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SCHOOL OF MEDICINE

Thesis

COMPARISON OF CHEMILUMINESCENT AND FLUORESCENT BLOOD DETECTION KITS

by

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B.Tech., VIT University, 2017

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2019

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ACKNOWLEDGMENTS

I would like to start by thanking the Boston University School of Medicine Biomedical Forensic Sciences program for equipping me with the knowledge and the resources to conduct my research. I owe a great amount of credit to all the members of my committee – Professor Amy Brodeur, Alexis Manolakis and Jennifer Sheehan for taking the time to help me with this project. I would also like to extend my thanks towards Dr. Robin Cotton without whom I could not have conducted my DNA experiments. I would like to thank my parents, my brother and my friends for their unending support.

COMPARISON OF CHEMILUMINESCENT AND FLUORESCENT BLOOD DETECTION KITS

MANAVI MURALIDHAR

ABSTRACT

Blood at a crime scene is not always detected by the naked eye, and hence requires the use of special reagents and alternate light sources. Two of these reagents – luminol and fluorescein – have been in use in forensic science both in their original forms and in the form of commercially produced reagents, Bluestar® and Hemascein™. The manufacturer of Hemascein™, the relatively newer product, states that the reagent exhibits lower amounts of cross-reactivity with bleach and is less detrimental to DNA as compared to Bluestar®. They also say that the reagent does not require complete darkness to function, unlike Bluestar®. These parameters, along with the impact of Bluestar® and Hemascein™ on pattern detail, were evaluated in this study. It was found that both reagents exhibited some positive reactions with bleach; however, Bluestar® had a less intense reaction with lower concentrations of bleach, while Hemascein™ showed a lower intensity for cross reactions with higher concentrations. A distinct difference was observed for the reagents with respect to retention of pattern characteristics in bloodstains, with Hemascein™ causing considerable diffusion of the pattern. The results also demonstrated that there was a dependence of the color of the substrate on the performance of Hemascein™, with lighter colored substrates far outperforming their darker counterparts. Hemascein™ performed better than Bluestar® in well-lit conditions, with a positive luminescent reaction observed in ambient lighting conditions. Additionally, neither of the reagents showed inhibition

during quantitative PCR, thus deeming them both appropriate for use when subsequent recovery of DNA is desired. There were differences in the amounts of DNA recovered from the treated blood samples, however, further studies and a larger sample size would be required to determine if these variations are related to the application of Bluestar® and Hemascein™. All DNA recovered was sufficient in quantity to expect successful DNA profiling if such analysis had been carried out.

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LIST OF ABBREVIATIONS

ALS	Alternate Light Source
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
IPC	Internal Positive Control
LCV	Leuco Crystal Violet
LMG	Leucomalachite Green
MI	Mean Intensity
µL	Microliter
mL	Milliliter
nm	Nanometer
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
SI	Substrate Intensity
TE	Tris EDTA

1. INTRODUCTION

Bloodstain evidence is vitally important for a great many reasons. It can provide assistance with crime scene reconstruction, hold critical DNA information and can reflect clean-up attempts at the scene. In some instances, blood left behind at the scene of a crime can be invisible to the naked eye. Such stains are referred to as 'latent' blood stains. Usually, in a search for latent biological stains, one of two methods is used – an alternate light source (ALS)[1] or chemical enhancement methods. Unlike most body fluid stains, however, bloodstains do not fluoresce with an ALS; thus chemical methods are usually employed. Latent blood detection techniques, therefore, must be chosen with caution so as to preserve any useful information from the crime scene.

1.1. Chemiluminescence and Fluorescence

Chemiluminescence is the property of an object to emit light in the form of electromagnetic radiation by releasing energy generated from jumping to a higher energy state[2]. Under basic conditions, luminol will produce chemiluminescence when combined with an oxidizing agent; the reaction is catalyzed by the iron present in hemoglobin, a component of blood[3]. The reaction involves the formation of an intermediate compound followed by emission of light[4].

Fluorescence is a phenomenon resulting from the emission of light of a longer wavelength following excitation by an external source resulting in a fluorophore jumping to an excited state and coming back to its relaxed energy state[5] [Figure 1]. Unlike

chemiluminescence, fluorescence is not the result of a chemical reaction, but the absorption of light.

Both of these mechanisms are often mistakenly interchanged, but it should be noted that the main difference between them lies in the usage of an external light source. While fluorescence is a jump in energy states due to external excitation, chemiluminescence does not require the same.

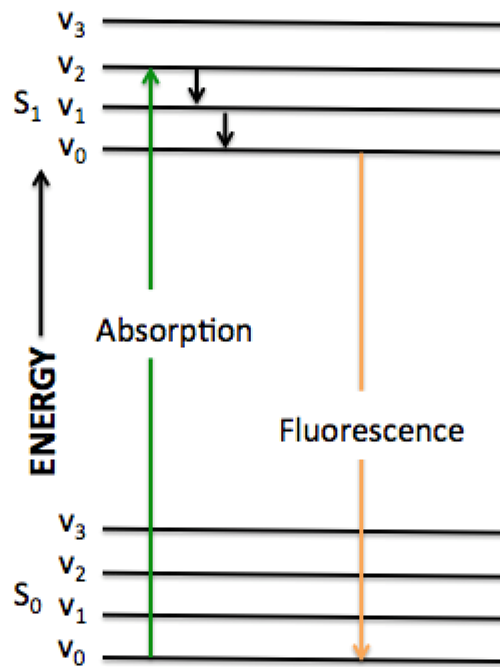

Figure 1. Jablonski Diagram Illustrating the Mechanism of Fluorescence. The particle jumps to a higher energy level upon absorption of energy from an external source and releases energy in the form of light upon returning to its ground state. (Source: https://commons.wikimedia.org)

1.2. History of blood chemical enhancement reagents

The term 'luminol' was first coined in 1934[6] by Huntress et al. It is used in forensics

for the detection of latent bloodstains. Aside from forensics, luminol has applications in the fields of clinical research for immunoassays and oncological research, as well as for protein detection following Western Blotting[7]. Several formulations of the compound have been used over time, but all involve the production of light when blood catalyzes the oxidation of luminol [Figure 2] in the presence of a chemical oxidant in basic solution. One formulation suggests the use of sodium carbonate as a base and sodium perborate as an oxidizing agent (Grodsky formulation)[8], while another formulation uses sodium hydroxide as the base and hydrogen peroxide as the oxidizing agent (Weber formulation)[9]. These preparations require multiple components to be measured and mixed in a laboratory setting, have a very short shelf life, and require complete darkness during use.

There are various commercially prepared formulations available as well, such as Bluestar® Forensic and its extra strength version Bluestar® Forensic Magnum (Bluestar, Monte-Carlo, Monaco), Luminol Dischaps™ (Sirchie, Youngsville, NC) and Luminosity Advanced Bloodstain Reagent (Arrowhead Forensics, Lenexa, KS), which have addressed some of the short comings of their predecessors [10]. Bluestar® Forensic is probably the most common luminol-based formulation and was first prepared in 2000[11], although the exact chemistry is proprietary.

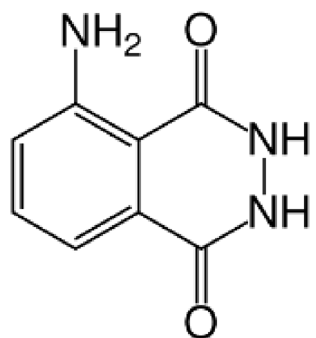

Figure 2. Chemical structure of luminol. (Source: https://commons.wikimedia.org)

Fluorescein sodium [Figure 3] is a fluorophore that is commonly used in the field of medicine, mainly ophthalmology, for treating angiographies and for resection of tumors[12]. It has been applied in the biomedical research field as well for fluorescence spectroscopy[13] and optical imaging[14]. For forensics, it must be used in conjunction with hydrogen peroxide, a 450 nm ALS and an orange barrier filter[15].

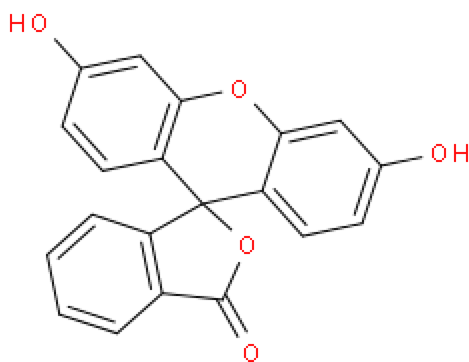

Figure 3. Structure of Fluorescein. (Source: www.chemspider.com)

Latent blood is first treated with fluorescin (reduced form), which is subsequently oxidized to fluorescein (oxidized form) after the addition of hydrogen peroxide. The reaction is typically viewed using an ALS around 450 nm. The subsequent fluorescence occurs at a peak of about 521 nm (in the yellow-green range of the spectrum)[16] and can be visualized with the help of an orange barrier filter[17]. This fluorescent reaction is catalyzed by heme[2]. Hemascein™ is a fluorescein-based latent blood enhancement reagent [16]. In the case of Hemascein™, the shelf life is reportedly longer compared to Bluestar® since the oxidizer (hydrogen peroxide) is applied independently of the reagent.

Many of the studies comparing luminol and fluorescein focus on sensitivity and specificity, although conflicting results for these parameters have been reported. These discrepancies arise from a number of variables such as concentration of blood samples, nature of substrate used and the color of the substrate.. Tobe et al. (2007) found that Bluestar® and luminol were able to detect up to 1:100,000 dilutions of blood[18]. Dilbeck (2006) found that when tested on vinyl tile, carpet, ceramic tile and maple wood surfaces, Bluestar® was easier to use compared to luminol and did not require complete darkness[10]. A comparison of Bluestar®, luminol and Hemascein™ showed that Hemascein™ produced longer lasting luminescence and was able to detect blood even when the sample was burnt[19]. Further, one of the initial sensitivity studies conducted by Lowis et al. (2012) involving Hemascein™ shows good reliability of the reagent between 1:1000 and 1:100,000 on a range of substrates[2].

