

#### INTRODUCTION

Forensic scientists rely on various blood screening tests to detect and identify the presence of blood at crime scenes. While many crime scene blood samples can be readily detected, there are circumstances when latent bloodstains need to be chemically enhanced; such as, when the crime scene has been deliberately cleaned in an effort to remove and or destroy any blood evidenced. In situations such as these, sensitive light-emitting blood enhancement reagents—luminol, Bluestar®, fluorescein and HemaScan™—are used to locate trace quantities of blood. Furthermore, arson-homicide scenes where the perpetrator has deliberately set a fire for the purpose of covering up the crime or destroying evidence creates significant challenges in detecting and identifying blood. In these circumstances the usage and effectiveness of blood enhancement reagents is not well documented.

Luminol is a sensitive blood enhancement reagent which exhibits a chemiluminescence-based, blue-light emission when it reacts with the heme group in hemoglobin; therefore, it is regularly used at crime scenes to detect trace quantities of blood. Another, blood enhancement reagent, Bluestar®, uses a modified form of the luminol molecule which affords a brighter, longer-lasting chemiluminescence emission. Luminol and Bluestar® must be prepared fresh just before use due to a short shelf life. Moreover, when utilizing these reagents the crime scene must be darkened in order to see the fleeting light emissions. Fluorescein is a different blood enhancement reagent which emits a fluorescence-based, yellow-green light when excited with intense blue light via a catalytic reaction that oxidizes fluorescein to fluorescein in the presence of heme and hydrogen peroxide. HemaScan™ is a sensitive, highly specific fluorescein-based product which also emits a yellow-green fluorescence when excited with blue light. Due to its short shelf life it is recommended that a freshly prepared fluorescein reagent is used at every scene; conversely, the HemaScan™ working solution has a long shelf life, several months when stored in a refrigerator.

In this study, fluorescein and HemaScan™ were quantitatively compared using a fluorometer for their sensitivity of detection and light emission characteristics when used to detect burnt and unburnt blood samples. There are two methods for preparing fluorescein, and HemaScan calls for hydrogen peroxide concentration of 1% to 3%; therefore, studies were completed to optimize these reagent test conditions. In addition, two different preparations of fluorescein were compared for their efficacy, and the optimal concentrations of hydrogen peroxide required for both fluorescein and HemaScan™ activity were determined. These findings were compared to the results obtained previously with luminol and Bluestar® enhancement reagents.

#### MATERIALS AND METHODS

##### Blood Samples\*

• Serial blood dilutions ranging from 1:10 to 1:1,000,000 were prepared with distilled water.

• Bloodstain smears of approximately 1 cm<sup>2</sup> were prepared on glass microscope slides using 5µl of 1:10 liquid blood dilution and burnt by direct exposure to the flame of an ethanol fire for 1, 3 or 5 minutes with temperatures ranging from 400-600 °C (Figure 1).

\*Canine blood in Vacutainer™ tubes with EDTA, a preservative, was used in this study for health and safety reasons, and for its similarity to human blood in red blood cell count and hemoglobin concentration.

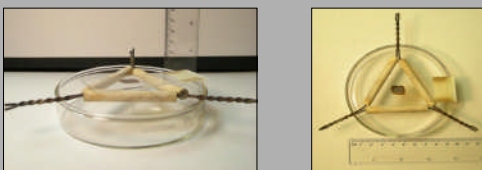


Figure 1a and 1b: Preparation of Burnt Bloodstains

Glass microscope slides with bloodstain smears were suspended face-down over a 10 cm diameter Petri dish filled with 3, 7 or 11 ml of absolute ethanol, depending on desired burn time.

##### Blood Enhancement Reagent Preparation

• HemaScan™ stock and working solutions were prepared according to the manufacturer's instruction. The 1% and 3% hydrogen peroxide solutions were prepared by diluting 30% hydrogen peroxide with distilled water.

• Fluorescein stock and working solutions, ethanol based and water based methods, as well as hydrogen peroxide solution were prepared according to the procedures provided by the Washington State Patrol Crime Lab, Spokane.

##### Emission Spectroscopy

• A Bio-Rad VersaFluor™ fluorometer with GAIN set to low was used to record light emissions. The light intensity in Relative Fluorescence Units (RFUs) was recorded every 7 seconds for 5 minutes.

