Article

Adaptation of Bluestar to Extreme Outdoor Cold Conditions[1]

Kelly Hatch [2]
Kim Lavoie [2]
Frank Crispino [3]

Abstract: The use of a blood enhancer (e.g., Bluestar) at crime scenes is required for the observation of latent bloody stains. This process is often necessary to understand a bloody crime dynamic, as well as to identify where DNA is likely to be present. Although Bluestar has a great capacity to adapt to environmental conditions, its effectiveness has not been tested on temperatures lower than -10 °C. The objective of this research was to adapt Bluestar so that it would not freeze at low temperatures, nor hinder DNA analysis. An ethylene glycol:Bluestar solution proved to be a viable alternative to the standard solution when the ambient temperature drops below 0 °C. This solution did not hinder DNA analysis.

Introduction

The use of a presumptive blood detection reagent is frequently required for the observation of latent bloodstains at crime scenes to help understand how a crime was committed, as well as to identify where DNA is likely to be found. Among the many existing presumptive blood detection reagents, luminol and Bluestar are the most used because of their good sensitivity and their ability to produce chemiluminescence in contact with blood [1]. A redox reaction occurs when the basic aqueous solution is in the presence of hydrogen peroxide and a catalyst, such as iron that is present in the hemoglobin of blood, which results in an emission of luminescence [2].

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[2] Université du Québec à Trois-Rivières (UQTR), Département de Chimie, biochimie et physique

[3] Laboratoire de recherche en criminalistique (UQTR)

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Bluestar Forensic was developed in 2000, with the aim of improving the results obtained with luminol, of which Bluestar is a derivative [3, 4]. The improvement and persistency of brightness emitted over time are among the advantages of Bluestar over luminol [5]. Moreover, because of its greater brightness, the complete darkness needed with luminol is not required when using Bluestar. Furthermore, DNA does not show any sign of degradation until 30 days after contact with a Bluestar solution [6], which means complete genetic profiles can be obtained for this entire period.

Solvated in water, Bluestar has shown good results under various environmental conditions [7]. However, its effectiveness was not tested in extreme outdoor cold conditions. A study conducted by Miles [8] showed blood samples react with Bluestar at controlled temperatures of 0, -5, and -10 °C. A positive result was also obtained for blood samples that had experienced periods of freezing and thawing or had been in contact with ice. Thus, the reaction between luminol, which is present in the Bluestar solution, and blood hemoglobin is not affected by cold temperatures down to -10 °C. However, the reaction of Bluestar with blood samples on snow and the vaporization of that solution in more extremely cold environments have not been investigated.

The aim of this study was to find a Bluestar preparation that would not freeze at low temperatures and would not alter DNA, yet would maintain the product's high efficiency in detecting bloodstains.

Material and Methods

Sterilization

All materials that were going to have contact with blood, including water used to dilute the blood samples or Bluestar solutions, were sterilized by a steam autoclave to minimize contamination with other DNA from the environment (e.g., bacterial DNA) [9–11]. This way, it would be possible to determine whether there was any degradation of the bloodstains' DNA after they had been sprayed with the different solutions being tested. Masks were worn in addition to other personal protective equipment, including gloves and goggles, while experiments were carried out in the presence of blood.

Blood Sample Preparation

Human blood that had been previously collected[1] with EDTA blood collection tubes was diluted at factors of 1:10, 1:50, 1:100, 1:500, and 1:1000. These dilutions were used to evaluate the reaction with blood of the various tested formulations. For each experimental formulation, the reaction with whole blood was also evaluated. A volume of 30 microliters for each of the various dilutions of blood was deposited on filter papers using micropipettes. Ultimately, each blood dilution was arranged at approximately equivalent distance from one another on each filter paper, each containing a spot of whole blood in the center. For each filter paper, the same arrangement of the dilutions and the whole blood was maintained, as shown in Figure 1. Then, these filter papers were stored in a freezer at -20 °C for a minimum of 1 week.