The effect of various substrates on the detection of latent blood has also been studied. One such study showed that upon comparison of 11 different fabrics, assays such

as luminol and fluorescein worked better on natural fabrics than semi-synthetics or synthetics[20]. A study by McCaskill (2010) showed that Hemascein™ had a greater sensitivity and a longer positive luminescent reaction on both black and white fabrics and ceramic tiles compared to Bluestar®, luminol and fluorescein[21].

Aside from chemiluminescent and fluorescent reagents, there are many other blood detection reagents that do not involve the emission of light. Protein stains such as Hungarian red, Coomassie blue and Amido black are commonly used in the enhancement of weak or partial bloodstain patterns. Similarly, catalytic color tests that cause a color change in the presence of blood are also frequently used in forensic laboratories and at crime scenes, particularly for pattern enhancement; examples of these reagents include leucocrystal violet and diaminobenzidine. Although these chemicals are important tools for forensic scientists, the focus of the current research is on light emitting assays, thus these color-based stains will not be further discussed.

1.3. Reactivity with Bleach

Bleach is a commonly encountered household item that is used in everyday cleaning activities, especially on ceramic surfaces frequently found in bathrooms. It has been shown to elicit a luminescent response from both Bluestar® and Hemascein™ resulting in false positive reactions, seemingly due to the presence of sodium hypochlorite[22]. A 2012 study by Seashols et al. showed that while Hemascein™ reacted with a variety of vegetables and cleaners, Bluestar® only showed a positive reaction with bleach[17]. In contrast, Tobe et al. observed cross-reactivity for Bluestar® from a variety of

substances, including those listed above[18].

Studies have been conducted to determine if there is a difference in the reaction obtained between Bluestar® and bleach and Bluestar® and blood. Quickenden and Cooper (2001) evaluated the false positive reactions caused by bleach and showed a slight difference, about 25 nm, between the wavelengths of light emitted upon reaction with blood and reaction with bleach[23]; this difference would likely be indistinguishable with the naked eye. In contrast, Blum et al. (2006) reported a qualitative difference between the reaction of Bluestar® with blood and with bleach, whereby the appearance of the light reaction observed could be distinguished by the examiner[24].

1.4. Pattern detail

The link between the retention of pattern detail and blood enhancement techniques has been explored through studies of the effects of dye stains on bloody shoe prints[25,26,27]. For example, leucocrystal violet (LCV) has proven to be useful through studies by Farrugia et al. (2010) and Bodziak (1996) to determine its efficacy in enhancing bloody shoe prints on alginate[26] and in large areas such as carpets in homes[25].

Chemiluminescent reagents have been criticized for distortion of pattern detail upon application to bloodstains, although the use of fixatives has been suggested to mitigate this[28]. Additionally, complicated photography procedures are necessary to document sufficient detail for comparison purposes[25]. Fluorescein was used for a pattern evidence study by Cheeseman and Tomboc (2001) to determine the efficacy of the reagent with various shoe treads. The authors found that the pattern was detectable in various instances, even when the print was latent[29]. Farrugia et al. (2011) compared LCV,

leucomalachite green, luminol and fluorescein and their effects on bloody footwear impressions and found luminol to be the best reagent both to provide contrast and to prevent diffusion of the impression on fabric. Fluorescein gave good visual results on dark colored fabrics and leather, but showed a poor contrast for denim[30].

1.5. Lighting conditions

Both fluorescein and luminol have been found to operate best in complete or near complete darkness, with fluorescein showing better results in partial darkness[31]. A study by Cheyne (2011) showed that Hemascein™ was successful in lighting conditions that did not involve complete darkness[28], which is in concordance with the claims of the manufacturer.

Due to the uncontrolled nature of crime scenes, it may be difficult to achieve complete darkness. For example, ambient light may be unavoidable in outdoor crime scenes being processed during daylight hours, a room with windows that cannot be blacked out or in places where overhead lighting cannot easily be turned off. Due to the dependence of these reagents on the emission of light in order to locate bloodstains, they may prove to be less effective in situations such as those detailed above.

1.6. DNA Detection

DNA information contained within bloodstain evidence can play an important role in almost any case[32], since bloodstain evidence is one of the most common types of evidence encountered at crime scenes. In the case of blood, DNA can be obtained only from white blood cells (WBCs) since red blood cells are anucleated. One consideration

with analyzing bloodstains for DNA is that the presence of hemoglobin can act as an inhibitor for Polymerase Chain Reaction (PCR)[33]. Extraction methods have been developed particularly for blood, such as QIAmp DNA Blood Mini by Qiagen (Hilden, Germany) and PDQeX foresicGEM Blood by Zygem (Charlottesville, VA), so as to chelate hemoglobin and any other potential inhibitors.

Several studies have been performed to evaluate the effect of blood detection reagents on subsequent DNA analysis, with variable and inconsistent results. In one such study, Budowle et al. (2000) used fluorescein and luminol on various surfaces and found the reagents to have no interference with development of profiles for 13 loci. However, it should be noted that the dilutions of blood used that resulted in a full profile varied from surface to surface, with porous surfaces giving poorer results[34]. In another study, Jakovich (2007) found that a bloodstained carpet treated with the Bluestar® reagent produced a full STR profile (13 loci), however, the same study yielded poor results for luminol[31].

2. MATERIALS AND METHODS

2.1 Preparation of Bluestar®

Bluestar® was prepared as per the manufacturer guidelines by first adding the white tablet followed by the beige tablet to 125 milliliters (mL) of distilled water. The white tablet contains hydrogen peroxide and urea[35] and the beige tablet contains sodium hydroxide[36].

The solution was prepared in spray bottles such as those typically available in most laboratories. A gentle swirling motion was employed to ensure the tablets completely dissolved in the water, being careful to avoid extreme agitation. This process took about 10 to 15 minutes, and the prepared solution is reported to be stable for approximately 3 hours. It is important to note that once mixed, the prepared Bluestar® reagent cannot be stored for future use.

2.2 Preparation of Hemascein™

Each Hemascein™ kit comes with a 5mL tube containing a powdered form of the Hemascein™ stock solution, two empty ABASpray® bottles, a Hematrace® immunoassay card and a transfer pipette. The stock solution was prepared by adding 5mL of distilled water directly into the powder. This solution can be stored for up to 15 months in the refrigerator. To create a working solution, 1mL of the stock was added to 100mL of water in one of the ABASpray® bottles provided. This solution is reportedly stable for 7 months if kept refrigerated. A 3% hydrogen peroxide solution (CVS Pharmacy, Woonsocket, RI) was added to the second ABASpray® bottle. The manufacturer recommends anywhere

between 1 to 3% hydrogen peroxide to be used in conjunction with Hemascein™.

2.3 Camera Settings

A Canon EOS REBEL T5i camera was used to photograph experiments for both reagents in conjunction with a photography stand (Sirchie, Youngsville, NC). Each reagent required the use of different camera settings, which were determined both through trial and error and guidance from the manufacturers [Table 1].

Table 1. Camera Settings. Camera settings used with each reagent.

	Bluestar®	Hemascein™
Aperture (f-stop)	f/4	f/9
Shutter speed (seconds)	30	4
ISO	400	200

2.4 Application of Bluestar®

Before the experiment was conducted, the camera was set to manual mode with the pre-determined Bluestar settings and mounted to the photography stand. The camera was placed about 8 inches from the test surface. The surface was first wiped clean and covered with fresh bench paper before placing the sample down. The lights were switched off and the nozzle of the reagent was depressed completely exactly one time, while simultaneously pressing the trigger of the camera.

2.5 Application of Hemascein™

Hemascein™ required additional steps of preparation prior to any testing in order to accommodate the ALS. A clamp stand was placed at a distance of 8 inches from the photography stand in order to anchor the lamp of the ALS utilized, a CrimeScope® CS-16-500W-15F (SPEX Forensics, Edison, NJ). The CrimeScope® was set at a wavelength of 455 nanometers (nm), which falls within the recommended range of 415 nm to 480 nm. As required with the use of an ALS, a Quantaray 58 mm Orange (YA2) barrier filter (Sigma Corporation, Japan) was used on the camera lens to filter out the excitation light. Each ABASpray® bottle was pumped to ensure sufficient build-up of pressure for proper and complete dispensing of the reagent. Following this, the nozzle for each spray bottle is was pressed for exactly 2 seconds at a distance of about 1-1.5 inches from the substrate.

2.6 Cross reactivity with Bleach

Commercially available bleach (Clorox®) was used to create three dilutions: 100%, 10% and 50%. The 10% dilution was created by adding 10mL of bleach to 90mL of distilled water, while the 50% dilution was created by adding 5mL of bleach to 5mL of distilled water. Using a transfer pipette, about 250μL (microliters) of each dilution was spotted onto black and white ceramic tiles and left to dry overnight in a fume hood. The next day, the reagents were applied to the tiles [Sections 2.4 and 2.5]. All testing was conducted in triplicate.

2.7 Loss of pattern detail upon application of reagents

Five different fabrics were purchased from a fabric store:

1. White cotton

2. Black cotton
3. Dark blue denim
4. White sweatshirt material
5. Black sweatshirt material

A 12 inch by 1 inch strip was cut from each cloth. A small quantity of blood that had been treated with ethylenediaminetetraacetate (EDTA) for preservation was dropped into a weigh boat with the use of a transfer pipette. The blood was spread evenly on the weigh boat by moving the drop around to create an even layer of blood. A stamp measuring approximately 1 square inch was dipped in the blood and pressed onto the fabric six times in succession to create a diminishing series, with a 1 inch space between each stamp [Figure 4]. These fabric strips were air dried and placed in manila envelopes between layers of tissue for a period of 25 to 26 days. They were then taken out and tested with Bluestar® and Hemascein™ as previously described [Sections 2.4 and 2.5]. All testing was performed in triplicate.

