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# **The forensic luminol test for blood: unwanted interference and the effect on subsequent analysis**

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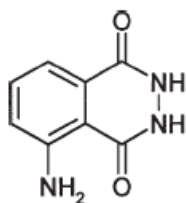
**Abstract:** When no bloodstains are found by visual inspection in crime scene investigations the chemiluminescence (CL) produced in the luminol test for blood is a useful aid. The complex reactions that give rise to the blue-green CL are facilitated by the catalytic activity of the hemoglobin in blood. The luminol test has a high sensitivity compared to other forensic blood tests. However it has a limited selectivity for blood. Different factors influence the potential to detect blood. Several compounds and substrates can trigger CL that can be mistaken for that of blood. One example is the sodium hypochlorite of bleaches. This is unfortunate because bleaches are sometimes used to clean areas at a crime scene. If a crime scene is allowed to air for one or two days this interfering effect diminish. Often are subsequent analyses of the blood visualized by the luminol test of interest. The test does not seem to affect subsequent use of presumptive blood tests like the phenolphthalein test. The test does not appear to have a severe inhibitory effect on neither PCR nor fluorescence based STR analysis. However prolonged exposure to the preparations used implicates a risk of DNA degradation. It should though be kept in mind that the often small amounts of blood visualized would not have been found at all by other reasonable means.

**Keywords:** forensic science, luminol, presumptive blood test, interfering catalysts, forensic DNA analysis

## Introduction

The reactions where luminol produces chemiluminescence (CL) has been known for many years. The luminol test for blood was first described by Walter Sprech in 1937 and its forensic use has since then greatly expanded [19]. In contact with the hemoglobin in blood luminol produces light with a blue green colour. Today, due to its great sensitivity the luminol test is used all over the world in crime scene investigations to visualize trace amounts of blood. The chemical structure of luminol is shown in fig. 1.

Blood is one of the most common physical evidence in investigations of violent crimes. Forensic analyses of the blood found at a crime scene supply in many ways valuable information that can be decisive in the solving of a crime. Questions that to some degree can be answered by the use of forensic serological techniques are: Is it really blood? Is the blood human? From whom does the blood come (identity and sex)? How did the blood get there?



**Fig. 1:** The chemical structure of luminol (3-amino-phthalhydrazide). [5]

Fresh bloodstains have a characteristic red colour through the erythrocytes, but when the stains age their colour change to brown through changes in the hemoglobin [18]. The stains can be hard to see on different substrates and difficult to separate from other materials. Since the mid of the 19<sup>th</sup> century many different blood tests have been described. They are often the first of several subsequent forensic analyses that are performed on a bloodstain. Often these tests are based on the ability of hemoglobin to catalyse the oxidation of a chromogenic compound which produces a colour change [14]. Today the forensic use of about twenty such presumptive blood tests has been described [14]. They have all in common that they produce false positive and negative results to some degree, and therefore they are only presumptive [14]. At the Swedish National laboratory of forensic science (SKL) leucomalachite green (LMG) is routinely used as a presumptive test for blood [23]. As a fast and practical way for presumptive species determination several immunochemical tests have been developed. These are in many cases based on immunochromatographic systems with antibodies against human hemoglobin [18].

An important part of the forensic analysis of blood is to find out from whom it is shed. For

many years different techniques like the ABO-system and characterization of serum proteins have been used for this [18]. These early methods have a limited certainty in the identification and hence in the recent decades they have almost been replaced by DNA-techniques. Today the use of PCR and typing of short tandem repeats (STR) markers has become routine at most forensic laboratories. Sexdeterminations can be done with these DNA-techniques [18].

The appearance, shape and placement of the bloodstains at a crime scene contain information. Under the 20<sup>th</sup> century blood pattern analysis has grown to a branch of forensic science. Here the physical and biological characteristics of blood are used together with mathematical models to decide the activities and mechanisms in the events that produced the pattern. The patterns and imprints in the blood at a crime scene can be only partially visual or altogether weak. Hence, to visualize latent and weak bloodstains on different substrates several enhancement techniques are available. The imprints that can be distinguished are amongst others footwear impressions, handprints or fingerprints [14]. Examples of some common blood enhance-ment methods are Amido Black, Coomassie brilliant blue and Hungarian red [14]. Many of these techniques simply dye the proteins in the blood (not only the hemoglobin) and hence they are therefore not used as a presumptive test for blood [9][14].

