Int J Legal Med (2013) 127:723–729
DOI 10.1007/s00414-012-0800-9

ORIGINAL ARTICLE

An evaluation of luminol formulations and their effect on DNA profiling

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Received: 3 July 2012 / Accepted: 22 November 2012 / Published online: 21 December 2012

Abstract Luminol is a presumptive test reagent used for the location of latent bloodstains. Various formulations are used by different forensic practitioners and commercial products are also widely available. There is little concurrence between authors with regards to the sensitivity limits of luminol which can vary significantly depending upon the substrate. We evaluated the sensitivity and stability of five different luminol formulations on a range of blood dilutions. All formulations showed an overall decrease in performance over 24 h though the effect was more gradual on a non-porous surface compared to porous. We found that BlueStar® Magnum showed the greatest sensitivity compared to other formulations and detected 50 μl of 1/100,000 blood dilutions on both porous and non-porous surfaces. Two formulations of luminol were selected based on the result of the sensitivity and stability study and were assessed for their impact on the DNA profiling process. There was a statistically significant improvement in DNA profile peak area from luminol-treated samples when compared to control samples of neat blood stains. However, at the weaker blood dilution of 1/1,000, the difference between control and luminol-treated samples was dependent on the substrate type with porous (fabric) samples showing a significant difference and non-porous (tile) swabbed samples requiring further work to conclusively ascertain the effect.

Keywords Luminol · BlueStar® Magnum · BlueStar® · Lumiscene · Blood · Forensic · DNA profiling

Introduction

The chemiluminescent property of luminol has been employed in forensic science for over 40 years as a presumptive test reagent to detect or enhance small, diluted latent bloodstains which are often invisible to the naked eye. It can be used to detect smears and wipe patterns from clean-up actions and patterns on clothing, and is particularly useful for searching large areas [1]. It is widely used in many countries for the detection of traces of blood, although its use in the UK and some other countries has been limited due to health and safety concerns [2]. It is clear from the current literature that luminol is an extremely sensitive tool and could provide valuable evidence in many cases.

The light-emitting pathway of luminol is complex and not entirely elucidated. The reaction is catalysed by an oxidising agent in the presence of a catalyst in an alkaline solution. The iron within the haem group of haemoglobin acts as the catalyst for the chemiluminescent oxidation of luminol in the presence of an oxidant such as hydrogen peroxide [1, 3].

The two most commonly used luminol formulations were developed by Grodsky in 1951 (occasionally referred to as Luminol I) and Weber in 1961 (occasionally referred to as Luminol II) [3]. The Grodsky formulation uses sodium carbonate as the base and sodium perborate as the oxidising agent, whereas the Weber formulation uses sodium hydroxide as the base and hydrogen peroxide as the oxidising agent [3, 4]. More recently, commercial luminol products have become available, namely BlueStar® and BlueStar® Magnum (Blue Star Forensic, Roc Import, Monte Carlo, Monaco) and Lumiscene (Loci Forensic Products, Amsterdam, Holland) which offer the advantage of being easier to prepare.

There is little concurrence between authors with regards to the sensitivity limits of luminol which can vary significantly

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depending on the substrate type. The published sensitivity of luminol varies from 1/200 [5] to 1/10,000,000 [6] blood dilution. Absorbent materials are more likely to retain significant amounts of blood in relatively undegraded form due to rapid drying in sheltered or covered environments, whilst non-absorbent surfaces such as glass and tile do not effectively retain blood, and the DNA is more prone to degradation [3]. The variation in sensitivity is likely due to the differences in reagent concentrations, differences in sample preparation and the substrate upon which the blood is deposited and differences in the recording of results (qualitatively rather than quantitatively).

