

## A NEW HIGH-PERFORMANCE REAGENT AND PROCEDURE FOR LATENT BLOODSTAIN DETECTION BASED ON LUMINOL CHEMILUMINESCENCE.

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### ABSTRACT

A new occult blood revealing agent was designed such that DNA material would be preserved and a DNA profile could be obtained. Indeed, there were uncertainties about previous chemicals that might alter blood DNA content and have adverse effects on DNA typing. BlueStar<sup>®</sup> is a chemiluminescent bloodstain detecting agent resulting from our research. It combines ease-of-use with minimum health risks and does not have any harmful effects on DNA profile determination.

### RÉSUMÉ

Résultat de nos recherches, le BlueStar<sup>®</sup> est un produit chimique de révélation de traces de sang latentes. Alors que l'action de certains produits ayant la même fonction reste incertaine sur l'ADN, celui-ci ne perturbe pas la détermination d'un profil génétique. A cette qualité essentielle s'ajoute une facilité d'utilisation avec des risques minimisés sur la santé de l'utilisateur.

### INTRODUCTION

Bloodstains can be found anywhere a violent crime has occurred. Bloodstain patterns on the floor (from a dripping wound, for example) or spattered on the walls can be interpreted for crime scene reconstruction. DNA typing analysis can establish the genetic profile(s) of the participant(s) in a violent crime. Consequently, bloodstains are among the most useful evidence for court.

This fact is becoming well-known, as criminals now often attempt to clean up the crime scene. A wide variety of chemicals may be used as presumptive tests to detect evidence of these cleanings. Blood detection methods are either based on the detection of hemoglobin and its derivatives (catalytic test) or on the detection of proteins and amino acids (non-covalently bound to proteins). However, the use of some chemicals may have adverse effects on subsequent Short Tandem Repeat (STR) DNA typing and also has been shown to be potential health hazards. For example, benzidine and ortho-tolidine have potential carcinogenic properties (1–2) and leucomalachite green does not allow for DNA typing (3).

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Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) has become the most popular presumptive test for blood in crime scene investigation (4–6). The chemiluminescent properties of luminol were first reported in 1928 by Albrecht (7). In aqueous medium, an oxidation system and an oxidative catalyst are required in addition to alkaline conditions. Transition metal cations, either free or complexed to organic or inorganic ligands, catalyze the luminol chemiluminescence oxidation. This is why heme-containing proteins and hemoglobin are able to catalyze the chemiluminescence of luminol in the presence of an oxidant. To establish a working assay, the choice of reactants (oxidant and alkaline medium) and reaction conditions (pH and reactant concentrations) must be carefully considered.

Two formulations exist, Luminol I described by Grodsky (8) and Luminol II described by Weber (9). Previous studies have shown that this test did not disturb DNA typing (10–12) but its use remained difficult due to the short duration of the chemiluminescence (13–14).

The goal of the present study was to develop a new Luminol formulation, BlueStar<sup>®</sup>, which would be easy-to-use at a crime scene or in a laboratory while affording a brighter and more long-lasting chemiluminescence emission, with no damaging effects on DNA typing, nor any hazard to the crime scene investigator. The first part of the study aimed to optimise the concentrations of the various components. The second part of the study looked at the performance of the newly formulated blood reagent.

## **MATERIALS AND METHODS:**

### **Reagents**

Luminol and hydrogen peroxide were supplied by Sigma-Aldrich (Lyon, France). Potassium carbonate, sodium carbonate, potassium hydroxide, sodium hydroxide and sodium perborate were purchased from Prolabo (Fontenay-sous-Bois, France). Aqueous solutions were prepared with distilled demineralized water.

### **Instrumentation and procedure for chemiluminescence measurements**

- *Light measurements for the optimization of the reaction conditions*

The assays were performed by adding 90  $\mu\text{L}$  of suitable reagents (oxidant, base and luminol) at the appropriate concentrations to 10  $\mu\text{L}$  of blood in the wells of 96 well plates from Nunc (purchased from Fisher Bioblock Scientific, Illkirch, France). The light intensity, expressed in arbitrary units, was measured using a plate reader luminometer (Luminoscope from Labsystems).

- *Light measurements using commercial bloodstain chemiluminescent detection kits*

Micro-drops (0.3  $\mu\text{L}$ ) of undiluted or diluted blood were deposited on white wall-tiles and dried at ambient temperature. For each kit, the chemiluminescent solutions were prepared as recommended by the supplier. The solutions were sprayed from a distance of 50 cm on the wall-tiles which were then immediately introduced in a high-sensitive cooled charge coupled device (CCD) light measurement system (Intelligent Dark Box II, Fuji Film). On the pictures obtained, light intensity was quantified in arbitrary units with a Fuji film image analysis program (Image Gauge 3.12).