Figure 4. An example of the pattern detail. The untreated diminishing series showing the pattern of the stamp. This example is the 1st, 2nd and 3rd stamp of the diminishing series on white sweatshirt material.

2.8. Efficacy of reagents under different lighting conditions

Plain white cotton fabric was cut into 1.5 inch squares. A 1:50 dilution of blood was made by adding 20µL of blood treated with EDTA to 980µL of distilled water. This dilution was chosen since it was still detectable by the naked eye, but dilute enough to warrant the use of latent blood reagents. Each fabric square was then spotted with approximately 75µL (one drop from the transfer pipette) of the dilution. The samples were left to air dry for one hour. Each of three lighting conditions was evaluated in terms of lumens, as recorded by the phone application 'Lux Light Meter'.

2.8.1. Evaluating in complete darkness

For this section, complete darkness refers to conditions under which only the reagent (and ALS, when applicable) are used without an external white light source. For Bluestar®, this refers to a lighting condition of 0 lumens. For Hemascein™, use of just the ALS at 455 nm in complete darkness was considered to be 0 Lumens. The original camera settings [Table 1] were used under these conditions. The reagent was sprayed on the samples and the reactions were noted. All testing was performed in triplicate.

2.8.2. Evaluating in laboratory lighting conditions

The overhead lighting in the laboratory was found to be between 90 and 120 lumens at the designated testing spot. Based on trial-and-error, the photography settings were

altered [Table 2] to accommodate the change in ambient light. Reagents were applied to the bloodstained fabrics [Sections 2.4 and 2.5] and the reactions were recorded. All testing was performed in triplicate.

2.8.3. Evaluating in low light conditions

The brightness in the area of the photography stand was adjusted to about 1000-1200 Lumens with the use of a desk lamp. Based on trial-and-error, the settings for photography were adjusted to suit the altered conditions. These settings were used for both Hemascein™ and Bluestar®. Both reagents were then applied to the diluted bloodstains as stated in the earlier procedures. All testing was performed in triplicate.

Table 2. Altered settings. Settings for the camera were altered to optimize the information obtained from the photographs taken under different lighting conditions.

	Laboratory light (90-120 lumens)	Desk light (1000-1200 lumens)
Aperture (f-stop)	5.6	9
Shutter Speed (seconds)	1/4	1/8
ISO	200	200

2.9. Evaluating impact of reagents on DNA

2.9.1. Sample preparation and extraction for testing inhibition

Each reagent was prepared as per the methods described previously [Sections 2.1 and 2.2]. In four separate 1.5 mL microcentrifuge tubes, 3μL each of Bluestar®, hydrogen peroxide, Hemascein™ and a combination of hydrogen peroxide and Hemascein™ were

pipetted in the form of neat reagents. To each tube, 30μL of Tris EDTA (TE) Buffer was added. These tubes were left to incubate at room temperature for 25 minutes. The tubes were then centrifuged at 14,000 revolutions per minute (rpm) for 5 minutes. From all tubes, 30μL of liquid was pipetted out and discarded, leaving behind approximately 3μL of liquid in the tube for subsequent testing.

2.9.2. Fabric sample preparation

A 1:10 dilution of blood was made by adding 100μL of postmortem blood treated with EDTA to a 1.5 mL microcentrifuge tube and mixing with 900μL of distilled water. This dilution was chosen as it was determined to contain sufficient DNA for analysis prior to treatment with the reagent.The tube was vortexed to ensure proper mixing as the blood tended to settle at the bottom of the tube.

Nine plain white fabric swatches were made by cutting fabric into 1.5 inch squares. Each swatch was spotted with approximately 10μL of the 1:10 blood dilution previously prepared. Following air drying, the swatches were divided into groups of three. The first group was treated as control sample and was not sprayed with any reagents. The second group was treated with Bluestar® and the third with Hemascein™. Care was taken to ensure even distribution of the reagents on the stain. These samples were left to air dry following application of the reagents for one hour. A cutting about 1 cm in diameter was taken from all swatches to ensure all of the stain was used. Each of the cuttings were then placed in separate 1.5 mL microcentrifuge tubes. To each tube, 500μL TE Buffer was added and left to incubate at room temperature for 25 minutes. All tubes were centrifuged

at 14,000 rpm for 5 minutes. Approximately 470µL of the remaining liquid in the tube was then pipetted out and discarded, leaving only the substrate and the pelleted cells in the tube.

2.9.3. Extraction of DNA using Chelex® 100

Approximately 120µL of 5% Chelex® 100 was added to each tube [Section 2.9.1 and 2.9.2] for a total volume of 123 µL or 150µL, respectively. The tubes were incubated at 56° C for 25 minutes, vortexed for about 10 seconds, and then placed in a boiling water bath for 8 minutes. Tubes were vortexed once more for 10 seconds and then centrifuged at 14,000 rpm for 3 minutes.

2.9.4. Quantitation of DNA in samples

Once the samples were prepared, the amount of DNA present was determined to document any notable differences between the quantities obtained before and after treatment by the reagents. This was done using quantitative PCR. Master Mix was prepared by mixing 10.5µL per reaction of Duo Primer Mix to 12.5µL per reaction of Duo Reaction Mix. The Master Mix tube was then vortexed and centrifuged briefly. To each reaction well, 23µL of Master Mix was added. Each sample was vortexed and centrifuged before use to ensure that the Chelex beads, which inhibit PCR, formed a pellet at the bottom of the tube. Following this, 2µL of the four inhibition samples [Section 2.9.2] and nine fabric extraction [Section 2.9.3] samples were added to the corresponding reaction well [Table 3]. The quantitation reactions were then run on a ABI Prism 7500. The HID Real-Time PCR Analysis Software was used to analyze the samples.

Table 3. Samples used for DNA analysis. This table summarizes the different samples used for the DNA analysis section of the experiment. Following independent sample preparation, each of these samples underwent the same extraction and quantitation procedures.

Sample Name	Sample Type	Number of replicates
Bluestar®	Liquid	1
Hemascein™	Liquid	1
Hydrogen peroxide	Liquid	1
Hemascein and hydrogen peroxide	Liquid	1
Blood on fabric	Fabric swatch	3
Blood on fabric treated with Bluestar®	Fabric swatch	3
Blood on fabric treated with Hemascein™	Fabric swatch	3

3. RESULTS

3.1. Cross reactivity with Bleach

All photographs taken were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD). A probative area of the photograph where the bleach stain showed luminescence was selected and the average intensity was calculated, along with the intensity of the substrate. The difference between the calculated intensity of the substrate and the stain was noted to remove potential background fluorescence that was being recorded. Each photograph was captured following the settings listed in Table 1. The intensity of the substrate and the bleach stain obtained from the three triplicates was averaged. The mean intensity of the stain (MI) and average substrate intensity (SI) were then subtracted to obtain the average relative intensity.

Mathematically,

$$\text{Average Mean Intensity (MI)} = \frac{(\text{MI1}+\text{MI2}+\text{MI3})}{3}$$

$$\text{Average Substrate Intensity (SI)} = \frac{(\text{SI1}+\text{SI2}+\text{SI3})}{3}$$

$$\text{Relative Intensity (RI)} = (\text{MI} - \text{SI})$$

The numerator represents the replicate samples. For example, the average mean intensity for application of Bluestar® to 10% bleach on white tile was calculated using the mean intensities obtained from each replicate matching those parameters. The calculations were performed to remove an element of subjectivity from evaluating the photographs and determining the intensity of the light reaction. Since each sample was tested in triplicate, there were sufficient data points to conduct a statistical analysis for the experiment.

Table 4. Cross reactivity with bleach on white tile. Results for both reagents and the intensity of reaction produced upon cross reaction with bleach have been noted. Intensity values are calculated in ImageJ by measuring the total number using red, green and blue pixels.

	Reagent	Average MI	Average SI	Relative Intensity
10% Bleach	Bluestar®	8.673	4.538	4.135
50% Bleach	Bluestar®	51.601	3.635	47.967
100% Bleach	Bluestar®	125.741	4.138	121.603
10% Bleach	Hemascein™	48.324	10.066	38.258
50% Bleach	Hemascein™	62.098	13.064	49.034
100% Bleach	Hemascein™	73.461	16.061	57.400

Table 5. Cross reactivity with bleach on black tile. Results for both reagents and the intensity of reaction produced upon cross reaction with bleach have been noted. Intensity values are calculated in ImageJ by measuring the total number using red, green and blue pixels.