There are a wide range of environmental, domestic and industrial substances which are able to affect luminol blood-induced chemiluminescence. However luminol does not give a positive reaction to other body fluids such as perspiration, saliva, semen and urine [7]. Similar to other presumptive tests, luminol is sensitive to substances displaying peroxidase activity [1]. The most common substances tested giving false positive results are plant peroxidases and iron or copper compounds such as cupric sulphate and ferric sulphate [8, 9]. The interference that has been most investigated is caused by sodium hypochlorite, a common additive to household and industrial detergents/bleaches [3]. This causes additional complications when attempts have been made to clean up scenes to remove blood evidence. Hypochlorite-induced luminescence displays an intense and short-lived reaction, similar to a bright spark or flash. Consequently, experienced scene examiners are able to differentiate between the reactions from hypochlorite interference and blood [1, 5, 9].

It is generally recommended that sampling for short tandem repeat analysis should be carried out before luminol testing [10]. However, this will not always be possible when invisible stains are located using luminol. Various studies have shown that luminol and BlueStar do not have a significant effect on DNA profiling [4, 11–15]. Here we compared the newest commercially available formulations with original formulations and examined their effect on DNA profiling methods in order to assess the suitability of the use of luminol in laboratory procedures for search and recovery of latent bloodstains.

Materials and methods

Formulation assessment

Five luminol preparations were compared in this study: BlueStar and BlueStar Magnum (BSM); Lumiscene, a formula developed by Weber; and a formulation provided by a police department in Holland which will be referred to as the Dutch formulation.

Sample preparation

Blood samples were obtained with informed consent from two donors and were serially diluted in sterile distilled water (SDW) to give 1/10, 1/100, 1/1,000, 1/10,000, 1/100,000, and 1/1,000,000 dilutions. Neat bloodstains of 1 and 50 μl of each dilution were tested in duplicate, per donor for each formulation, on a porous (dark cotton fabric) and non-porous surface (ceramic tile). Replicates of this sample set were tested in order to examine the stability of formulations at 4, 6 and 24 h after solution preparation. All stains were allowed to dry at room temperature for a minimum of 12 h before testing by spraying with a luminol solution using the Eco-Spray atomiser (BlueStar Forensic, Roc Import, Monte Carlo, Monaco).

Formulation preparation

BlueStar, BSM and Lumiscene were prepared according to manufacturers' instructions. Weber formulation was prepared by mixing 10 ml of three solutions A, B and C into 100 ml of SDW. Stock solution A consisted of 6 g sodium hydroxide in 100 ml SDW; B solution consisted of 5 ml 30 % hydrogen peroxide in 245 ml of SDW; C solution consisted of 177 mg luminol in 31.25 ml of stock solution A and made up to 250 ml with SDW. The Dutch formulation consisted of 0.36 g sodium hydroxide for consistency, 0.2 g luminol and 1 ml 30 % hydrogen peroxide in 200 ml SDW.

Scoring results

The chemiluminescence reactions were scored in terms of their intensity with 0=no reaction; 1=weak/faint; 2=strong/intense; 3=very strong/very intense. To ensure consistency in the amount of solution sprayed, all stains were sprayed once from approximately the same distance. Approximately 7 min was allowed for dark adaptation (of eyes) before each testing session was conducted. Two operators observed the reactions and in case of any discordance, the lower score was recorded.

DNA profiling

Fifty microlitres of neat and diluted (1/1,000, 1/10,000 and 1/100,000) bloodstains were tested in duplicate, per donor for each formulation, on porous and non-porous surfaces generating a total of four samples per formulation and substrate type.

All stains were allowed to dry at room temperature for minimum of 12 h before 20 μl of luminol solution or SDW (control) was applied. BSM and the Dutch formulation

were selected for this assessment based on the results of the sensitivity and stability testing.

Stains on fabric were excised (approximately 1 cm^2), and the stains on tiles were swabbed using wet and dry mini-pointed swabs (Tetra Scene of Crime®) which were combined for extraction.