- *Performance of the Bluestar<sup>®</sup> bloodstain detection kit and comparison with commercial luminol solution*

In order to estimate the sensitivity of the Bluestar<sup>®</sup> chemiluminescent bloodstain detection kit, different dilutions, ranging from 1:5 to 1:10 000, of blood in isotonic solution

were prepared. Afterwards, 0.3 µl of diluted blood were deposited on white wall-tiles and dried at ambient temperature. After spraying the Bluestar® solution and the commercial luminol solution on the bloodstains, the light intensity was measured. These tiles were introduced in the charge coupled device (CCD) light measurement system and the light emission was monitored for 10 minutes.

## **Instrumentation and procedure for DNA typing**

- *DNA Extraction and Quantification*

All DNA extractions were performed using the organic extraction method (Proteinase K with an organic solvent). All extracted DNA samples were quantified by slot blot analysis (15) using the Quantiblot kit (Applied Biosystems, a division of Perkin Elmer, Branchburg, NJ).

- *STR Amplification and DNA typing*

Polymerase chain reaction (PCR) amplification of template DNA was performed using the Perkin Elmer GeneAmp 9700 system (Applied Biosystems, a division of Perkin Elmer, Branchburg, NJ). Approximately 0.5 ng of DNA was amplified using AmpFISTR® SGM Plus® PCR Amplification Kit (Applied Biosystems). This kit co-amplifies ten short tandem repeats (STR): D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, FGA, plus amelogenin. DNA typing was performed by capillary electrophoresis on the ABI Prism™ Genetic Analyzer (Applied Biosystems) according to manufacturer's recommendations. The system was prerun for approximately 180 seconds (15 000V, 0.1 mA, 4.5 W, 60°C) prior to sample injection. The system was allowed to run for approximately 1500 seconds (15 000 V, 0.1 mA, 4.5 W, 60°C). Allele sizes were analyzed in real time using the local Southern Method by GeneScan® Analysis Software Version 3.7 (Applied Biosystems).

## **Chemistry**

- *Choice of the oxidizing agent*

For bloodstain detection, perborate and hydrogen peroxide are the most frequently used oxidants. However, due to the instability of the perborate solutions, measurements with this oxidant are not reproducible. Consequently all experiments were performed with hydrogen peroxide as the oxidizing agent.

- *Choice of the alkaline solution and pH*

The maximum light intensity can be obtained in a pH range varying from 10.5 to 13, depending on both the catalyst and the oxidizing reagent used (16). Such strong alkaline conditions can be obtained using carbonate, in the form of either sodium or potassium salt, or a strong base such as sodium hydroxide or potassium hydroxide. However, to allow DNA typing on bloodstains detected with a chemiluminescent method, the pH of the sprayed solution must be around 11.5. In order to avoid problems that could occur with an excessively high pH, a value of 11 was chosen and different alkaline solutions were prepared having a pH close to this specified value. The exact pH value was measured in the final reacting solutions containing hydrogen peroxide and luminol in addition to the alkaline compound. The different solutions were sprayed on identical dried bloodstains and the results were compared by measuring the maximum light intensity.

- *Optimization of the luminol and hydrogen peroxide concentrations*

To determine the optimum concentrations of luminol and hydrogen peroxide, solutions containing 25 mM NaOH and varying concentrations of luminol (1–10 mM) and hydrogen peroxide (5–100 mM) were prepared.

## DNA typing procedures

- *BlueStar® Preparation*

BlueStar® tablets (all tablet kit) were diluted in 125 mL of deionised water. The mixture was sprayed with an atomizer at a distance of about 50 cm. Two controls were included to check each preparation: a positive control (a piece of material with three blood deposits at dilution ratios of 1:10, 1:100, 1:1000) and a negative control (a piece of material without blood or other biological products).

- *Micro volumes sampling, detection with BlueStar® and genotyping*

Minute volumes of blood were taken with a pipette tip by capillary action. This technique was used in cases **A**, **B**, **C**:

**A and B**: two identical tests which consisted of three deposits of blood, performed in order to ensure the repeatability of the process.

**C**: Six deposits of blood.

Five other volumes of blood were tested (0.1 µL, 0.5 µL, 1 µL, 2.5 µL and 5 µL) in order to estimate the influence of blood amount on DNA typing (Table 2 and Figures 3 and 4)

- False positive test

Six different tests with varying blood and bleach ratios (Table 3) were repeated three times. These 18 spots were extracted and analysed by DNA typing.