	Reagent	Average MI	Average SI	Relative Intensity
10% Bleach	Bluestar®	2.092	1.672	0.420
50% Bleach	Bluestar®	47.872	0.358	47.515
100% Bleach	Bluestar®	68.080	0.764	67.316
10% Bleach	Hemascein™	1.940	1.395	0.545
50% Bleach	Hemascein™	3.030	0.933	2.097
100% Bleach	Hemascein™	10.724	0.820	9.904

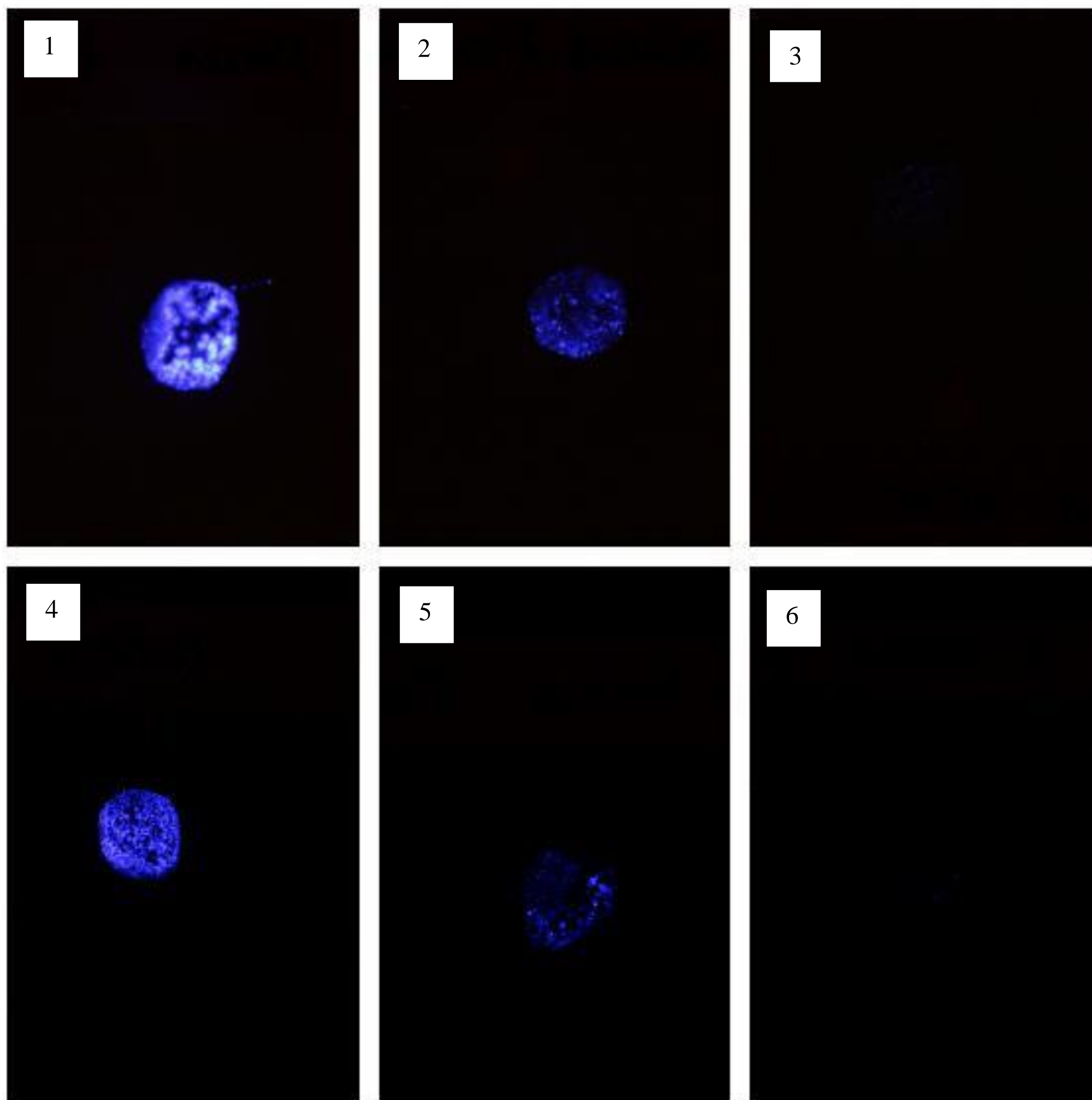

Figure 5. Cross reactivity of Bluestar® with bleach. 1. 100% Bleach on White Tile; 2. 50% Bleach on White Tile; 3. 10% Bleach on White Tile; 4. 100% Bleach on Black Tile; 5. 50% Bleach on Black Tile; 6. 10% Bleach on Black Tile

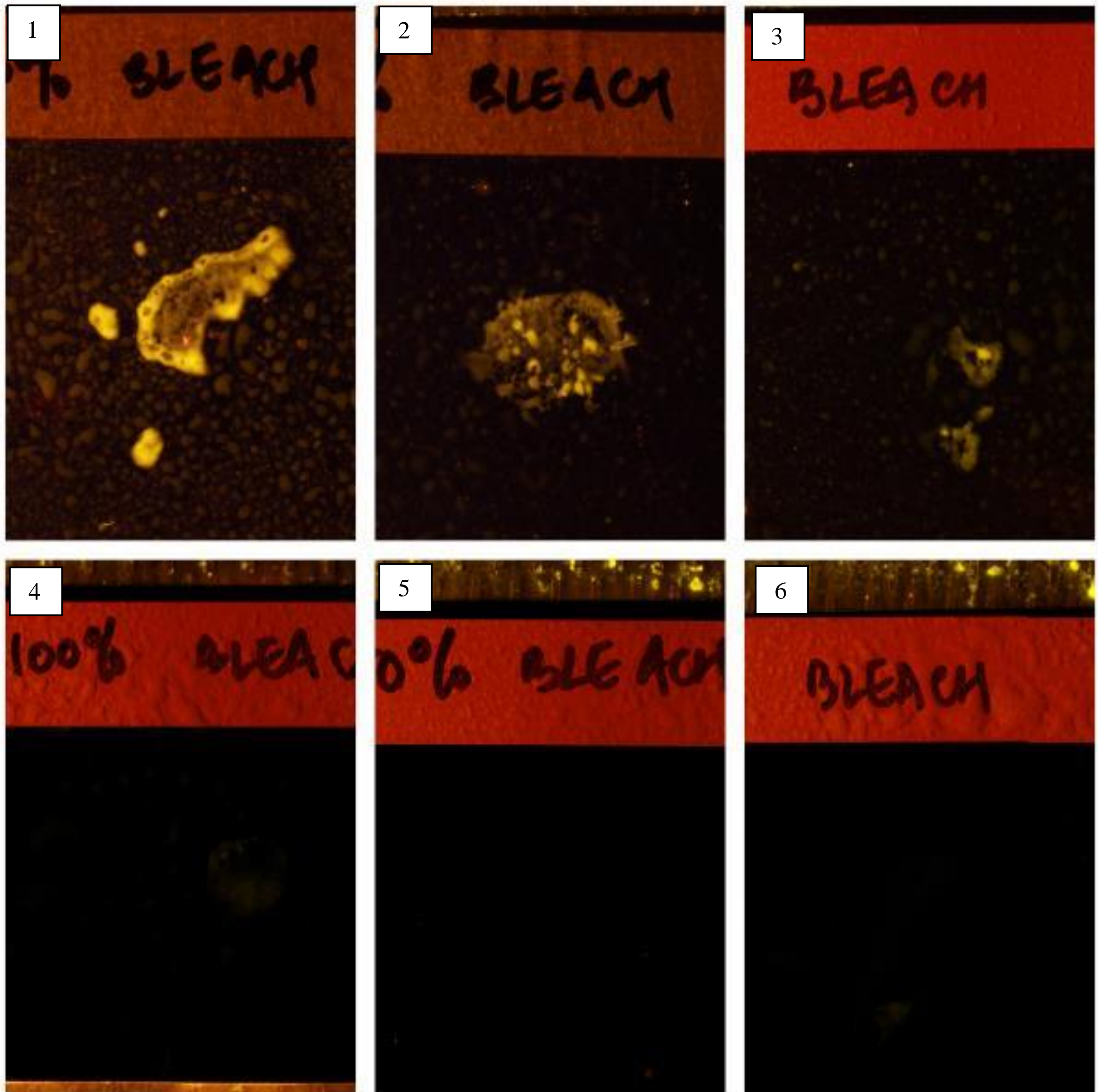

Figure 6. Cross reactivity of Hemascein™ with bleach. 1. 100% Bleach on White Tile; 2. 50% Bleach on White Tile; 3. 10% Bleach on White Tile; 4. 100% Bleach on Black Tile; 5. 50% Bleach on Black Tile; 6. 10% Bleach on Black Tile

Table 6. Statistical results for comparison of black and white tile. The relative intensity results obtained for each reagent where compared using an unpaired t-test to determine if there was a statistically significant difference.

Reagent	p-value
Bluestar®	0.6485
Hemascein™	0.0021
Bluestar® and Hemascein™	0.1364

An unpaired t-test determined the statistical significance of the results of the RI data [Table 6]. The RI values for the black versus white tiles for each reagent were analyzed using a t-test calculator. It was found that the difference between the values for Bluestar® was not statistically significant. The unpaired t-test results for Hemascein™ showed that the difference was statistically significant (p value = 0.0021). The mean and standard deviation for the black tiles were almost equal. This data can be confirmed qualitatively [Figures 5 and 6] since the difference in brightness in the photographs between black and white tile with Hemascein™ is much greater than that of Bluestar®.

3.2. Pattern loss upon application of reagents

A stark difference was observed between use of the two reagents on bloodstain patterns made on fabric. Due to the nature of the pattern and the results obtained, all analyses were qualitative [Table 7].

One of the interesting observations made from this experiment was that the intensity of the positive luminescent reaction observed did not always reflect the

preservation of the pattern. A bright reaction indicated the presence of blood, but often showed no particular shape for the stain. As shown in Figure 7, the positive luminescent reaction for the Hemascein™ samples on white cotton is very bright, but the pattern is practically unrecognizable. In contrast, the Bluestar® samples show a fainter reaction, but the shape of the stamp is still visible in certain parts.