Samples were extracted on an EZ1® Biorobot (Qiagen, Crawley, UK) and quantified using Quantifiler® Duo (Applied Biosystems, Warrington, UK) using internally validated protocols in accordance with the manufacturer's recommendations, except that the reaction volume was halved. The samples were amplified using the AMPFlSTR® SGM Plus™ (Applied Biosystems, Warrington, UK) PCR amplification kit at 28 and 34 cycles using a Tetrad PTC-225 (MJ Research) thermo cycler. Samples with DNA concentration below 0.04 ng/μl were amplified at 34 cycles. Those samples with a higher concentration were amplified at 28 cycles using 3 ng of template DNA in 25 μl PCR reactions. PCR fragments were resolved on 3130xl genetic analyser (Applied Biosystems, Warrington, UK) and analysed using GeneMapper® software v3.2 (Applied Biosystems, Warrington, UK).

Results

Sensitivity and stability of formulations

Sensitivity

On both substrate types tested, the intensity of the reaction increased as blood became more dilute peaking at 1/100 to 1/1,000 dilution before reducing again. This was true for all formulations though the level of intensity varied between formulations.

On fabric, BSM gave the strongest results in terms of intensity with a majority of stains receiving a score of 2 or 1. The formula giving the weakest reaction was Weber, where a majority of stains scored either 1 or 0. Only Lumiscene gave a weak reaction with 1/1,000,000 dilution though only with one out of four replicates (Fig. 1).

The results from tile were similar to those found on fabric. The most intense reactions were from stains of 1/100 to 1/1,000 dilutions. BSM reacted the most intensely with the majority of reactions scoring 2 or 3. The poorest formulation was Weber which consistently scored 1 for a majority of dilutions with occasional samples scoring 2 or 0 (Fig. 2). BlueStar, BSM and the Dutch formulation also produced strong reactions with several samples scoring 3.

Overall stains on tile generated higher scores than on fabric (data not shown) with BlueStar and Dutch formulations giving the highest intensity scores for stains on tile alone, whereas BSM produced the most intense chemiluminescence on both substrate types.

Stability

It was important to determine the stability of the solution for the intended purpose of using in a laboratory rather than the scene of a crime. There was an overall reduction in performance over 24 h, although the decrease was inconsistent (data not shown); specifically, BSM, Weber and Dutch formulations performed slightly better at 6 h than at 4 h for stains on fabric. On tile, the decline in performance was to a lesser extent than that observed on fabric with BSM giving a very strong reaction for several samples even after 24 h from preparation.

Optimal results were achieved between 0–4 h after preparation for a majority of sample types and formulations. The decline in performance was more gradual on tile than fabric, where there was a smaller difference in the number of samples reacting at 0 h and after 24 h.

Effects on DNA profiling

As expected, all of the neat samples gave sufficient quantity of DNA to allow 28-cycle amplification. The remainder of samples were processed using 34-cycle methodology. The control swab samples yielded the highest DNA concentration, an average of 18.03 ng/μl, whilst BSM fabric samples gave an average of 2.22 ng/μl. At dilutions of 1/10,000 and 1/100,000, only a few samples across the different sample sets provided a concentration value. A greater number of samples from 1/1,000 dilution yielded a quantitation value; the highest average of 0.005 ng/μl was obtained from the Dutch fabric samples and the lowest of 0 ng/μl from Dutch tile samples. The neat sample set and the most dilute sample set were further analysed in order to test the extremes.

Bloodstains at lower dilutions of 1/10,000 and 1/100,000 were initially tested but provided very partial profiles for luminol treated and control samples; therefore, they were not evaluated further in this study.

Peak areas of all DNA samples were statistically evaluated using Mann–Whitney test, which determines whether there is a significant difference between the medians of the two data groups under test. A comparison was carried out between peak area values obtained at each dilution (Table 1). Neat BSM-treated fabric samples did not show a significant difference when compared to the control; however, Dutch-treated samples at these dilutions showed an increase in peak area compared to control samples, thereby demonstrating a positive effect on DNA profiling. Overall, the results suggest that luminol begins to have an adverse effect on DNA profile peak areas at lower dilutions of blood. At 1/1,000 dilutions, there was a significant difference between