- *Testing on different kinds of support materials*

Various support materials were tested: absorbents (wallpaper, various kinds of paper, wool, cloth for lining, sheet, various woods, fitted carpet, various cardboards), non-absorbents (cork, tube, different kinds of plastic, glass bottle, various plants, leaves (dead), emery paper, a piece of metal with acrylic painting, chisels, rusted metal, gravel), and porous (terracotta ware, earthenware, sugar, shell). Each support material was divided into three areas. On two of the areas, a bloodstain (20 µL) was deposited, while the third area remained empty. Two identical tests, which consisted of three deposits of blood were used to allow for recreation of the testing procedures. BlueStar® was then spread over the three areas, the third one serving as a negative control.

## RESULTS

### Chemistry

- *Choice of the alkaline solution and pH*

As shown in Table 1, the light intensity measured in the presence of 25 mM NaOH was higher than in the presence of other alkaline compounds. More precisely, the intensity of the emitted light according to the nature of the alkaline compound was in the order: NaOH > KOH > Na<sub>2</sub>CO<sub>3</sub> > K<sub>2</sub>CO<sub>3</sub>. These results are in agreement with other measurements performed at different pH values and for different values of the other reactant concentrations.

TABLE 1

Maximum light intensity measured after spraying different alkaline chemiluminescent solutions on dried bloodstains. Each solution contained 5 mM luminol and 50mM hydrogen peroxide

Alkaline compound	K <sub>2</sub> CO <sub>3</sub> 0.47 M	Na <sub>2</sub> CO <sub>3</sub> 0.47 M	KOH 25 mM	NaOH 25 mM
pH measured in the final solution	10.95	10.90	11.10	11.10
Light Intensity (a.u.)	26400	30970	34300	39740

TABLE 2

Optimal STR detection obtained upon genotyping different quantities of bloodstains (variation of blood volume deposited)

Volume	A : 3*	B : 3*	C : 6*	0.1 µL	0.5 µL	1µL	2.5 µL	5 µL
Number of STR detected	10	10	10	10	10	10	10	10
Amelogenin	1	1	1	1	1	1	1	1

\* number of micro bloodstain deposits

As a matter of fact, at a given pH value and whatever the reaction conditions, the light intensity measured in the presence of sodium or potassium hydroxide was higher than in the presence of sodium or potassium carbonate. Moreover, sodium ions, either with hydroxide solutions or with carbonate solutions, seemed to produce a higher light emission intensity than potassium ions in the corresponding solutions (Table 1).

- *Optimization of the luminol and hydrogen peroxide concentrations*

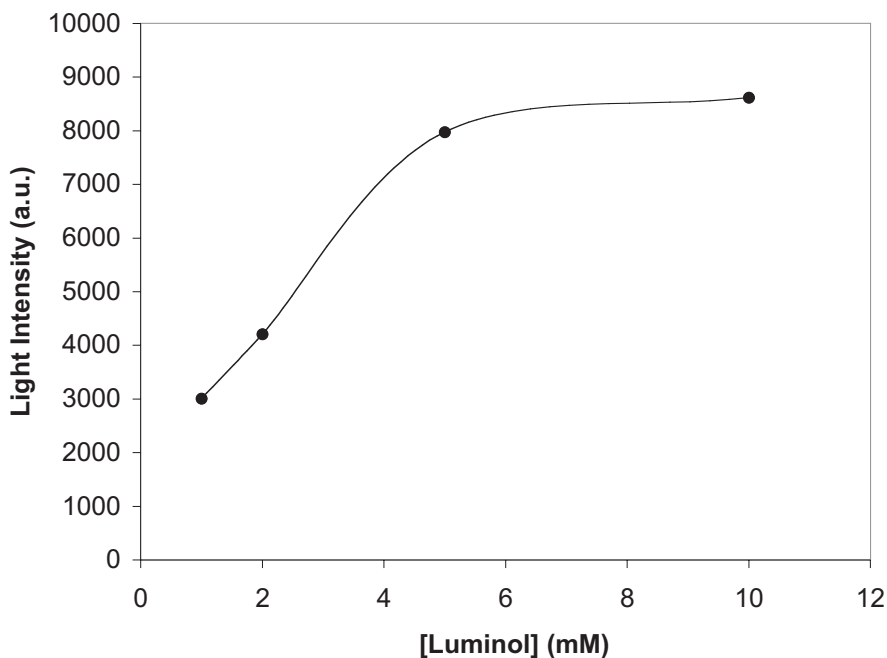
The selected results (Fig. 1–2) show that the optimum light measurement conditions were obtained with a hydrogen peroxide concentration of 50 mM and a luminol concentration of 10 mM. However, for this latter compound, a two-fold increase of the luminol concentration, from 5 mM to 10 mM produced an increase of the light intensity of only 8%. Consequently, a 5 mM luminol concentration was chosen.

Finally the optimum reaction conditions determined for bloodstain detection were a 25 mM NaOH solution containing 5 mM luminol and 50 mM hydrogen peroxide. With the alkaline conditions chosen (pH=11), the preferable reactant final concentrations are : Luminol : 5 mM, NaOH : 25 mM, H<sub>2</sub>O<sub>2</sub> : 50 mM (17).