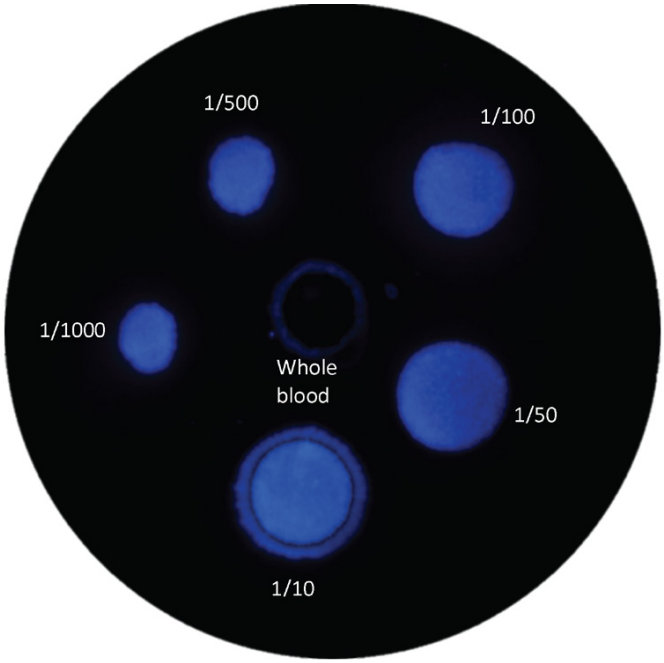


Figure 1

Filter containing all blood concentrations that were sprayed with a solution of Bluestar in the dark.

[1] The experiments were in accordance with the Helsinki Declaration addressing ethics in research.

Formulations of Bluestar-Based Solutions

Six Bluestar solutions were prepared according to the manufacturer's instructions. To each one, a volume of either ethylene glycol or propylene glycol was added to provide different formulations of Bluestar containing 15, 25, and 35% of either ethylene glycol or propylene glycol[2]. (The greater the amount of a glycol product, the lower the freezing temperature of a solution.)

Another Bluestar solution was prepared as per the manufacturer's instructions except that the distilled water was heated to 90 °C. By the time of its use, the temperature of the solution had decreased to between 45 °C and 65 °C.

Another Bluestar solution was prepared as per the manufacturer's instructions and then the solution was heated on a hot plate to a temperature of 85 °C. By the time of its use, the temperature of the solution had decreased to between 45 °C and 65 °C.

Bluestar Application and Photography Procedures

The various developments were evaluated and compared under the same experimental conditions: filter papers were stored in a -20 °C freezer right next to a walk-in refrigerator set to maintain 4 °C. They were brought one by one from the freezer to the walk-in refrigerator to be sprayed while being being protected from any light and they were immediately photographed. Each formulation was sprayed by the same person on three separate filter papers. To optimize the comparison process, a Nikon D3200 camera with a 55 mm lens was used according to the photographic settings recommended by Bluestar manufacturers: the ISO was set at 400, f/stop at 5.6, and the exposure was for 30 seconds.

Chemiluminescence Results Analysis

Using Adobe Photoshop Extended CS6, numerical values from normalized histograms were collected to compare the light intensities of the chemiluminescence reaction for each of the filter papers. After each photograph was scaled, converted to gray scale, and the levels (histogram) auto-adjusted, the contour of the luminescent zones corresponding to the different dilutions of blood was plotted using the magnetic lasso tool [12] to facilitate the selection of bright pixels. A luminosity histogram was obtained for each of these luminescent zones. The values on

[2] To avoid intoxication by propylene glycol or ethylene glycol, protective masks were worn when vaporizing Bluestar.

the luminosity histograms ranged from 0 to 255. The higher the value, the stronger the intensity of the reaction of chemiluminescence [13].

Outdoor Experiments

Outdoor experiments were performed with 20 mL 1:100 diluted blood sprayed on snow, very early in the morning while the external temperature was -6 °C. A further dilution of the blood preparation was expected due to the diffusion process in snow, assessed between 5 to 10 [8], thus obtaining an approximate concentration after diffusion of up to 1:1000. The standard Bluestar solution, the hot water solution, and both 15 and 35% ethylene glycol solutions were sprayed on the diluted blood sample.