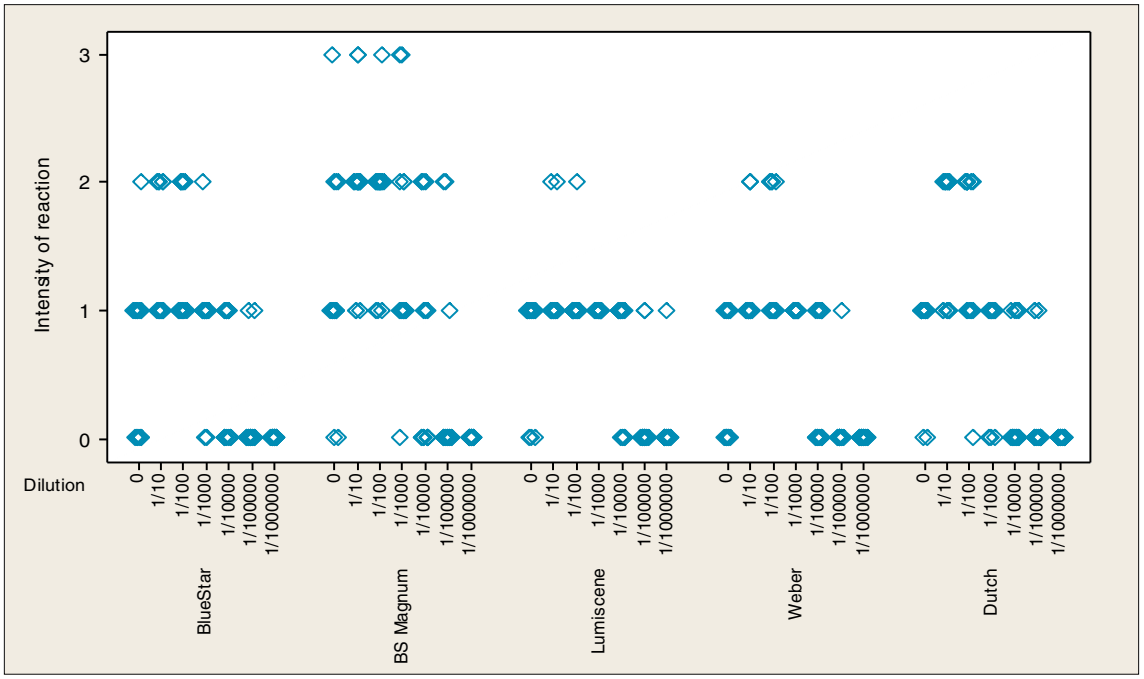

Fig. 1 Comparison of the formulations on fabric at a range of dilutions over 24 h from preparation (*x*-axis: 0=no reaction; 1=weak/faint; 2=strong/intense; 3=very strong/very intense). Darker data points indicate multiple scores for that variable

the values obtained from control samples compared to samples exposed to both luminol treatments.

Statistical analysis of peak area information from tile samples showed that luminol formulations had a positive effect on samples. Both formulations showed an increase in peak areas compared to the control that was statistically significant with neat samples. At the lower dilution of 1/1,000, the Dutch formulation did not show a significant difference compared to the control, whereas it was difficult to determine a clear statistical significance with BSM-treated samples.