• Limit of quantization for the fluorometer is 20,000 RFUs.

• A 460 ± 5 nm excitation filter and a 520 ± 5 nm emission filter were used for fluorescein and HemaScan™ blood tests.

• For all blood analysis experiments, a 25 µl aliquot from one of the blood dilutions was added to 2.0 ml of reagents in a UV-transparent plastic cuvette and mixed. (See Optimization, Sensitivity and Burnt Bloodstain Sections)

##### Optimization

• HemaScan™  
A 25 µl aliquot of a 1:4,000 liquid blood dilution and a specified amount of either 1% or 3% hydrogen peroxide (See Table 1) were simultaneously added to a cuvette containing a specified amount of HemaScan™, then immediately mixed via rapid pumping of mechanical pipette.

• Fluorescein  
A 25 µl aliquot of a 1:4,000 liquid blood dilution and a specified amount 3% hydrogen peroxide were simultaneously added to a cuvette containing a specified amount of either fluorescein-ethanol base or fluorescein-water base, then immediately mixed via rapid pumping of mechanical pipette.

##### Sensitivity Study

• A 25 µl aliquot of varying blood dilutions (1:10, 1:10<sup>2</sup>, 1:10<sup>3</sup>, 1:10<sup>4</sup>, 1:10<sup>5</sup>, 1:10<sup>6</sup>) and 0.20 ml of a 1% hydrogen peroxide solution for HemaScan™ and a 3% hydrogen peroxide solution for fluorescein were simultaneously added to a cuvette containing 1.80 ml of blood enhancement reagent, then mixed via rapid pumping of mechanical pipette

##### Burnt Blood Stain Analysis

• A cotton swab moistened with distilled water was used to remove individual burnt stains from the glass slides. The collected stain and 0.20 ml of hydrogen peroxide solution were simultaneously placed in a cuvette containing 1.80 ml of blood enhancement reagent, then immediately mixed via rapid pumping using a mechanical pipette.

#### RESULTS AND DISCUSSION

##### Optimization

• HemaScan™ and hydrogen peroxide in a ratio of 9:1 was determined to be the optimal conditions with both a 1% and 3% hydrogen peroxide solution; however, the 1% hydrogen peroxide solution produced rapidly increasing fluorescence over the first 60 seconds, then leveling off remaining almost constant for the rest of the five minute test period. Therefore, a 9:1 ratio of HemaScan™ to 1% hydrogen peroxide was used for Sensitivity and Burnt Bloodstain studies. (Figure 2)

• Fluorescein, water base and ethanol base, and 3% showed the highest light intensity (Figure 3). Furthermore, the water base method had higher RFU values over all ratios than the ethanol base; maximum RFU value 300 (results not shown). Therefore, the water base version in a ratio of 9:1 was used for Sensitivity and Burnt Bloodstain studies.

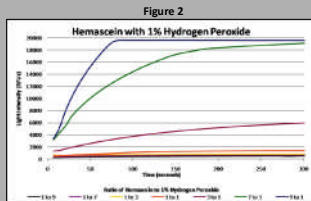


Figure 2

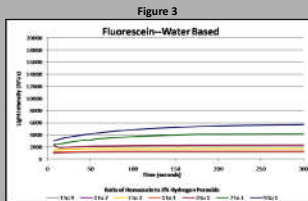


Figure 3

##### Sensitivity

• Liquid blood assayed with HemaScan™ produced a rapidly increasing light intensity within the first 60 seconds, followed by a gradual increase, and leveling off towards the end of the 5 minute test period (Figure 4).

• Blood assayed with fluorescein showed a nearly constant light intensity over the entire 5 minute test period. (Figure 4).

• Other liquid blood dilutions assayed with HemaScan™ and fluorescein produced similar results. See Table 2 for maximum light intensity values for all blood dilutions and for comparison values for luminol and Bluestar®.

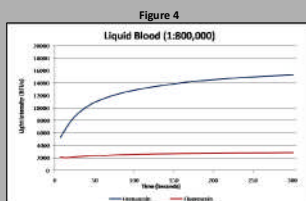


Figure 4

Table 2: Summary of the maximum light emissions obtained for liquid blood, and burnt bloodstains tested with fluorescein and HemaScan™.