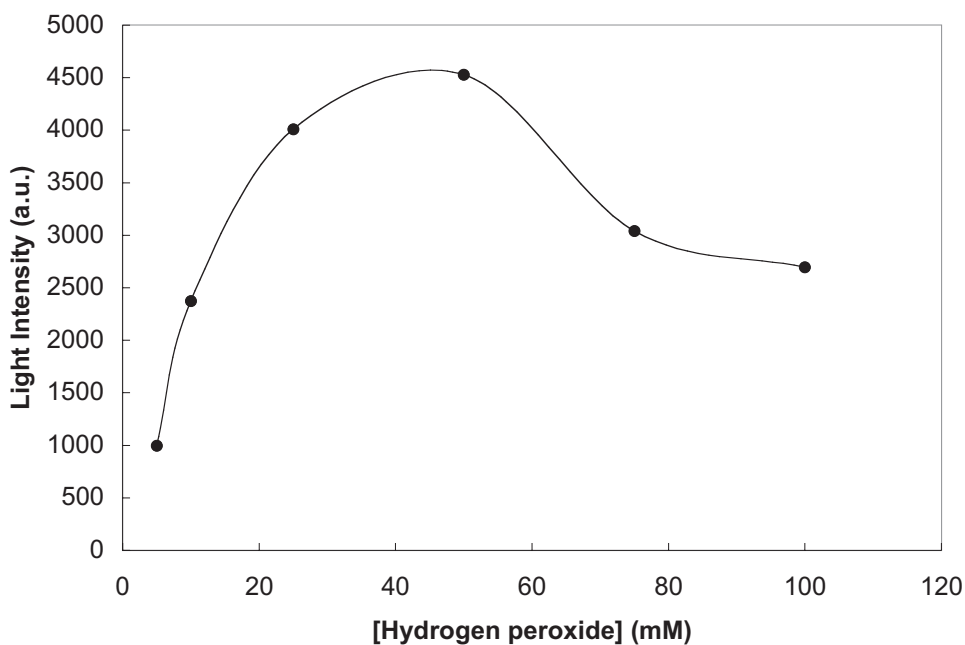
- *Microvolume detection with BlueStar® and genotyping*

In all of these tests, the bloodstains were clearly visualized at the crime scene with BlueStar® and samples could be taken from those detected bloodstains. In addition, it appeared also possible to use BlueStar® in the laboratory so as to detect micro-bloodstains on different kinds of objects.

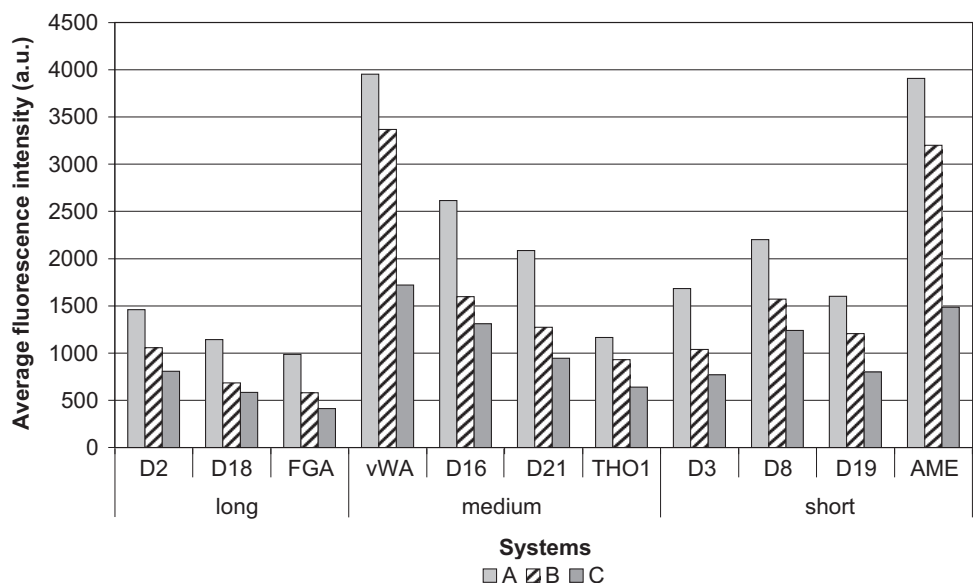
Figures 3 and 4 show the average fluorescence intensity as a function of the 10 Short Tandem Repeats (STR) and amelogenin in the presence of different volumes of blood. Successful STR detection was obtained upon genotyping bloodstains with different quantities of blood (cases A, B & C and with different volumes : 0.1 µL, 0.5 µL, 1 µL, 2.5 µL and 5 µL). This detection allowed DNA typing of 10 STR and amelogenin with satisfactory intensities (Fig. 5).