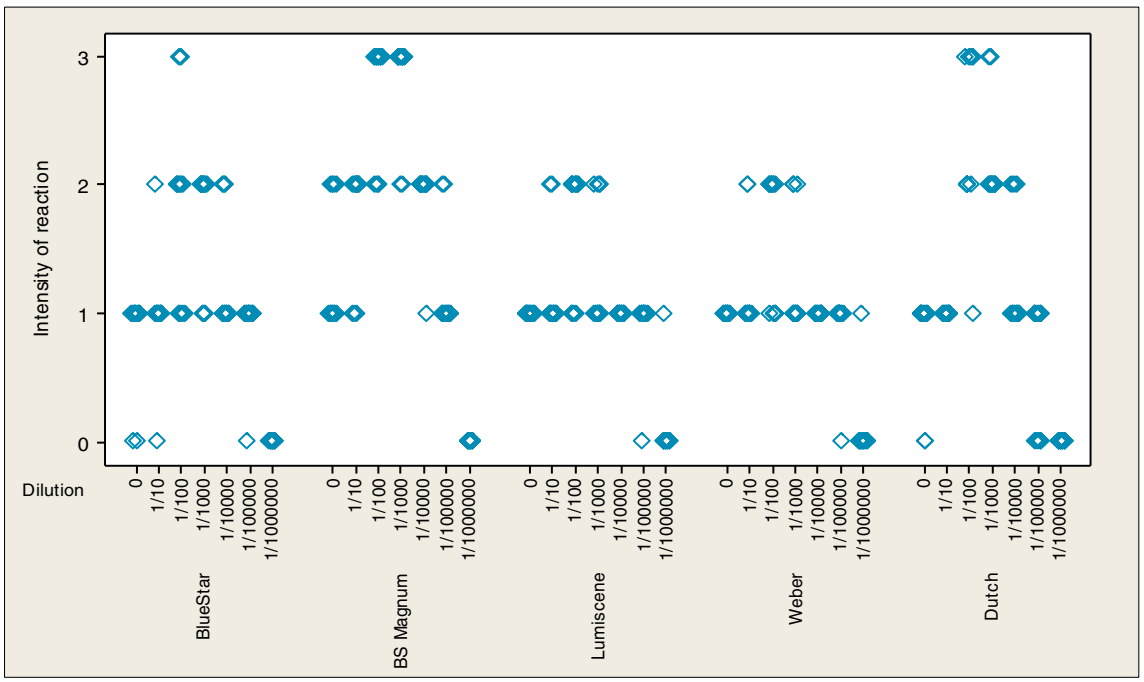

Fig. 2 Comparison of formulations on tile at a range of dilutions over 24 h from preparation (*x*-axis: 0=no reaction; 1=weak/faint; 2=strong/intense; 3=very strong/very intense). Darker data points indicate multiple scores for that variable

Table 1 Results from Mann–Whitney test to determine statistical significance between total peak areas obtained from luminol-treated samples versus controls on fabric and tile substrates

Dilution	BSM vs. control fabric Fabric	Dutch vs. control fabric Fabric	BSM vs. control tile Tile	Dutch vs. control tile Tile
0 (neat)	Not significant	Significant (Dutch)	Significant (BSM)	Significant (Dutch)
1/1,000	Significant (control)	Significant (control)	Significant (unclear)[a]	Not significant

Stronger sample set is indicated in parentheses

[a] A significant difference is indicated by the confidence interval (CI) enclosing 0 and a p value below 0.05. In this comparison the p value was below 0.05 but the CI did not enclose a 0 value

Figure 3a, b shows the total peak area from stains on fabric and swabs from tile. A comparison of undiluted samples shows that luminol-treated samples produced higher peak areas than the control samples. There was a marked difference between control and treated samples swabbed from tiles whereas with fabric samples, this difference was less defined. At a dilution of 1/1,000, there was a notable difference in the peak areas obtained from the two substrate types. The control samples produced significantly higher peak areas than both luminol-treated samples on fabric. Samples from tiles do not show a substantial difference between treated and control samples.

Discussion

The observation of reduced luminescence from the more concentrated samples could be due to a quenching effect of excess haemoglobin in these samples. This was also observed in an unpublished internal study in which a halo effect was observed with 1/10 dilution samples; the luminescence was observed as a ring around the edge of the samples where the bloodstain became thinner. As the iron within the haem group of haemoglobin acts as a catalyst for the chemiluminescent oxidation of luminol in the presence of an oxidant [1, 2], the more dilute samples, which showed the most intense reactions, such as 1/100 and 1/1,000 may have contained the optimal levels of haemoglobin for the reaction to occur. Moreover, with further diluted stains, the amount of catalyst required for the reaction decreases, resulting in a reduced intensity reaction.