Sample Type	Sample & Test Conditions		Maximum Light Intensity (RFUs)			
	Blood Dilution*	Burn Time	Luminol	Bluestar®	Fluorescein	HemaScan™
Liquid Blood	1:800	-----	20,000**(*)	20,000**(*)	20,000**	20,000**
	1:8,000	-----	18,525(*)	20,000**(*)	20,000**	20,000**
	1:80,000	-----	4,368(*)	9,364(*)	20,000**	20,000**
	1:800,000	-----	361(*)	146(*)	2,856	15,363
Burnt Bloodstains	1:4,000	1 min	481(*)	11202(*)	3,524	6,393
	1:4,000	3 min	NT	1128(*)	2,494	4,027
	1:4,000	5 minutes	NT	785(*)	2,616	2,567

(\*) Luminol and Bluestar® figures obtained from previously recorded data. (1)

(\*\*) Dilution values for blood in the test buffers

(\*\*\*) Light intensity exceeded instrument's quantization limit

NT = Not detected or below 100 RFUs (the light intensity which would not be visible to the naked eye)

ND = Not Tested

##### Burnt Bloodstains

• As reported in a previous study, luminol only yielded detectable light emissions for bloodstains subjected to a 1 minute burn and Bluestar® showed considerable light emissions for bloodstains subjected to 1, 3, and 5 minute burns (Figures 5, 6 and 7).

• HemaScan™ showed significant light emissions which gradually increased over the entire test time for 1, 3 and 5 minute burnt bloodstains (Figures 5, 6 and 7).

• Fluorescein like HemaScan™ yielded a gradual increase in light emissions over the entire test time for 1, 3 and 5 minute burnt bloodstains (Figures 5, 6 and 7). However, the HemaScan™ RFUs were considerably higher than those for fluorescein except for the 5 minute burn where HemaScan and fluorescein showed similar RFU values (Figure 7).

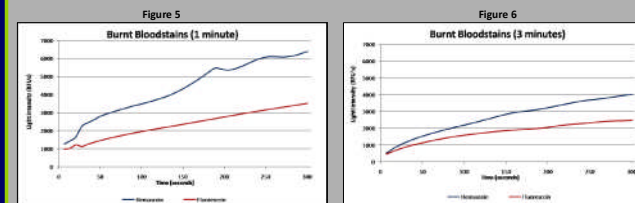


Figure 5

Figure 6

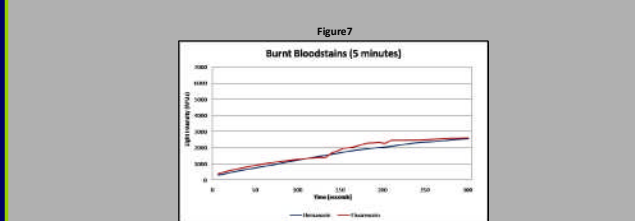


Figure 7

#### CONCLUSION

The results show that fluorescein and HemaScan™ have similar fluorescence characteristics for liquid blood and burnt bloodstains; in addition, the light intensity values for blood tested with HemaScan™ either equaled or surpassed those of fluorescein. Moreover, the preparation of HemaScan™ requires less equipment and reagents, streamlining the method making it more user friendly.

When comparing the results for fluorescein and HemaScan™ obtained in this study to those of luminol and Bluestar® as reported in a previous study, fluorescein and HemaScan™ taken as a whole outperformed luminol and Bluestar®, with regards to sensitivity of detection of liquid blood dilutions and burnt bloodstains.

This study indicates that not only does HemaScan™ show a greater sensitivity for liquid blood, detecting blood in a 1:800,000 dilution, it was also able to detect blood in stains that had been exposed to very high temperatures of a fire, with light emissions above 2,000 RFUs. Furthermore, the above results and the properties of HemaScan™; high sensitivity, ease of use and long shelf life, suggest the potential benefit of using it to detect fire compromised bloodstains at crime scenes. A study is presently underway to optimize the collection and testing of bloodstain evidence, and apply these techniques in situations typically encountered at crime scenes.

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