A special group of presumptive blood tests give CL or fluorescence in contact with blood. Here it is the peroxidaselike catalytic activity of hemoglobin that gives rise to the complex reactions that produces the CL or fluorescent compounds [14]. They often require that the test is carried out in darkness. Luminol produces blue-green CL in contact with blood and need no light source. In test like the one with fluorescein fluorescent species are formed by haemoglobin and an alternate light source is needed to trigger the fluorescence. Both tests are often used as a last resort when no blood is found by visual inspection or only weak partial patterns are found. Often they are used to visualize blood at rather large areas at

specific sites, where whiteness descriptions or experience indicate that blood should be present. Due to there great sensitivity they can detect blood patterns or drag marks even though deliberate cleaning attempts have been made to conceal the blood [10]. They can also be used to visualize blood that has flown between floor cracks or stains that has been deposited on substrates where they are hard to see. See Appendix A.

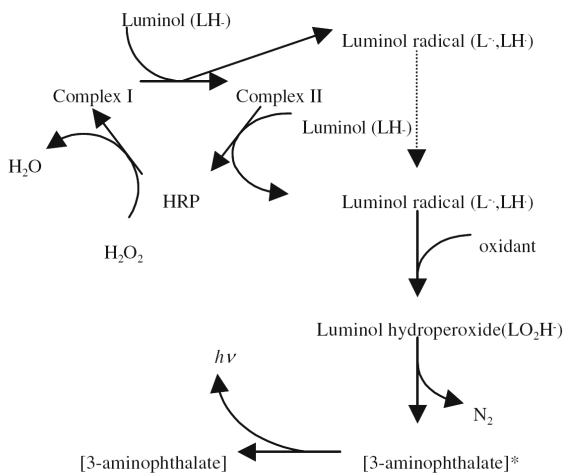
The use of CL for detection of blood has at the present a narrow use at the Swedish National laboratory of forensic science (SKL) and by the Swedish police authorities [23]. The increased sensitivity in other forensic techniques, such as DNA-analysis, has made the small amounts of blood detected important. The use of these forensic tests is therefore expected to increase in Sweden. The luminol test for blood has been used for many years and is a relatively dependable method and hence at SKL it is important to gather scientific knowledge in the subject [23]. In this report the chemical reactions behind the CL of the luminol test is encapsulated. It also describes common interferences with the test when it is used in different crime scenes. The effects on following analyses of the detected blood are also processed.

## **The chemistry behind luminol's chemiluminescence**

The chemistry of the CL of luminol is important in many fields of biochemistry. Many compounds besides from hemoglobin give rise to the CL of luminol in different reactions. The chemical reactions that lead to the CL of luminol are complex and they are in many cases probably not known in detail yet [1][5]. In the case of blood it is the activity of the heme groups in the hemoglobin that facilitates the reactions that result in strong CL, however the precise mechanisms are still not well known. In large the reaction used in the forensic blood test implicates luminol in an alkalic solution and the presence of an oxidant. The alkalic condition and catalytic activity of hemoglobin lead to oxidation of luminol, which transcend through several intermediate steps to an excited intermediate (3-aminophthalat) from which light is radiated

[17]. A relatively well established description of the reactions between luminol and hydrogen peroxide in the presence of a heme-enzyme are shown in fig. 2.

During the many years that luminol has been used to detect blood several preparations have been described in order to give optimal forensic usefulness [14]. Two commonly used preparations, where different oxidants and basic compounds are used, were described by Grodsky et al. 1951 and by Weber 1966 [14]. Both preparations give different advantages and disadvantages.



**Fig. 2:** A schematic representation of the reactions between luminol and hydrogen peroxide (oxidant) catalysed by horseradish peroxidase (HRP) in an alkalic solution. The catalytic mechanisms of hemoglobin are similar [24]. The enzyme facilitates the initial reactions of oxidation by hydrogen peroxides through complex I and II. [17]

The reactions, in the forensic preparations, where luminol produce CL can be triggered by different compounds. Other so called catalysts like metals, other heme-proteins or oxidizing agents such as hypochlorite also give CL with luminol in these solutions [17]. The mechanisms in these reactions are not well known, but it is thought that the formation of reactive intermediate complexes or radicals play an important role [5]. The matter is complex, hence when luminol preparations are applied on a substrate at a crime scene complex solutions are created where systems of reaction paths, defined by the compounds present, arise. Many outcomes are possible.