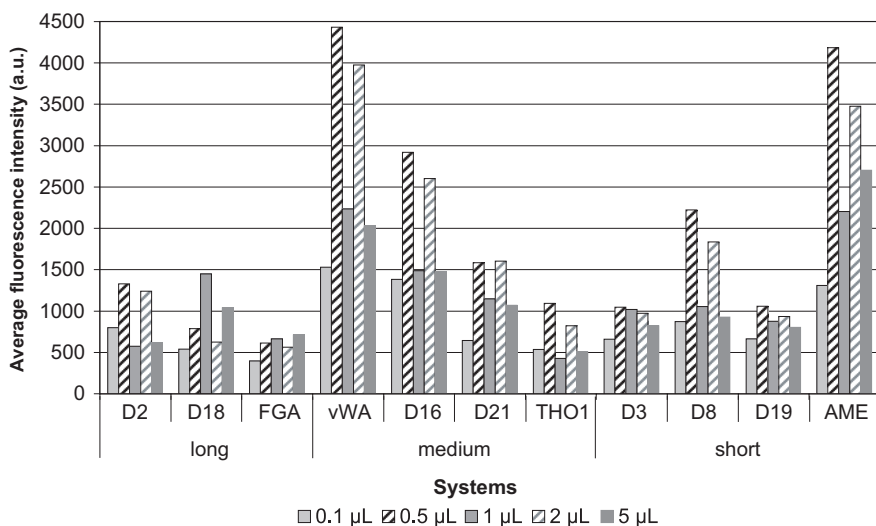
**Figure 1.** Light intensity as a function of luminol concentration in the presence of 50 mM hydrogen peroxide and 25 mM NaOH. Light was measured after spraying the different chemiluminescent solutions on identical dried bloodstains.



**Figure 2.** Variation of the light intensity as a function of the hydrogen peroxide concentration in the sprayed solution. The chemiluminescent solutions containing 25 mM NaOH and 5 mM luminol were sprayed on identical dried bloodstains.



**Figure 3.** Average fluorescence intensity as a function of 10 short tandem repeats (STR) and amelogenin in the presence of different volumes of blood (Table 3).

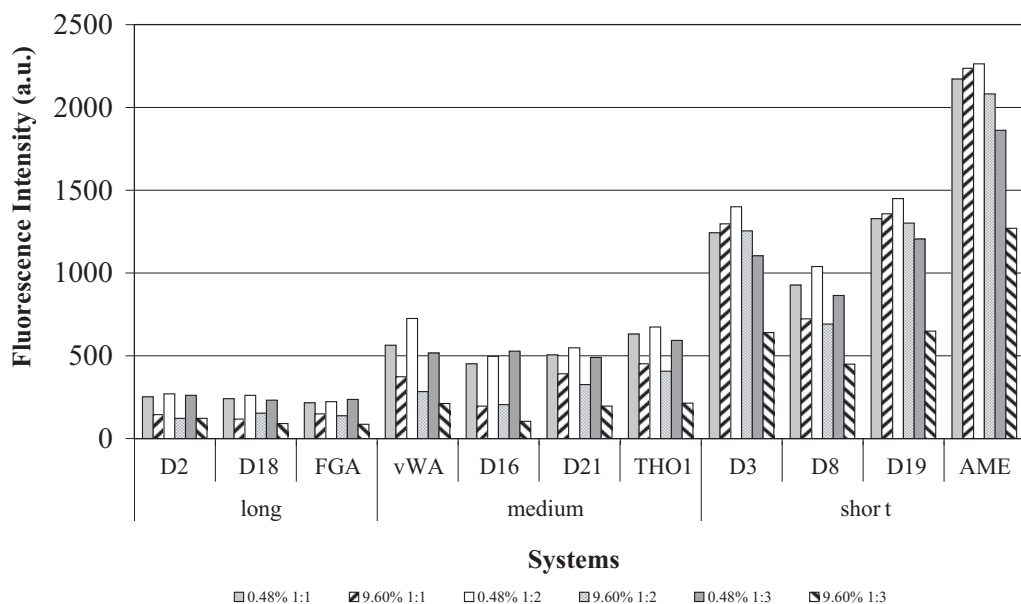


**Figure 4.** Average fluorescence intensity as a function of 10 short tandem repeats (STR) and amelogenin in the presence of different volumes of blood (Table 2).