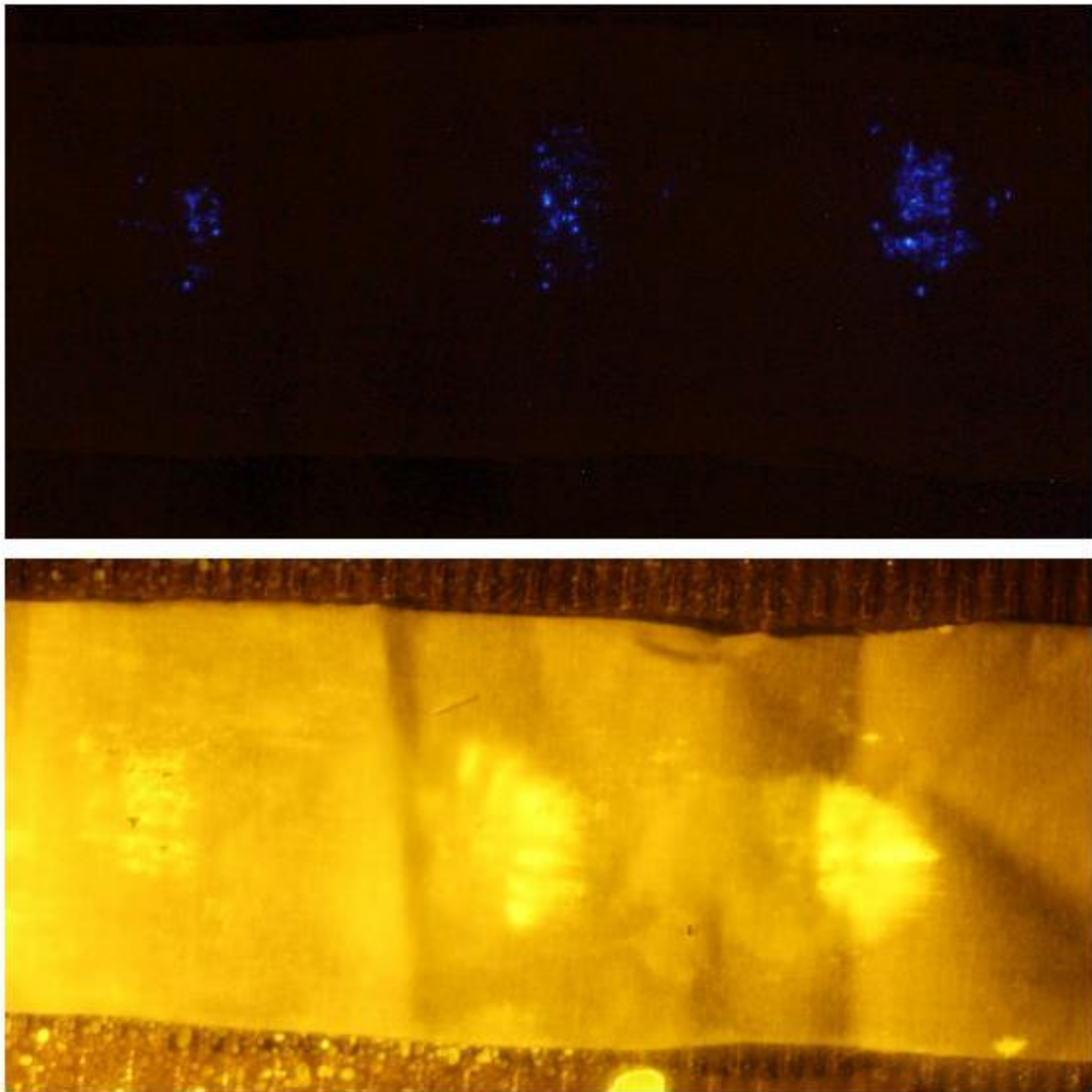

Figure 7. White cotton pattern detail. The figure represents the 4th, 5th and 6th stamps of the diminishing series on white cotton. Top – Bluestar® , Bottom – Hemascein™.

There were some observable differences between the results on the black and white cotton. White cotton showed a brighter light reaction, while black cloth exhibited a quenching effect, resulting in a dimmer light reaction [Figure 8].

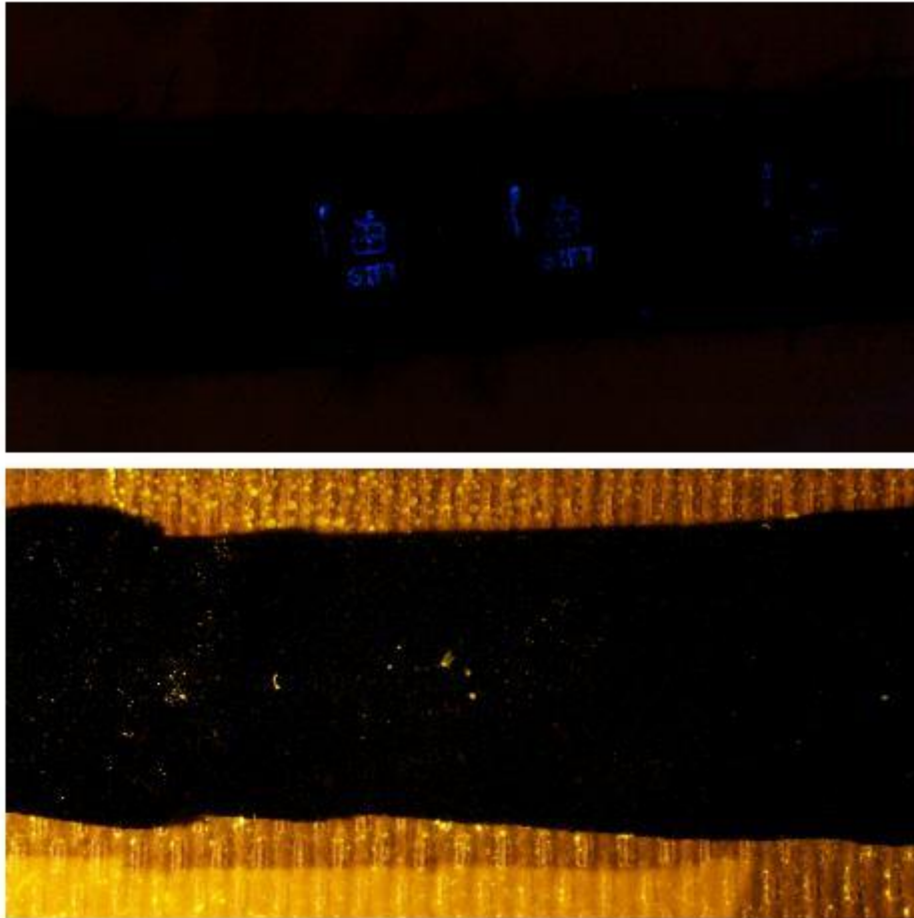

Figure 8. Black cotton pattern detail. The figure represents the 4th, 5th and 6th stamps of the diminishing series on black cotton. Top – Bluestar® , Bottom – Hemascein™.

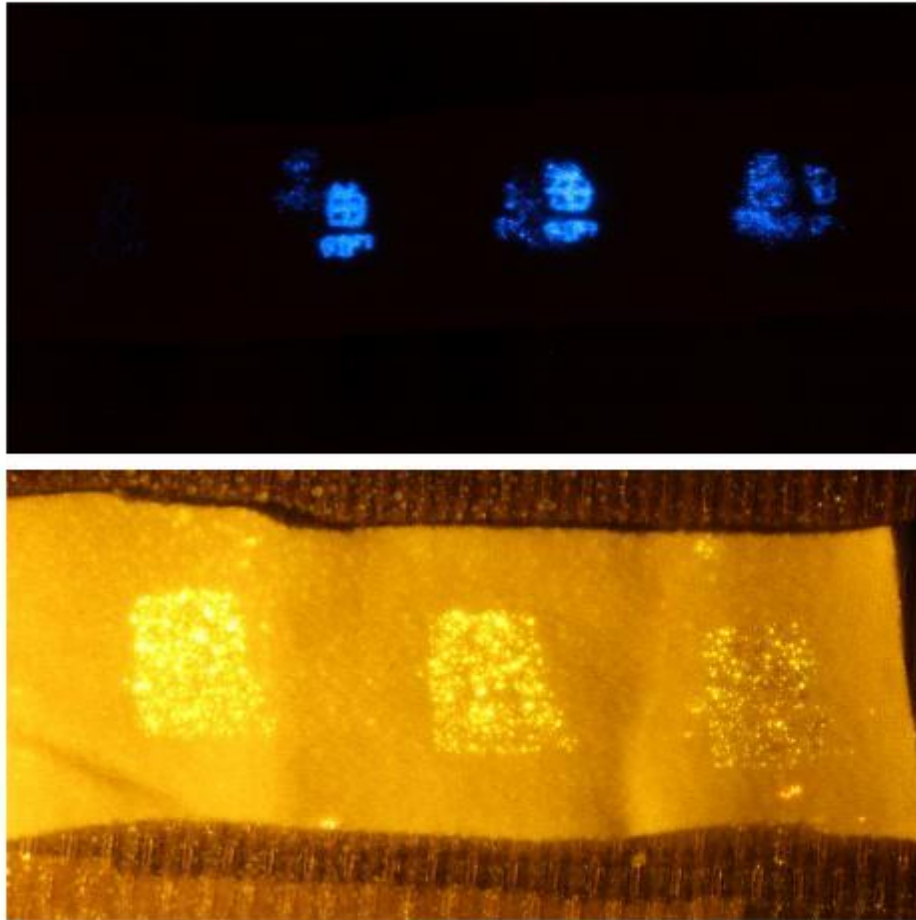

Figure 9. White sweatshirt pattern detail. The figure represents the 4th, 5th and 6th stamps of the diminishing series on white sweatshirt. Top – Bluestar® , Bottom – Hemascein™.

The 4th to 6th iterations of the diminishing series were used as these bloodstains on the cloth were not easily visible by the naked eye in this part of the series for all fabrics, thus making chemical enhancement a logical step. A fair amount of blood was visible in the 1st to 3rd stamps of the series.