### Interference of the luminol test for blood

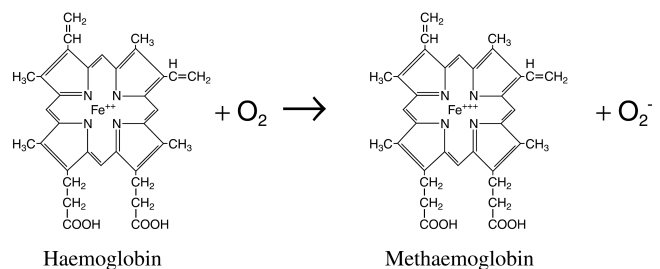
The luminol test for blood has a very high sensitivity for blood, which makes it as a

useful tool in forensic investigations [22]. However the tests usefulness is reduced by its limited selectivity for blood. Several factors can interfere with the test.

### The effect of bloodstain age and preheating

Different chemical and biological processes take place in blood when it dries and age. These changes lead to the formation of methemoglobin from hemoglobin when the iron in the heme prosthetic groups oxidizes from Fe(II) to Fe(III) [18]. This affects the bloods catalytical properties in the reactions where luminol's CL is produced. Old bloodstains have been reported to give more intense and longer lasting CL than fresh [7][14]. The fact that the forensic luminol test can be used repeatedly seems to be accepted [14]. However no quantitative investigation seems to have been done in this matter.

Quickenden et al. [21] describes how the luminol test is affected by preheating of the tested blood. Here series of test were executed to mimic the temperature cycles that blood is exposed to in motor vehicles. Hemoglobin solutions (with concentrations comparable to that of blood) were placed on glass slides and exposed to constant temperatures between 20 °C to 120 °C under 3 h to 48 h, whereupon CL were measured under normal conditions at room temperature. When the hemoglobin solution was preheated in the given interval the measured CL increased considerable with rising temperature. Quickenden et al. [21] describes how these changes probably are given by a formation of methemoglobin from hemoglobin produced by the provided heat. See fig. 3.



**Fig. 3:** One of the reactions which change the charges of the iron in the heme groups of hemoglobin resulting in methemoglobin. [21]

### *Mimicking interference of the luminol test for blood*

Already when Walter Sprechert 1937 described the forensic use of the luminol test for the first time it was known that not only blood give CL in the solution applied [14]. The tests specificity for blood has been described in several studies since then. It was found that amongst others haemenzymys like peroxidases, iron and copper species and the sodium hypochlorite of bleaches give CL [20]. See Appendix B. Many of these older studies are based on qualitative descriptions of the CL and they do not give a complete overview of the different substrates and compounds that mimic positive reactions [5]. To mitigate this problem a group of scientists at the University of Western Australia in collaboration with local law enforcement agencies performed and published a series of comprehensive quantitative investigations of interferences with the luminol test for blood [5][6][19][20][21][22]. In these studies the same luminol preparation (0.1g luminol, 5.0g Na<sub>2</sub>CO<sub>3</sub> and 0.7g NaBO<sub>3</sub>4H<sub>2</sub>O in 100ml distilled water) and solutions of hemoglobin (same concentrations) was used. The same equipment including a photomultiplier tube was used for accurate quantitative measurements of the intensity and the spectral distribution in the CL. In 2003 Creamer et al. [5] published a comprehensive study where the blood mimicking behaviour of 250 different substrates and compounds, common at crime scenes, had been measured on. In these test only 9 kinds of substrates or compounds were reported to give strong enough CL to be easy mistaken for blood. These were preparations of turnip, parsnip, horseradish, bleaches (hypochlorite), copper metal, enamel paint, certain spray paints, furniture polish and interior fabrics in motor vehicles. In a separate study Quickenden et al. [21] examined the interference with the luminol test for blood in motor vehicles. Also in motor vehicles only a few materials gave strong CL, without the presence of blood or hemoglobin solution. These were among others parts of seatbelts, a gear knob and the interior lining of a boot. In the different publications by the Australian group of scientist there are informative tables

displaying intensities and wavelength shifts in the CL produced by the diverse materials.

There are probably no large and useful differences in the spectral distribution of wavelengths in the CL from luminol, catalyst by hemoglobin or other compounds, when the test is used at a crime scene [5]. The differences in wavelength maxima are in many cases too small and depend on local conditions [5]. Comparatively large spectral shifts, like the one between the red hemoglobin and the uncoloured hypochlorite, is believed to depend on the absorption of light by species in solution of the reactions. This produces a kind of inner filtering effect [16]. Also fluorescent compounds are believed to influence the spectral distribution. This is thought to occur if the wavelengths in the CL match the absorption of the fluorescent compound [5]. Spectral equipment can therefore probably not be used at a crime scene investigation as an easy and general way to rule out the CL triggered by other catalysts than hemoglobin in the common forensic luminol test preparations.