DNA Profiling

A DNA-IQ kit was used for the digestion and extraction phases. Briefly, the method involved cutting a small square of about 0.5 cm^2 with a sterile blade inside the nondiluted bloodstain on the filter papers that had been sprayed. Whole blood was selected for DNA analysis to ensure that complete genetic profiles would be obtained. Otherwise, there may have been some risks of obtaining only partial profiles by using one or the other of the blood dilutions, which would be inconclusive to determine any possible degradation of the DNA following its vaporization by the different Bluestar formulations. The risks of obtaining partial profiles are indeed present with dilutions of 1:100 and become quite significant with dilutions of 1:1000.

The Promega Plexor kit (Promega Corp., Madison, WI) was used to perform the cross-linked fluorophore quantitation, and the Promega's PowerPlex 16HS kit was used for DNA amplification. The extracted and amplified DNA was then analyzed using a polyacrylamide gel. However, to more reliably determine the presence of degradation, the amplified DNA was also sent to the Laboratoire des Sciences Judiciaires et de Médecine Légale (LSJML, provincial forensic laboratory), located at Montreal in Quebec, to obtain the complete genetic profiles. The genetic profiles from the blood that had been in contact with the new formulations were then compared with those obtained following the extraction of DNA of whole blood that had no contact with any of the Bluestar solutions.

Results and Discussion

The areas of the bloodstains on the filter papers were smaller as the blood concentrations increased. No correlation could be established between the difference in area and the luminosity intensities that were observed. However, there appeared to be a slight correlation between the light intensity that was emitted and the increase in the dilution of the blood solutions that were used, which had already been observed in a prior study by Patel and Hopwood [1]. A decrease in light intensity was observed for more concentrated blood solutions and for whole blood, which would be explained by a quenching effect of the reaction because of an excessive amount of hemoglobin, and thus too much iron, the catalyst for the reaction. This excess of hemoglobin can also be observed by the presence of a halo (Figure 1) on vaporized filter papers for whole blood and for blood dilution of 1:10.

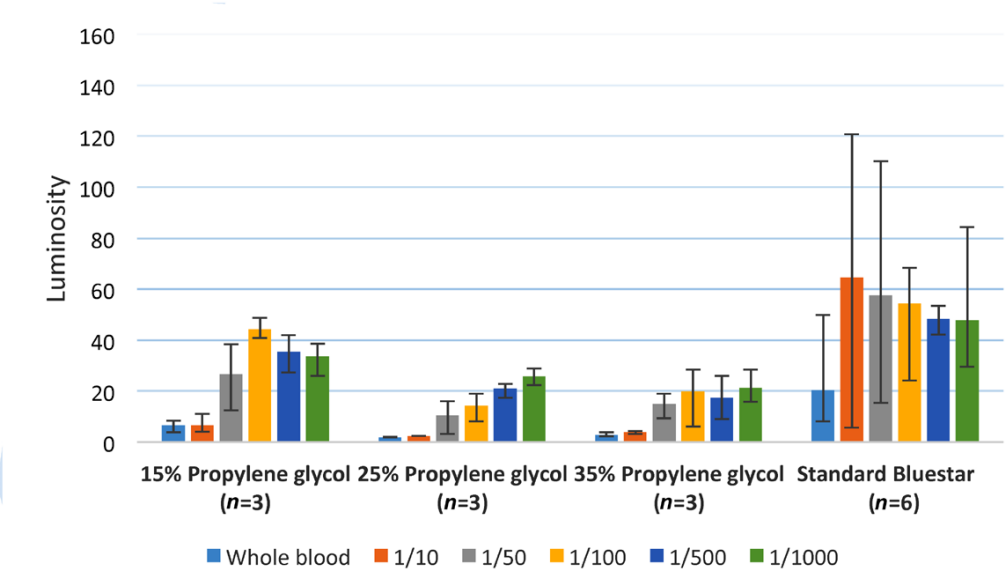
The bars in Graphs 1, 2, and 3 show average luminosity on each blood-diluted spot; the error bars represent the observed maximum and minimum of light intensity for their related spots. The letter *n* represents the number of filter papers tested for each solution. Numeric values were generated by Photoshop and represent a light intensity value. Thus, a value of 255 would represent white; 0 would represent black.

Water and Propylene Glycol-Based Formulations

Because propylene glycol is less toxic than ethylene glycol [14], it seemed appropriate to start with this product. The results of the luminescence intensities at 4 °C, depending on the blood dilutions, are presented in Graph 1.