**TABLE 3**

**Parameters chosen to test the influence of bleach concentration and blood: bleach ratio on bloodstain detection.**

Bleach Type		9.6%			0.48%		
Blood: Bleach Ratio	(1:1)	(1:2)	(1:3)	(1:1)	(1:2)	(1:3)	
Bloodstain Volume (μL)	20	20	20	20	20	20	
Bleach Volume (μL)	20	40	60	20	40	60	



**Figure 5.** Influence of the bleach concentration and blood: bleach ratio on the DNA typing fluorescence detection.

- *False positive test*

Bleach gives a positive reaction with BlueStar®. However, this reaction is visually different from the reaction with a bloodstain. An experienced eye is able to see immediately the difference between a bleach stain (diffused aspect) and a bloodstain (stain clearly defined). Moreover, the bleach dilutes the blood and makes the detection of the latter easier. In 18 cases (Table 3) the bleach with blood produced chemiluminescence with BlueStar®. In all cases, 10 STRs and amelogenin gave a positive result with an intensity for amelogenin averaging 1980 a.u (Fig. 5).

The bleach concentration did not impair DNA typing, and this chemical prevents neither BlueStar® detection nor DNA typing.

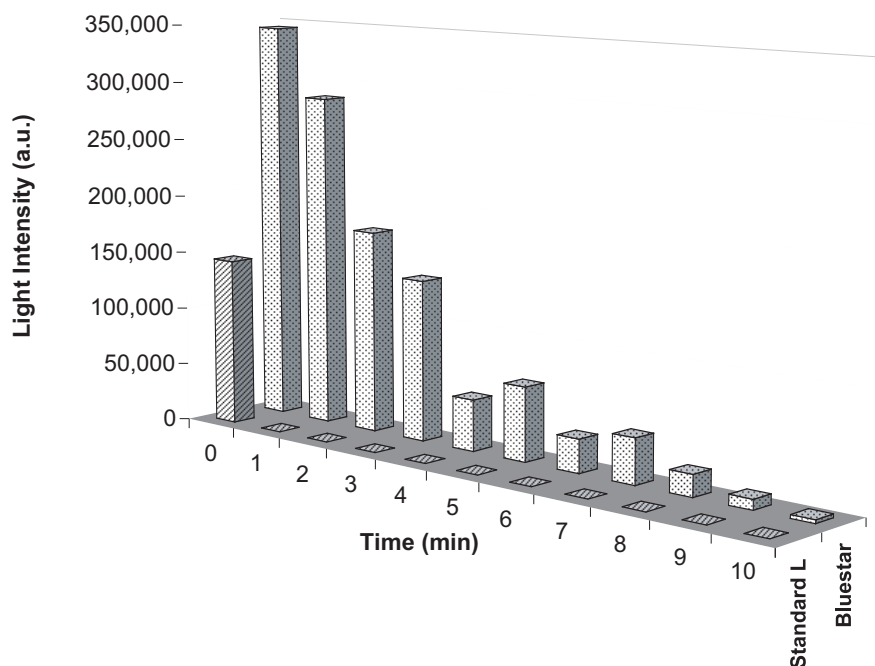
- *Testing on different kinds of support materials*

Bloodstains can be detected with BlueStar® on different materials. However, special attention must be paid with hydrophobic materials because the spraying of BlueStar® leads to blood dispersion. The technician's competence is then crucial to accurately localize the bloodstain.

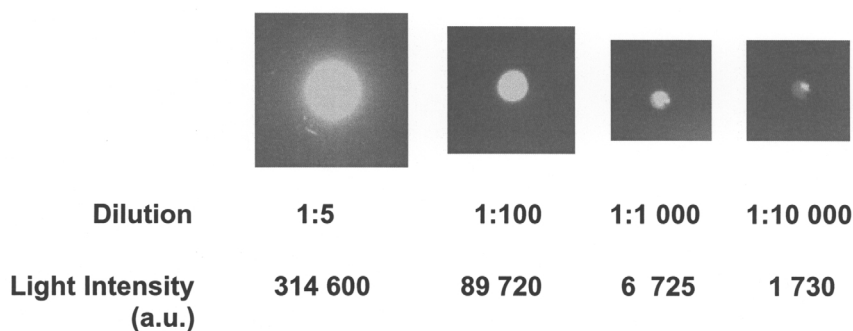
## Comparison with other commercial luminol solution

The light intensities at  $t_0$ , i.e. immediately after spraying, were 344 600 a.u. with the BlueStar® solution and 143 500 a.u. with a standard Grodsky formula-based commercial kit. The results (Fig. 6) show that after 1 minute, the light intensity was close to 0 with the standard commercial kit whereas with the BlueStar® solution, the light intensity was 83% of the initial value measured at  $t_0$ . Moreover, after 7 minutes with the BlueStar® solution, the measured light was still 10% of the initial value and after 10 minutes, even if the luminous signal was only 1% of the initial one, it was still quantifiable (3 100 a.u.).





**Figure 6.** Light intensity as a function of time measured with a Grodsky formula-based standard kit (Standard L) and the Bluestar<sup>®</sup> formula. Both chemiluminescent solutions were sprayed on identical bloodstains.