The finding that luminol formulations were more sensitive with samples on a non-porous substrate could be a result of porous surfaces being able to absorb blood deeper into the substrate, whereas non-porous substrates will retain the blood on the surface, making all of the haemoglobin

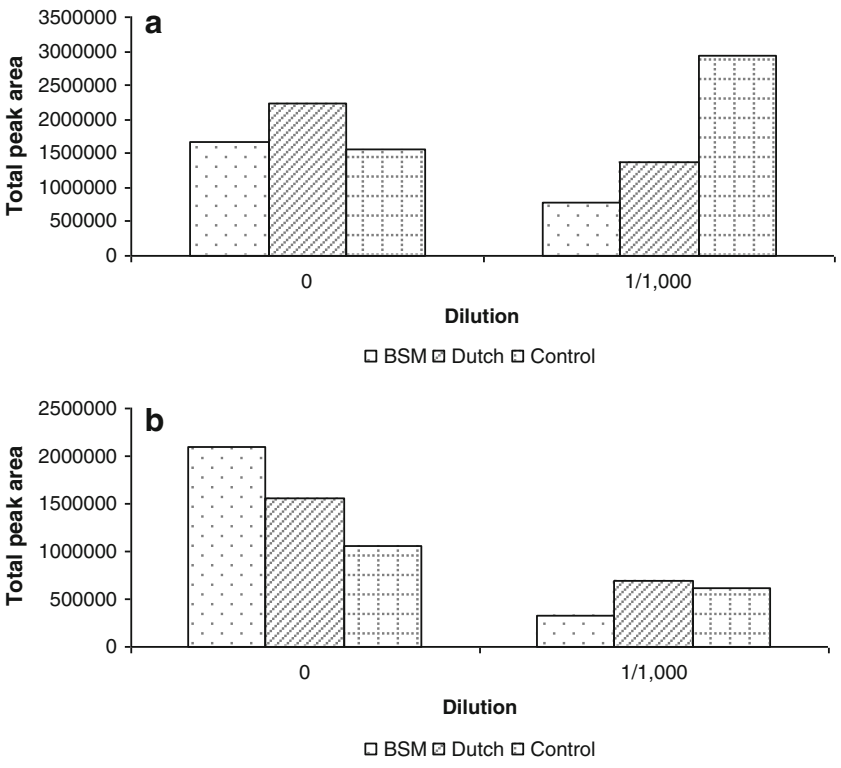

Fig. 3 **a** Comparison of total peak areas for luminol-treated and control samples from stains on fabric. **b** Comparison of total peak areas for luminol treated and control samples from stains on tiles

available to react. The study by Budowle et al. showed a thousand-fold increase in sensitivity with non-absorbent surfaces when compared to absorbent surfaces [11] similar to the findings from this study. This observation could be further investigated by examining different types of surfaces such as fabrics with deeper weaves (e.g. woollen), denim, leather and carpet.

The qualitative scoring of the reaction is subjective and prone to some variation due to the visual nature of the method. The variation in the intensity of the reaction at different time points could be due to observational differences. It was important for this study to develop a method that could be used to differentiate between formulations; however, in practise luminol is likely to be used as a simple positive or negative test since an inference cannot be made from the intensity of the reaction.

Our findings showed that luminol samples exhibited a positive effect on DNA profiling in that greater peak areas were obtained with treated samples than control. Quinones et al. reported similar findings where a formulation similar to the Dutch formulation produced higher percentage profiles than control [4]. This is an interesting observation that could be investigated further.

The commercial products (BlueStar, BSM and Lumiscene) are supplied in tablet form which are dissolved in water or solution provided with the kit. These products were noted to be easier to use than Weber and Dutch formulations which involved mixing several chemicals to make the test solution.

Luminol is primarily used as a presumptive test at scenes of crime. In these scenarios, its cross-reactivity has been widely studied, particularly for those substances with peroxidase activity [3, 7–9]. However, this study was carried out in order to investigate its use in a laboratory environment for the search and recovery of latent bloodstains on items. It would be useful to investigate the effect of general background luminescence on worn items and study the effect these potentially interfering substances may have on the visualisation of true reactions. This could be combined with varying the size of bloodstains, creating blood spatter for example, to assess whether true reactions are distinguishable from false positives or general background luminescence.