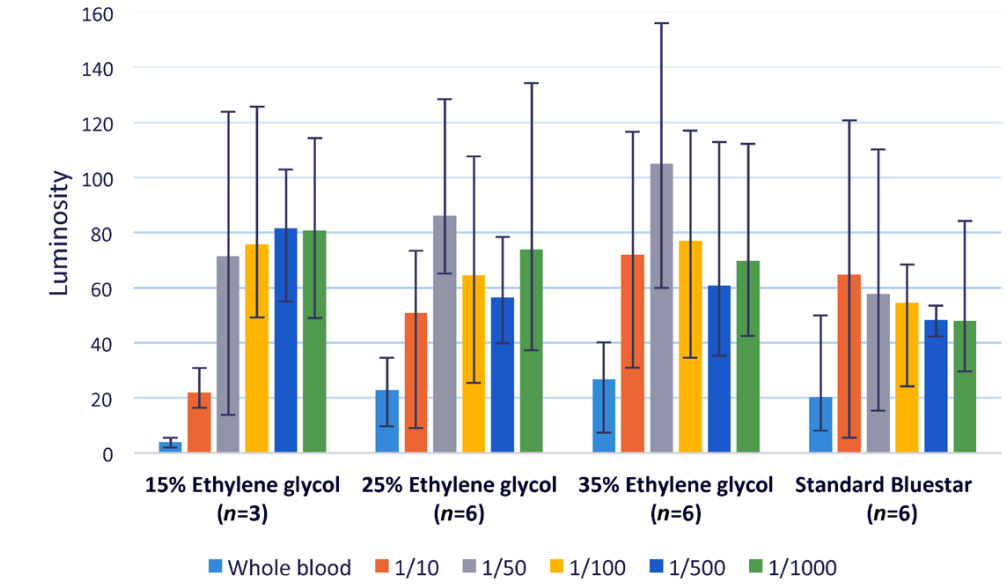
When working with the 25% solution of propylene glycol, it was impossible to observe any luminosity for blood dilution of 1:10 and for whole blood in two of the three trials. Thus, the single available measure of luminosity was used at the next step of comparisons. Also, as seen in Graph 1, both the luminosity averages and the maxima intensities that were obtained following the use of propylene glycol solutions are significantly lower than those obtained following the use of standard Bluestar at 4 °C.

Following those results, formulations based on different percentages of ethylene glycol were evaluated. The results of the luminosity intensities at 4 °C produced are shown in Graph 2.



Graph 1

Bluestar formulations at different concentrations prepared from a mixture of water and propylene glycol compared to the standard Bluestar evaluated in a 4 °C environment.



Graph 2

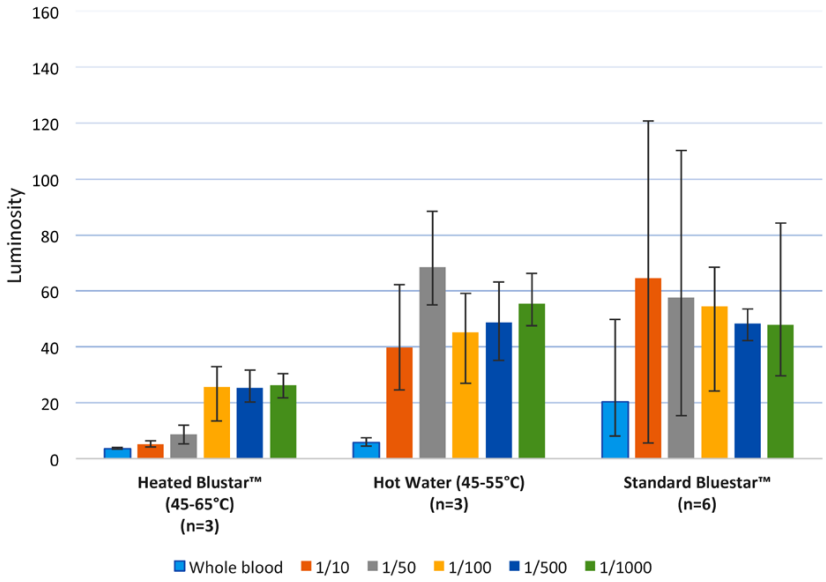
Bluestar formulations at different concentrations prepared from a mixture of water and ethylene glycol compared to the standard Bluestar evaluated in a 4 °C environment.