**Figure 7.** Photographs of the light emission obtained upon spraying the Bluestar<sup>®</sup> chemiluminescent solution on bloodstains of different dilutions.

As expected, the lower the dilution factor, the lower the light intensity (Fig. 7). It is noteworthy to observe that for the highly diluted blood (1:10 000), light emission was still quantifiable (1 730 a.u.).

### Performance of the Bluestar<sup>®</sup> bloodstain detection kit

BlueStar<sup>®</sup> allows detection of diluted bloodstains up to 1:1000 (blood diluted in sterile water). However, DNA typing is possible with dilutions from 1:250 to 1:500. In conclusion, the information given by BlueStar<sup>®</sup> is very promising for the detection of bloodstains.

## CONCLUSION

This new BlueStar<sup>®</sup> luminol mixture enables occult blood detection in washed areas.

Whether or not the substrate is porous and whatever the cleaning agent used, BlueStar<sup>®</sup> gives a positive reaction; the reaction is observed, under dim conditions, in the form of a short yet renewable blue chemiluminescence that is longer and stronger than the Grodsky formula-based commercial kit. Technicians can thus take pictures under optimum conditions (Appendix 1).

False positive reactions may be eliminated after DNA analysis is carried out. There remain some limitations due to the quantity of blood and to the thresholds of each technique. The threshold for detection of DNA is higher than the threshold of this new formula. Therefore, a visual detection does not ensure a successful DNA typing.

In conclusion, this new luminol reagent has been shown to be very efficient in forensic fields to localize washed/dilute bloodstains and is now used routinely in France.

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## APPENDIX 1: BLUESTAR® PROCEDURE FOR BLOODSTAIN DETECTION

BlueStar® has been used in different situations (crime scenes or laboratory) in France and has not interfered with subsequent DNA typing. The procedure is quite simple, but must be followed step by step.

### **Preliminary steps :**

- *Crime scenes*

First and foremost, the area must be darkened, although there is no need to seal off all ambient light. Total darkness, which can be hazardous for the technician moving around the scene, is not necessary.

Proper safety measures must be followed. Face masks should be worn when spraying in a confined area. Sterile officer protection suits are compulsory, not only to protect oneself, but also to prevent contamination due to the technician's DNA.

After making the active solution, the technician should check it using a known sample of blood as a positive control.

- *DNA typing laboratories*

An area dedicated to BlueStar® manipulations is recommended. Proper safety measures must be followed (technician wears gloves, face mask, labcoat, mobcap). Moreover, this area should be cleaned before and after manipulation. First and foremost, the area must be darkened, but not totally.

### **Spraying and sampling :**

- *Crime scenes*

The mixture is sprayed from a distance of about 50 cm. A bluish luminescence indicates a positive reaction. This will fade after about one minute, but the surface can be sprayed again. To serve as a blank or negative control, the technician should apply the solution to an unstained surface.

Tests to confirm the stain as blood must be carried out in the laboratory, as must be the DNA analysis. Sampling at the scene must be carried out as with any biological traces. Using swabs may be inadequate due to the low concentration of blood that may be present at a scene that has been cleaned. It may be preferable to excise or remove the sample. BlueStar® has been used at several crime scenes, and DNA profiles have been successfully obtained.

- *Laboratory*

In the laboratory, the potential bloodstains are often very small and/or washed. Therefore, a uniform and fine spraying has to be done in order to detect such bloodstains. When BlueStar® is applied to the sample, an efficient light emission is observed both in terms of time and intensity. However, some limitations exist when BlueStar® is used on a non-porous material. In such a situation, BlueStar® solution is not absorbed, leading to a dilution of the bloodstain.

Under these conditions, the accurate localization of bloodstains is more difficult. Some extra tools such as a pencil, chalk, or self-adhesive disc are helpful in localizing and sampling the bloodstains.

## Photographic techniques

Specific photographic conditions have been developed and successfully applied for the capture of chemiluminescence at crime scenes (18–19).

Natural diffuse light seems to produce better results than artificial light which sometimes produces pale yellowish or greenish pictures.

The following equipment is recommended: a camera with "B" setting for time exposures, a wide-angle lens (i.e. 24 mm) to cover large areas, a tripod to keep the camera steady during long time exposure, and ISO 400 film. A digital camera with a memory card may also be used.