Conclusions

Testing of various luminol formulations has shown that BSM produced the most intense chemiluminescence across a range of dilutions on tile and fabric. Stains on tile showed an increase in luminescence when compared to fabric. All of the formulations detected blood stains from neat to dilutions of 1/10,000 but peaked in intensity for dilutions between 1/100 and 1/1,000. The sensitivity of luminol for neat and concentrated blood was reduced probably due to an excess of haemoglobin concentrated into a small surface area.

The formulations showed a reduction in performance at 24 h from preparation on diluted stains. Most formulations showed a reaction up to 24 h after the solution was prepared though the intensity reduced over time. In order to achieve optimal results (most intense), the formulations should probably be used within the first 4 h of preparation.

The impact of luminol on DNA profiling was not wholly conclusive based on these results. At stronger concentrations of blood, neither of the formulation tested showed a significantly negative effect on DNA, but did show an increase in peak area compared to the control which was statistically significant. At a weaker dilution of blood (1/1,000), the effect of luminol seemed to become detrimental to the DNA profiling process. In order to prevent biasing, any samples showing poor morphology were not repeated; therefore, an improvement in the profiles could be obtained if optimisation was carried out.

As was apparent in this study, the appearance of the luminol reaction will depend on several variables such as background colour, substrate and presence of inhibiting substances; therefore, the differences noted in this study may be less apparent under true casework conditions [4].

Acknowledgments The authors wish to thank Andrea Grout and Robert Greener for their invaluable contribution to this study.

References

1. Siegel JA, Saukko PJ, Knupfer GC (2000) Encyclopedia of forensic sciences, vol 3. Academic Press, Waltham, pp 1323–1325
2. Larkin T, Gannicliffe C (2008) Illuminating the health and safety of luminol. Sci Justice 48(2):71–75
3. Barni F et al (2007) Forensic application of the luminol reaction as a presumptive test for latent blood detection. Talanta 72(3):896–913
4. Quinones I, Sheppard D, Harbison S, Elliot D (2007) Comparative analysis of luminol formulations. J Can Soc Forensic Sci 40(2):53–63
5. Fregeau CJ, Germain O, Fourney RM (2000) Fingerprint enhancement revisited and the effects of blood enhancement chemicals on subsequent profiler plus fluorescent short tandem repeat DNA analysis of fresh and aged bloody fingerprints. J For Sci 45 (2):354–380
6. Garofano L et al (2006) A comparative study of the sensitivity and specificity of luminol and fluorescein on diluted and aged bloodstains and subsequent STRs typing. Int Congr Ser 1288:657–659
7. Lytle LT, Hedgecock DG (1978) Chemiluminescence in the visualization of forensic bloodstains. J For Sci 23(3):550–562
8. Tobe SS, Watson N, Daeid NN (2007) Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA. J For Sci 52(1):102–109

9. Quickenden TI, Creamer JI (2001) A study of common interferences with the forensic luminol test for blood. Luminescence 16:295–298
10. Eversdijk M, Gelderman R (2000) Shedding fresh light at the scene of the crime. Inf Bull Shoeprint/Toolmark Examiners 6(1):11–17
11. Budowle B et al (2000) The presumptive reagent fluorescein for detection of dilute bloodstains and subsequent STR typing of recovered DNA. J For Sci 45(5):1090–1092
12. Barbaro A, Cormaci P, Teatino A (2008) Forensic Science International: Genetics Supplement Series, Prog Forensic Genet 1 (1):11–12
13. Breniaux A (2001) The effect of BlueStar™ blood reagent on DNA typing, Criminal Research Institute of the French Gendarmerie Nationale, French Defence Department, Nantes CHU Validation Study. Available via http://www.bluestar-forensic.com/pdf/en/IRCGN_DNA_Bluestar.pdf. Accessed 15 April 2009
14. Della Manna A, Montpetit S (2000) A novel approach to obtaining reliable PCR results from luminol treated bloodstains. J For Sci 45 (4):886–890
15. Blum LJ, Esperanca P, Rocquefelte S (2006) A new high-performance reagent and procedure for latent bloodstain detection based on luminol chemiluminescence. J Can Soc Forensic Sci 39(3):81–100