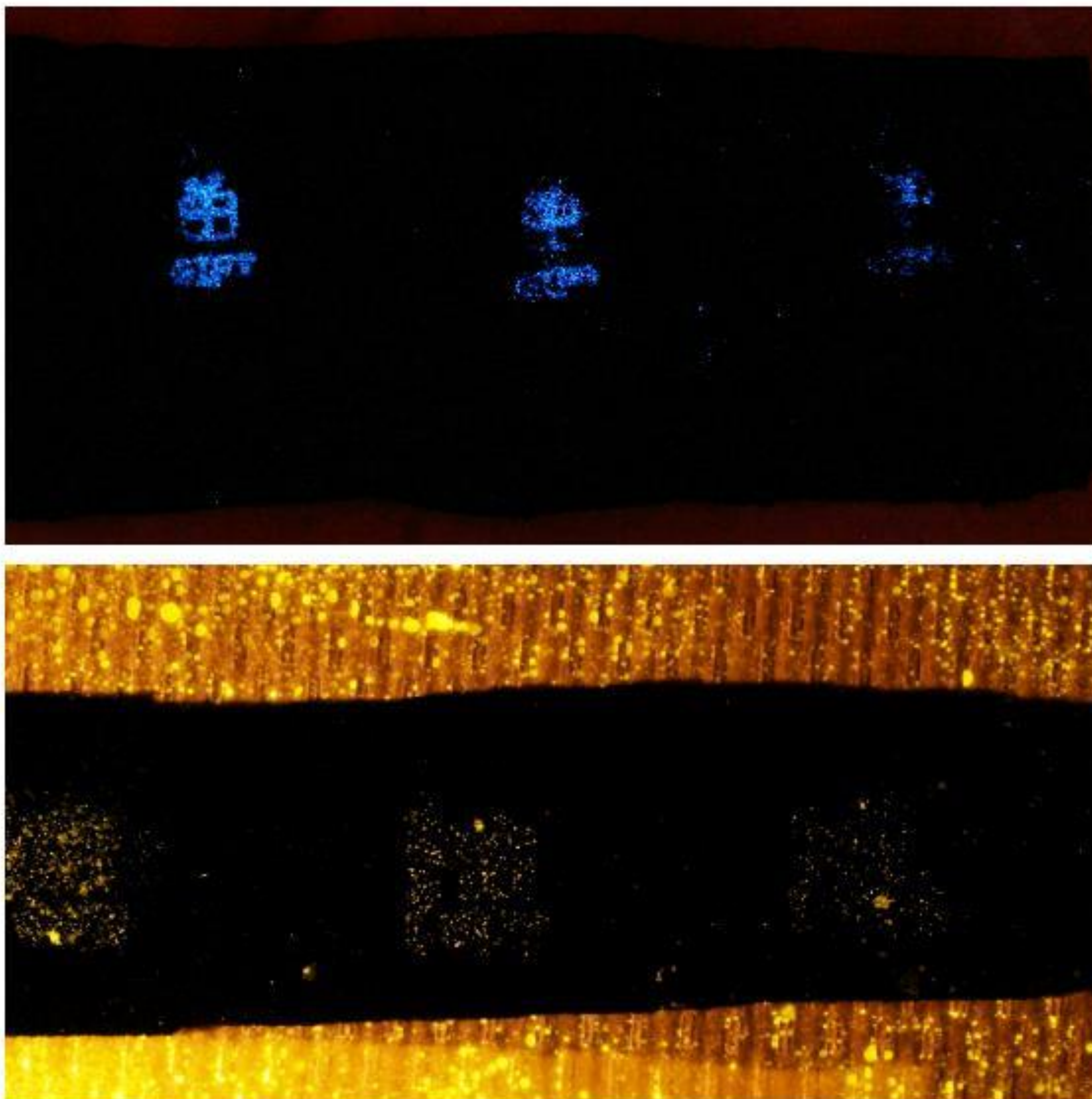

Figure 10. Black sweatshirt pattern detail. The figure represents the 4th, 5th and 6th stamps of the diminishing series on black sweatshirt. Top – Bluestar® , Bottom – Hemascein™.

For both black and white sweatshirt material, Hemascein™ appeared to have settled on the surface in the form of microdroplets, rather than absorbing into the fabric. Therefore, the resulting luminescent reaction showed no traces of the pattern that had been deposited

on the cloth. Additionally, there was a distracting amount of background noise on the bench paper with the Hemascein™ samples [Figure 10].

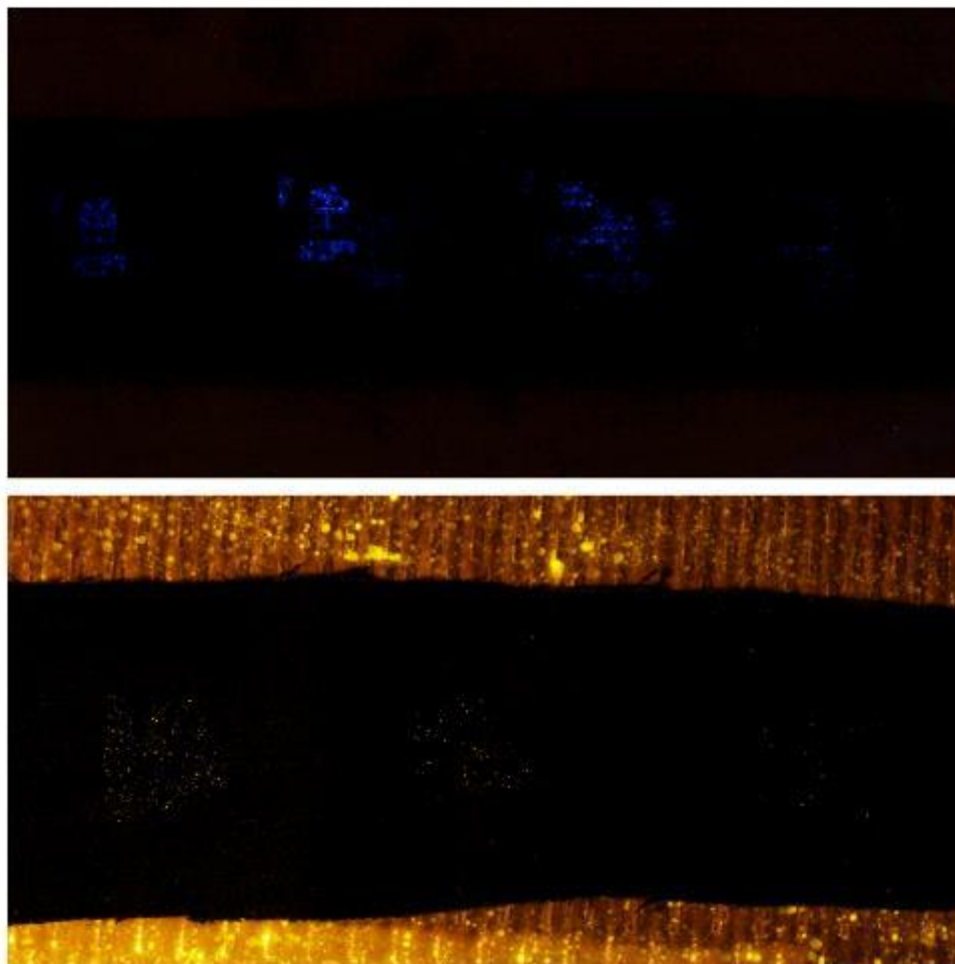

Figure 11. Denim pattern detail. The figure represents the 4th, 5th and 6th stamps of the diminishing series on denim. Top – Bluestar® , Bottom – Hemascein™.

Denim showed the poorest results of all the fabrics in the case of Hemascein™. In addition to the fact that the reagent was not absorbed by the denim, it was also noted that the reaction was fainter as compared to the other fabric types. Bluestar® also showed a fainter reaction, but was still visible and captured in the image [Figure 11].

Table 7. Summary of pattern detail results. The table demonstrates the results on various fabrics when treated with the reagents.

Fabric	Visible Reaction		Pattern Detail	
	Bluestar®	Hemascein™	Bluestar®	Hemascein™
White Cotton	Yes	Yes	No	No
Black Cotton	Yes	Yes, faint	Yes	No
White Sweatshirt	Yes	Yes	Yes	No
Black Sweatshirt	Yes	Yes, faint	Yes	No
Denim	Yes	Yes, faint	Yes, faint	No

3.3. Effect of lighting conditions on efficiency of reagents

There was an observable difference between the two reagents when exposed to various lighting conditions. As can be seen in Figure 12, the Bluestar® chemiluminescent reaction is not visible in the well-lit conditions. The perimeter of the diluted bloodstain can be seen with the naked eye, but a positive luminescent reaction could not be detected.

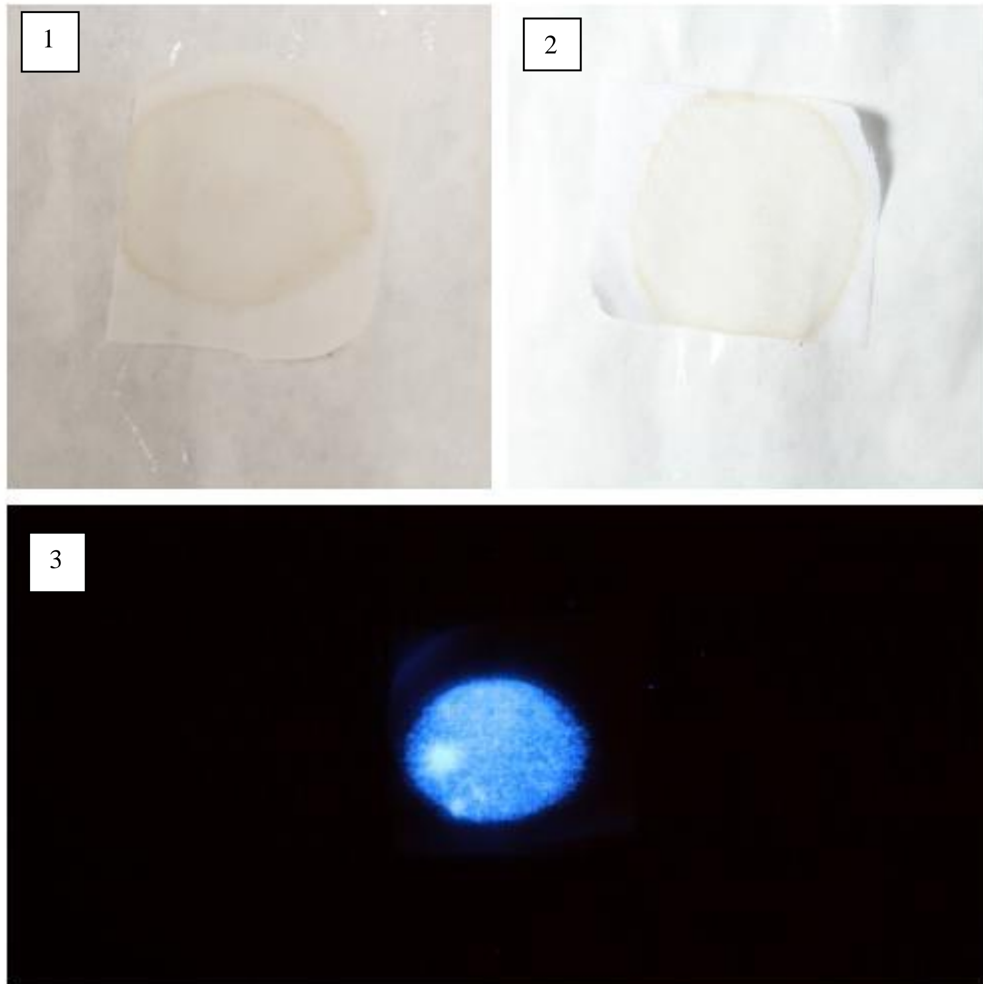

Figure 12. Light variation with Bluestar® . 1. Bluestar® under ambient laboratory lighting conditions; 2. Bluestar® with ambient lighting and desk light; 3. Bluestar® in complete darkness