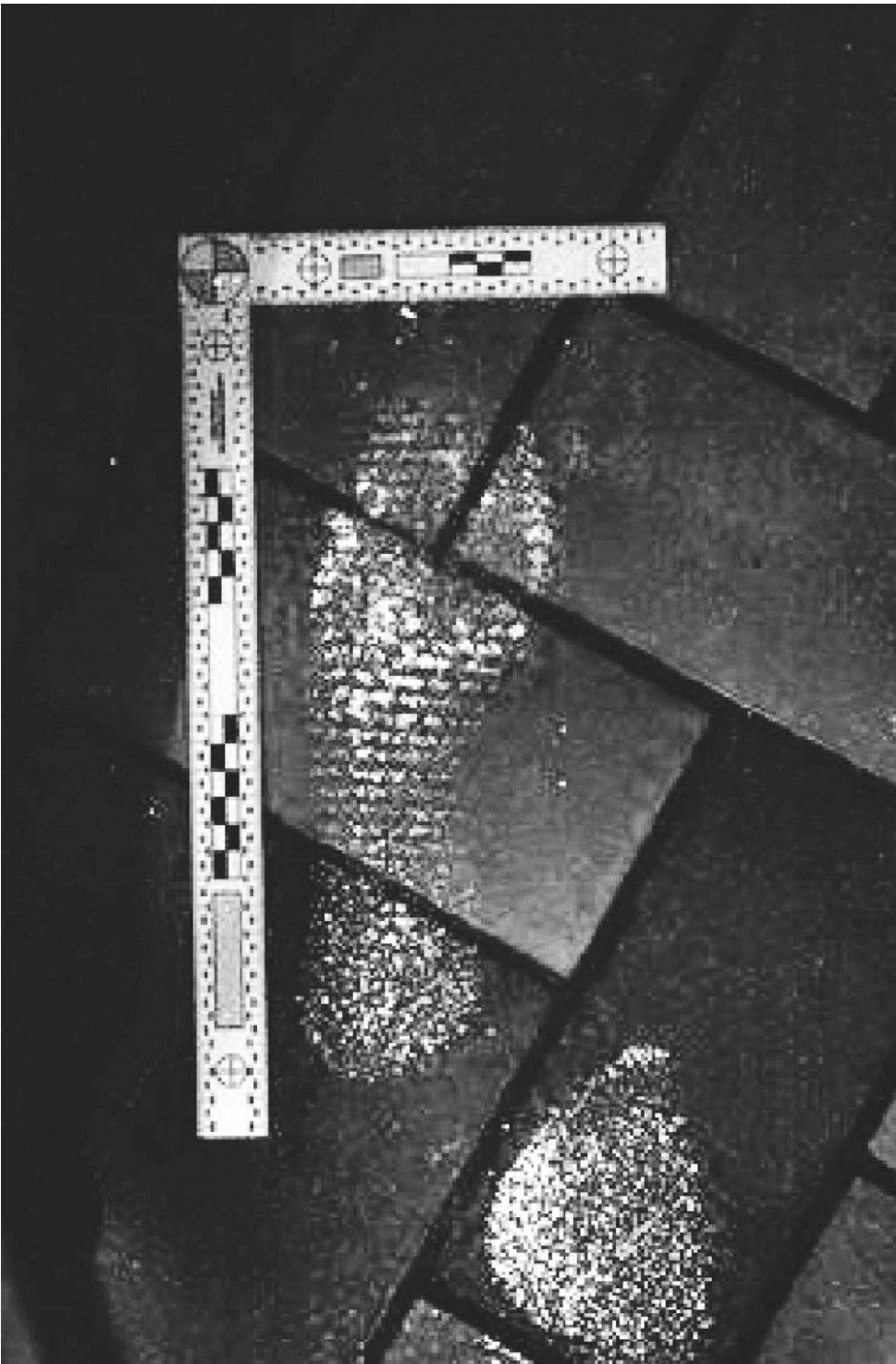
The camera is set up on the tripod and is adjusted to be perpendicular to the surface being photographed, to keep depth of field considerations to a minimum. Spraying with the camera in place and ready to take photographs avoids the need for overspraying. The aperture is set at f-2.8 to provide the largest aperture opening to capture the low level of luminescence. The shutter is set to "B", with the best time exposure being 30 seconds. With these settings, the use of a flash is not necessary.

When spraying the suspected area, the technician may pass through the field of view of the camera, but this may not be a problem given the long time exposure as long as she/he keeps moving. Wearing dark clothing and using a black atomizer are advised.

This method makes it easy to photograph exterior scenes at night, even under moonlight. Determining the photographic parameters is relatively easy to do, and the equipment is inexpensive. Examples of chemiluminescence (Figures 8–13) were taken with a FUJI-FILM Finepix S1 Pro digital camera, but use of digital equipment is not compulsory.



**Figure 8.** Latent swiping on tile floor



**Figure 9.** Luminescent latent footwear impressions





**Figure 10.** Close-up photograph of latent luminescent traces on the footwear





**Figure 11.** Evidence of clean up of blood developed on tile floor.



**Figure 12.** Latent swiping on a armchair back



**Figure 13.** Evidence of a clean up of blood on a bathroom floor.

