature on the ABAcard® HemaTrace® kit by testing the ABAcard® HemaTrace® kits against a dilution series. It was found that an increase in storage temperature and exposure time resulted in a decrease in the effective working range of the ABAcard® HemaTrace® kits. Furthermore, the stability test performed at 40°C demonstrated that the shelf life of the ABAcard® HemaTrace® kits was greatly reduced in response to elevated storage temperatures. In conclusion, this study successfully demonstrated that an increase in storage temperature could potentially explain the false negative results seen in northern WA. This study also highlights the importance for forensic practitioners to adhere to the recommended storage guidelines, which recommend that ABAcard® HemaTrace® kits should be stored below 28°C. Therefore, it is advisable that WA Police officers should refrain from storing the ABAcard® HemaTrace® kits inside police vehicles. Furthermore, in the event that the ABAcard® HemaTrace® kits have to be removed from storage to be used at a crime scene, kits should be placed in a thermally insulated containers to prevent elevated temperature exposure, especially during the Australian summer months. Although this study has demonstrated the degradative effects of temperature on the ABAcard® HemaTrace® kits, multiple other factors could also contribute to the occurrences of false negatives, such as substrate effects and environmental factors. Thus, further research may be required to determine whether these other factors may also play a role in the production of ABAcard® HemaTrace® false negative results.

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DISCLAIMER

The authors do not endorse any products for the purpose of blood identification. The authors declare that they have no conflicting interests.

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