The various solutions were each tested on three filter papers. Additionally, six filter papers were sprayed with a 25% ethylene glycol solution, a 35% ethylene glycol solution, and the standard Bluestar solution (Graph 2). These additional filter papers (six instead of three) were tested because it seemed surprising to obtain better results with the ethylene glycol solutions than with the standard Bluestar. However, these further tests simply confirmed the previous results: the average luminosity intensities were slightly higher for ethylene glycol formulations than for the standard Bluestar evaluated at a temperature of 4 °C, as well as the maximums and minimums of luminosity intensity.

Because propylene glycol and ethylene glycol have toxic properties, it was appropriate to evaluate the standard Bluestar solution after being heated or prepared with hot water, without any toxic reagent added to the standard solution. The luminosity intensity results at 4 °C for these formulations are shown in Graph 3.

For the heated Bluestar solution, both the averages and the maximums of luminosity intensity were lower than the values for the standard Bluestar. However, for the hot water-based Bluestar formulation, the average luminosity intensity had very similar values to those of the standard Bluestar solution. On the other hand, the maximum values obtained with the solution prepared from hot water were generally lower and the minimum values were higher than those obtained with the standard Bluestar solution.

The two antifreeze optimal operational solutions, consisting of 35% of the chosen diol (glycol) products in a standard Bluestar preparation, were put in a freezer for a week at -20 °C to confirm that they did not freeze. The formulation of Bluestar containing 35% of ethylene glycol did not freeze. The formulation of Bluestar containing 35% of propylene glycol remained liquid, although it showed some signs of freezing, confirming the performed calculations, which foresaw that a solution of 35% propylene glycol would freeze at about -16 °C to -18 °C , whereas a 35% solution of ethylene glycol would freeze at about -20 °C.



Graph 3

Bluestar heated and Bluestar prepared with hot water compared to Bluestar made from room temperature water evaluated in a 4 °C environment.

Effects on DNA Profiling

Because the heated solution of Bluestar and the hot water solution of Bluestar generated lower maximums of luminosity intensities, these formulations were not evaluated for possible DNA degradation. Nevertheless, it should be noted that DNA is not degraded by a standard formulation of Bluestar and that it does not degrade at temperatures below 100 °C [15].

For the other formulations, polyacrylamide gels were made with the previously extracted and amplified DNA of whole blood on filter papers that were sprayed. The results of these polyacrylamide gels developments are shown in Figure 2.

The pattern of bands was the same for all the wells of each polyacrylamide gel, and the corresponding bands had approximately the same respective molecular weights. Such high molecular weights would suggest that the DNA was not degraded. Otherwise, bands with lower molecular weights, due to degraded DNA fragments, therefore smaller lengths, would have been observed [16]. Also, the visible bands on the three polyacrylamide gels were well defined (i.e., there were no trailing marks in the bottom of each band that might suggest that the DNA could have been degraded) [17].

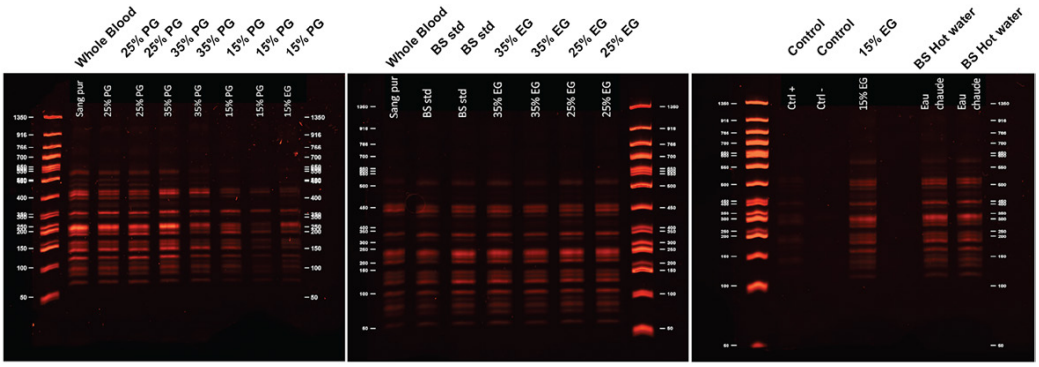
Subsequently, a complete genetic profiling was performed by the LSJML, and all of the profiles that were obtained contained the same alleles for the 16 markers that were studied. In addition, all alleles that were detected had very high relative fluorescent units (RFUs) and therefore were of great intensity. Normally, the intensity of the alleles of a profile where DNA has been degraded is lower for peaks of higher weighted alleles, resulting in a downward-looking profile of allele intensities [16]. Hence, the observations support the hypothesis that DNA was not degraded.