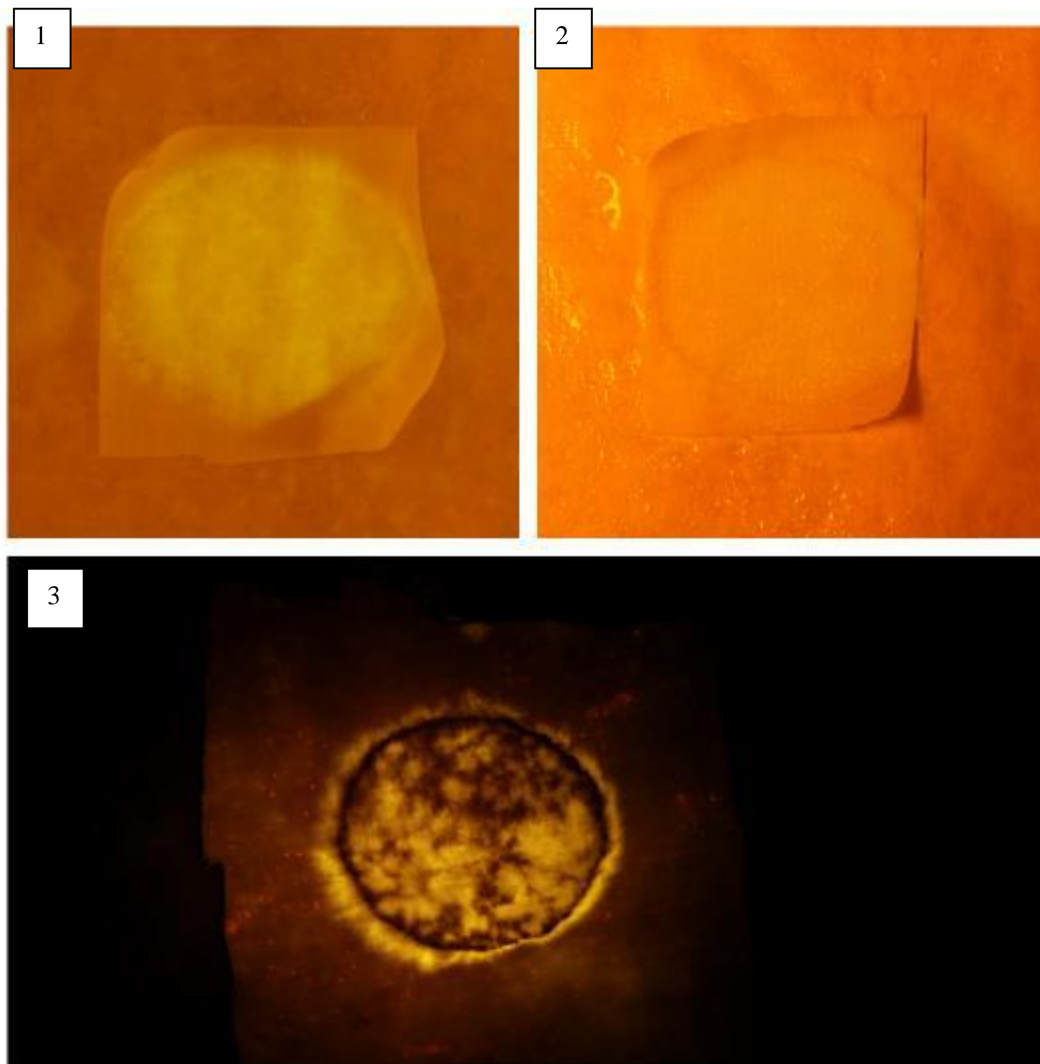

Figure 13. Light variation with Hemascein™. 1. Hemascein™ under ambient laboratory lighting conditions; 2. Hemascein™ with ambient lighting and desk light; 3. Hemascein™ in complete darkness

It should be noted that 'complete darkness' in the case of Hemascein™ still required the use of an ALS and an orange barrier filter.

3.4. Effect of reagents on DNA analysis

A total of nine samples were used to detect the amounts of DNA in the bloodstains following application of the reagents, and four samples were used to test the inhibitory effects of the reagents on DNA. The Internal Positive Control (IPC) value was recorded for all samples and was found to be within the normal range. The data was rounded to two significant figures and is listed in Table 8. A normal IPC value for a kit functioning as expected has a cycle threshold (C_T) value that lies between 28 and 31; a conclusion of inhibition is drawn when the C_T value exceeds 31[37].

Table 8. Inhibition Results. The information in the table shows that the IPC performed as expected for all samples and no inhibition was indicated.

Reagent	**IPC Detection Cycle**
Bluestar®	29.06
Hemascein™	29.15
Hydrogen Peroxide	28.99
Hemascein™ and Hydrogen Peroxide	29.00

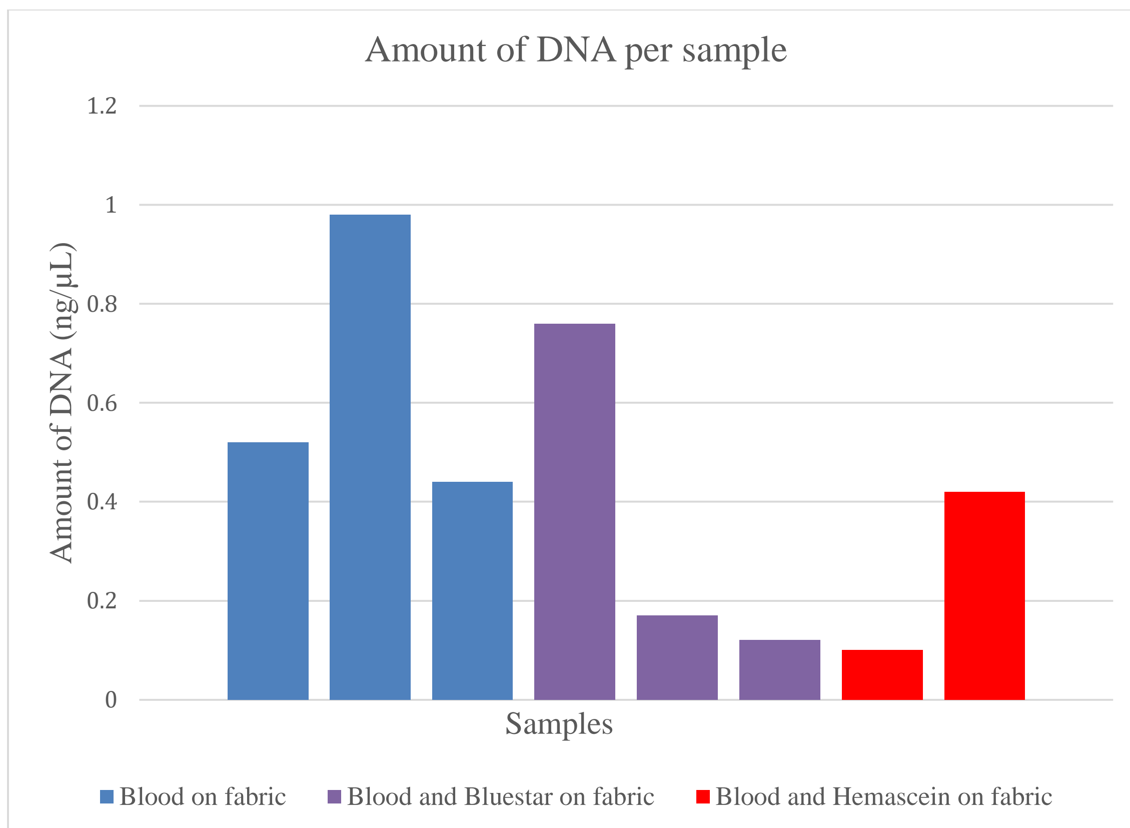

Figure 14. Graph for DNA. Graphical representation of the quantitative values of DNA obtained from the experiment.

For all fabric samples, the cycle threshold value obtained from the instrumentation was used to determine the amount of DNA present in the samples [Table 9].

Mathematically,

$$\text{Amount of DNA} = 10^{(C_T - 29.44782)/(-3.39065)}\ \text{ng}/\mu\text{L}$$

where C_T is the cycle threshold value, or the first cycle of detection for DNA in the sample, -3.39065 is the slope value noted for the standard curve and 29.44782 is the constant for the standard curve.

All results of the equation were then rounded to two significant figures. It should also be noted that the blood on the fabric is the same as the 1:10 dilution discussed in materials and methods.