Outdoor Experiments

A standard Bluestar solution froze instantly at the outlet of the nebulizer, forming a layer of ice, which confirmed the impossible use of the manufacturer's formulation of Bluestar at cold temperatures. The use of the hot water Bluestar was not tested because of the assumption that, although the solution is hot in the canister, once it is nebulized, the solution would also freeze instantly at the nozzle.

Formulations containing 15% and 35% ethylene glycol were tested to determine whether they would also freeze at the outlet of the nebulizer when vaporized in cold conditions, jeopardizing the use of standard Bluestar. Solutions were vaporized for 15 seconds, and they did not show any signs of freezing. Both concentrations gave satisfying results in luminosity intensities (Figure 3).

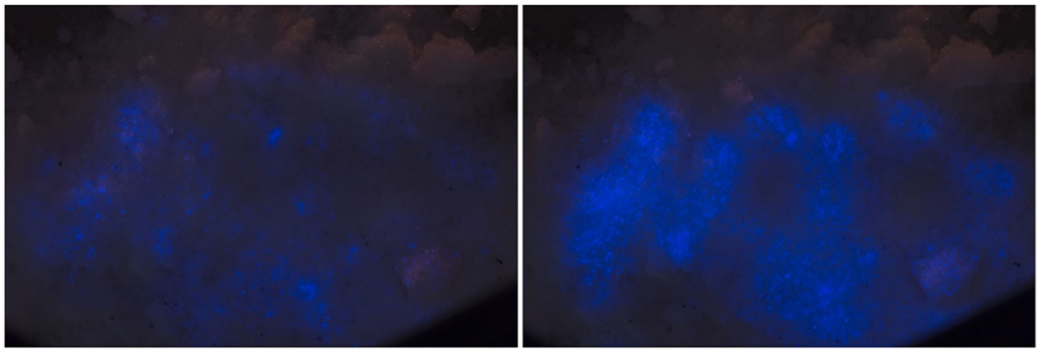
Solutions at the three concentrations of propylene glycol were not tested in the snow because they were considerably less effective than a standard Bluestar solution during the prior 4 °C testing.



BS = Bluestar, PG = Propylene glycol, EG = Ethylene glycol

Figure 2

Polyacrylamide gels obtained using pure whole blood from different filters vaporized with the new Bluestar formulations.



(a) *(b)*

Figure 3

Bluestar solution prepared with (a) 15% and (b) 35% ethylene glycol in snow at a temperature of -6 °C.

Conclusions

This project aimed to find a new solvent composition that could replace the one currently used for the preparation of Bluestar, to allow its use on blood samples down to -20 °C.

An ethylene glycol:Bluestar standard solution (15–35:100) proved to be a viable alternative to the standard Bluestar solution. The ethylene glycol solution showed the same magnitude of luminescence intensity, or even slightly better, than the one obtained with the standard Bluestar solution. In addition, outdoor testing demonstrated the high effectiveness of formulations containing ethylene glycol on blood dilutions in the snow when the ambient temperature dropped to -6 °C.

Heating the Bluestar solution is not recommended because the light intensity that was obtained was significantly lower than what can be achieved by preparing the solution from hot water. However, even though this last option makes it possible to obtain light intensity equivalent to the one obtained with a standard Bluestar solution, it is very likely that water-heated formulations will rapidly freeze at the nebulizer's nozzle when sprayed in a cold environment.

Finally, genetic profiles obtained from blood that had been in contact with the new formulations support the hypothesis that DNA was not degraded.

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For further information, please contact:

Frank Crispino
Université du Québec à Trois-Rivières (UQTR)
Département de Chimie, biochimie et physique,
3351 Bd des Forges, Trois-Rivières (QC) G9A 5H7
frank.crispino@uqtr.ca

References

1. Patel, G.; Hopwood, A. An Evaluation of Luminol Formulations and Their Effect on DNA Profiling. *Int. J. Legal Med*. **2013**, *127* (4), 723–729.
2. Lautz, J.; Webb, S. *A Comparison Study on the New Formula of Bluestar Latent Bloodstain Reagent and its Effects on DNA Typing/Amplification*. Clinical Laboratory Sciences Department, Saint Louis University: Saint Louis, 2011; p 1–18.
3. Dilbeck, L. Use of Bluestar Forensic in Lieu of Luminol at Crime Scenes. *J. For. Ident*. **2006**, *56* (5), 706–720.
4. James, S. H.; Nordby, J. J., Eds. The Use of Luminol Photography for Bloodstain Pattern Analysis. In *Forensic Science: An Introduction to Scientific and Investigative Techniques,* 2nd ed.; CRC Press: Boca Raton, FL, 2005; pp 206–207.
5. Blum, L. J.; Esperança, P.; Rocquefelte, S. A New High-Performance Reagent and Procedure for Latent Bloodstain Detection Based on Luminol Chemiluminescence. *Canadian Soc. For. Sci. J*. **2006**, *39* (3), 81–99.
6. de Almeida, J.; Glesse, N.; Bonorino, C. Effect of Presumptive Tests Reagents on Human Blood Confirmatory Tests and DNA Analysis Using Real Time Polymerase Chain Reaction. *For. Sci. Int*. **2011**, *206* (1–3), 58–61.
7. Gardner, E. T. Ability of Bluestar Forensics and Luminol to Reveal Latent Bloodstains Exposed to Extreme Outdoor Conditions. *J. For. Ident*. **2017**, *67* (4), 581–599.
8. Miles, H. F. Bloodstain Pattern Analysis: Developing Quantitative Methods of Crime Scene Reconstruction Through the Interpretation and Analysis of Environmentally Altered Bloodstains. Doctoral Thesis, University College London: London, U.K., 2014.
9. Ballantyne, K. N.; Poy, A. L.; van Oorschot, R. A. H. Environmental DNA Monitoring: Beware of the Transition to More Sensitive Typing Methodologies. *Australian J. For. Sci*. **2013**, *45* (3), 323–340.
10. Poy, A.; van Oorschot, R. A. H. Beware: Gloves and Equipment Used During the Examination of Exhibits Are Potential Vectors for Transfer of DNA-Containing Material. *International Congress Series* **2006**, *1288* (0), 556–558.
11. Taylor, D.; Abarno, D.; Rowe, E.; Rask-Nielsen, L. Observations of DNA Transfer Within an Operational Forensic Biology Laboratory. *For. Sci. Int.: Genetics*, **2016**, *23*, 33–49.

12. El-Bassouny, D. R. The Validation of Photoshop-Based Quantitative Analysis of Digital Electron Micrographs of Developing Erythrons. *Egyptian J. Histology* **2013**, *36* (4), 940–951.
13. Adobe Photoshop Manual (French). Affichage d'histogrammes et de valeurs des pixels (Display histograms and pixel values). Adobe Inc. 2013, San Jose, CA., pp 373–377.
14. New Hampshire Department of Environmental Services. Environmental Fact Sheet. *Ethylene Glycol and Propylene Glycol: Health Information Summary*; ARD-EHP-12; State of New Hampshire: N.H., 2006.
15. Karni, M.; Zidon, D.; Polak, P.; Zalevsky, Z.; Shefi, O. Thermal Degradation of DNA. *DNA & Cell Biol.* **2013**, *32* (6), 298–301.
16. Clayton, T. M.; Whitaker, J. P.; Sparkes, R.; Gill, P. Analysis and Interpretation of Mixed Forensic Stains Using DNA STR Profiling. *For. Sci. Int.* **1998**, *91* (1), 55–70.
17. Lahiri, D. K.; Schnabel, B. DNA Isolation by a Rapid Method from Human Blood Samples: Effects of $MgCl_2$, EDTA, Storage Time, and Temperature on DNA Yield and Quality. *Biochem. Genet.* **1993**, *31* (7–8), 321–328.