Table 9. Effect of reagents on DNA Quantitation. The results of the qPCR conducted for samples on white cotton have been noted.

Sample	**Cycle (C_T)**	**Amount of DNA (ng/µL)**
Blood on fabric (1)	30.41120529	0.52
Blood on fabric (2)	29.48503494	0.98
Blood on fabric (3)	30.65951729	0.44
Blood and Bluestar® on fabric (1)	29.84410858	0.76
Blood and Bluestar® on fabric (2)	32.07797623	0.17
Blood and Bluestar® on fabric (3)	32.55330276	0.12
Blood and Hemascein™ on fabric (1)	32.79529572	0.10
Blood and Hemascein™ on fabric (2)	30.71431541	0.42

All controls and reagent blanks behaved as expected and each number in the parentheses represents a replicate. However, one of the Hemascein™ samples was excluded as it showed a high amount of male DNA indicative of contamination since the donor sample was known to be female. The first Bluestar® replicate showed a similar

problem, however, the contamination was found to be minute compared to the total amount of DNA present, so the data was considered usable.

4. DISCUSSION

4.1 Cross reactivity with bleach

Both reagents were found to have a linear relationship between the concentration of bleach and the relative intensity of the brightness. The highest RI was observed at the 100% concentration and lowest at 10%. Hemascein™ showed a low amount of cross-reactivity at all concentrations on black tile, but there was a noticeable luminescent reaction with white tile for all concentrations, and the 10% and 50% concentrations showed a brighter light reaction compared to the same concentrations tested with Bluestar®. It is interesting to note that both reagents showed a lower intensity value for black tile as compared to white, especially in the case of Hemascein™. This may be due to a quenching effect occurring because of the black background, which absorbs all wavelengths of light.

4.2 Loss of pattern detail upon application of reagents

A few important aspects to examine with respect to pattern loss are the nature of the fabric being used, the color of the fabric and the diffusion of the pattern on fabric after the application of the reagent. Both color and type of fabric impact the detection of the bloodstains. Some colors can result in a quenching of the luminescent reaction and the more absorbent fabrics tend to mix the blood from the pattern and the reagent applied and result in a distortion.

There was a difference noted between both the reagents in terms of pattern visibility, with Bluestar® outperforming Hemascein™. While there was some diffusion observed in the patterns in the case of Bluestar®, Hemascein™ barely detected the

presence of blood on the fabrics. There could be a number of reasons for this difference. For one, Hemascein™ was applied using the ABASpray® bottles provided with the kit. The nozzles on these bottles were difficult to depress after pumping and produced a fine mist of the reagent that did not always appear to be absorbed into the fabric. The instructions recommend that the user not directly apply the reagents on the testing surface, but instead allow them to settle like a fine mist to allow even distribution. Upon following these instructions, it was found that this method resulted in an increase in background fluorescence which interfered with the quality of the image being captured. The background fluorescence observed on the bench paper may be caused by the light from the ALS reflecting on the unabsorbed individual droplets created from spraying Hemascein™. In cases of pattern analysis, the photograph quality has a huge impact on the value of the evidence; the photo is often the best evidence available to compare to a reference pattern. Since the fine mist had a tendency to adhere to the surface of the fibers rather than absorbing into the fabric itself, this resulted in a loss of most pattern detail present on the item.

Even with only the faint luminescence, there was a difference between the white and black fabrics for Hemascein™. The white cotton samples showed a brighter luminescent reaction, however, the pattern was completely obscured, while black samples of the same fabric showed little to no luminescence. Comparatively, sweatshirt material for both colors showed a brighter luminescent reaction than the black cotton sample. The denim material showed no light reaction.

Within the Bluestar® samples, it was noted that the white sweatshirt material performed the best, showing the most luminescence and pattern detail, while a very low amount of luminescence was observed with the denim sample. No noticeable changes could be observed in terms of colors.

Sensitivity comparison between the two reagents for the diminishing series was difficult due to the challenges in identifying pattern characteristics with Hemascein™. The microdroplets showed a reaction occurring, but an estimation of the brightness of the reaction could not be made. In the case of Bluestar®, the brightness steadily decreased further into the diminishing series for all fabrics.

4.3. Evaluating effect of lighting conditions on reagents

Based on the results obtained, it was found that as per the manufacturer's claims, Hemascein™ performed better in lighted conditions and stains were more visible by eye when wearing the orange barrier filter glasses. At 90-120 lumens, the light reaction could be captured by the altered camera settings as well. At 1000-1200 lumens, however, the camera was unable to capture the reaction, although the luminescence was visible briefly. No light reaction was observed with Bluestar® in the altered lighting conditions even with the naked eye, and therefore no images of a light reaction were captured.

4.4 Evaluating impact of reagents on DNA analysis

Inhibition was not detected, since the Internal Positive Control (IPC) behaved as expected, including for the samples containing only liquid reagents. Almeida, Gleese and Bonorino (2011) found that Bluestar® degraded DNA to some extent, but not enough for

it to be detrimental to the subsequent STR profiling[38]. The current study did not find any indication of degradation, since the IPC detection values for all the samples was within the 28.9-29.9 range, which would be considered normal for the QuantDuo® kit. Further, the cycle detection value for the IPC of the positive control was found to be within the same range.

All blood dilutions yielded a positive quantitation result. Each of the values for the qPCR conducted with the reagents along with fabric swatches yielded sufficient quantities of DNA for downstream analysis.

Table 10. Statistics for DNA. The mean and standard deviation observed with the DNA samples.

Sample	Number of samples	Mean (ng/µL)	Standard Deviation
Blood on Fabric	3	0.65	0.24
Bluestar® on Fabric	3	0.35	0.30
Hemascein™ on Fabric	2	0.26	0.16

An important factor to note is that even though the mean reflects that Bluestar® outperformed Hemascein™, the sample sizes for both were different, and the absolute population sizes are quite small. There is also some difference between the mean amount of DNA obtained in the untreated blood samples and in the samples treated with the reagents, which may not necessarily be attributed directly to the effect of the reagents on the DNA. Further research would be required to verify the DNA results, including a larger

sample size, varying the time between application of reagent and DNA testing, and different dilutions of blood.

Despite the variation between all samples in absolute amounts of DNA, all of the samples can be used for purposes of amplification since the amounts of DNA recovered are sufficient for downstream analysis based on the protocols used at the laboratory where this testing was conducted.

5. CONCLUSIONS

5.1 Cross reaction with Bleach

The cross-reactivity of each reagent with bleach varied from concentration to concentration, with Bluestar® performing better than Hemascein™ at lower concentrations. However, there was a difference between black and white tiles, showing that some degree of quenching occurs with dark substrates, and this quenching was more pronounced in the case of Hemascein™. For both reagents, there was no qualititative difference between the luminescent reaction observed with blood and the luminescent reaction observed with bleach.

5.2 Loss of pattern detail upon application of reagent

The results show that to retain the maximum amount of detail from a patterned latent blood stain, it would be more effective to use Bluestar® . The ABAspray® system could also use some improvement such that the stream is more dense and less mist-like, allowing more effective absorption. This could address the problems faced with respect to the amount of background fluorescence seen in the case of Hemascein™.

5.3 Use of reagents in altered lighting conditions

As per the manufacturer's claims, Hemascein™ outperformed Bluestar® in terms of detection of latent blood in well-lit conditions. Hemascein™ would be a more useful reagent to use in circumstances where there is ambient light that cannot be filtered out. Bluestar® cannot be used effectively unless there is complete darkness or near complete

darkness. Further testing must be conducted with Hemascein™ to determine if it can be successfully used in full daylight.

5.4. Evaluating impact of reagents on DNA analysis

All samples yielded high enough DNA concentrations to support downstream amplification and analysis. In addition, no inhibition was detected with either reagent. Based on the studies conducted, there is no disadvantage to either reagent in terms of DNA testing.

6. FUTURE DIRECTIONS

This study raised a number of questions that can be addressed by further research. It would be beneficial to learn what component of bleach causes it to cross-react with Hemascein™ and Bluestar®, and explore how this cross-reaction can be addressed with this knowledge. In terms of the pattern analysis, the pattern chosen for this study was small in size and one of the more likely patterns to be observed in forensic casework would be footwear impressions. The performance of the reagents should be evaluated on larger patterns on a variety of surfaces. It should also be noted that the difference in the spray bottles for the two studies could be a contributing factor to the difference between the two reagents. Hemascein™ may perform better when used with regular spray bottles instead of those provided by the kit. It would also be interesting to note the performance of Hemascein™ in outdoor sunlit conditions since indoor lighting is different from sunlight, even if the intensity is similar.

In a more practical scenario, such as in routine casework, samples are sometimes stored for a long period of time after collection, thus resulting in a large time difference between when the samples are treated with the reagent and when they undergo genetic analysis. A study examining the impact of this time lag could be beneficial to crime labs. A complete understanding of the effect on subsequent DNA studies could be obtained with a more thorough study involving different dilutions of bloodstains and different substrates on which these stains are present. In addition, it would be worthwhile to further investigate the performance of Hemascein™ and Bluestar® on dark substrates and additional porous and non-porous surfaces.

LIST OF JOURNAL ABBREVIATIONS

Chem Phys Lett	Chemical Physics Letters
Forensic Sci Int	Forensic Science International
Int J Legal Med	International Journal of Legal Medicine
J Am Chem Soc	Journal of the American Chemical Society
J Crim Law Criminol Police Sci	Journal of Criminal Law, Criminology and Police Science
J Can Soc Forensic Sci	Canadian Society of Forensic Science Journal
J Forensic Identific	Journal of Forensic Identification
J Forensic Sci	Journal of Forensic Sciences
Proc Nat Acad Sci USA	Proceedings of the National Academy of Sciences of the United States of America
Sci Justice	Science and Justice
Trans Faraday Soc	Transactions of the Faraday Society
Trends Anal Chem	Trends in Analytical Chemistry

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