### *Attempted cleaning of bloodstains*

The fact that hypochlorite give strong CL, in the preparation used for the luminol test, can be seen as a cruel fate. Hypochlorite is a common component in bleaches and cleaners, which are often used to remove blood from crime scenes. Hence the interference of the luminol test for blood by hypochlorite has been studied thoroughly over the years. Hypochlorite is an oxidant and it was one of the first compounds used by chemists to oxidize luminol in order to produce CL. Hypochlorite is even used in education to demonstrate luminol's CL [8]. However hypochlorite is volatile and comparably fast evaporates from a surface [6][15]. Creamer et al. [6] showed that when ten drops of water based solution with 125 g/L and 10 g/L hypochlorite was applied to a surface the CL from the applied forensic luminol preparation (without hemoglobin) was negligible after 8h and none were measured after 16 h. These results are in largely verified by similar experiments in another publication by Kent et al. [15]. Creamer et al. [6] also describes the

effect on the luminol test for blood given by repeated washing of bloodstains with either water or bleach on glazed terracotta tiles. When only water was used, when washing the bloodstains several times, the measured CL decreased until it was negligible. When similar washings was preformed with bleach the CL decreased initially but then after a few washes started to increase until it reached a stabilized value comparable to that of blood. In other words the hypochlorite in the bleach gave a CL comparable to that of the blood that was washed away on this surface.

If a crime scene is allowed to air for one to two days most of the hypochlorite will probably vaporize, but when no delays can be afforded the selectivity of the luminol test can be enhanced chemically [16]. By using a solution (Grotsky preparations) adjusted to pH 12 with glycine (0.05mol/L) the unwanted CL from hypochlorite can be significantly decreased [16]. At this high pH-value the reaction rate between hypochlorite and glycine is faster then that of the interfering reaction between hypochlorite and luminol. However the effect of glycine and the high pH-value on subsequent analyses of the detected blood has yet not been evaluated [16].

Harris et al. [12] investigated the effect deliberate attempts to clean bloodstained areas have on the ability to obtain results from DNA analyses. Blood samples where applied on several different surfaces whereupon cleaning was performed with thick chlorinated bleach, general pine disinfectant or non-chlorinated liquid detergent. DNA was extracted using a Chelex 100 protocol and STR analyses were performed with the AmpF/STR SGM Plus PCR amplification kit (Applied biosystems) and capillary electrophoresis. These tests showed that cleaning with bleaches gave DNA degradation and it had the most adverse effect on the ability to obtain complete DNA profiles. However DNA quality was found to be appreciably high despite the use of either chlorinated or non-chlorinated cleaning agents. The effect on the forensic luminol test given by attempts to clean bloodstained areas (stained with hemoglobin solution) in motor vehicles with water, soap solution and a

cleaner was examined by Quickenden et al. [21]. In motor vehicles there are a range of different materials and surfaces, where attempts to clean bloodstained areas give different results. Quickenden et al. [21] showed that on many surfaces the CL of the luminol test, after washing attempts, depend on the absorbtivity of the material and the solvability of hemoglobin in the cleaning solution used. The resulting CL after cleaning varied between different types of cars and it also much depended on how the washing was done. This affected the reproducibility of the measurements.

### **The luminol test in comparison with other presumptive blood tests**

At the present more or less all used forensic blood tests has a limited selectivity and are presumptive. Due to the lower selectivity of the luminol test positive reactions should be verified by other presumptive blood tests like the phenolphthalein test [14]. Cox et al. [4] conducted a study where it was shown that the used preparations of neither phenolphthalein nor LMG produced false positive results when exposed to household fruits and vegetables. Other investigations of these tests have shown similar high selectivity for blood [14].

Webb et al. [22] conducted a study where the luminol test was compared to four other forensic blood tests. These tests where phenolphthalein, LMG, Hemastix (Bayer) and a forensic light source. The luminol test used was found to have by far the greatest sensitivity. Under laboratorial conditions CL was detected from luminol treated stains of the used hemoglobin solution (corresponding to blood) diluted up to  $5 \cdot 10^6$  times. A comparably high sensitivity of the luminol test has been reported in other studies [22]. However the sensitivity is probably not as great under the conditions found at a crime scene and here, depending on several factors, perhaps one may "only" see blood diluted to about 1:10000 [14].

Garfano et al. [10] compared the sensitivity and the specificity of the luminol and fluroscein tests qualitatively. The tests had

comparable sensitivity. It was also found that the two tests show different detection capabilities depending upon the substrate.

## **The luminol test's effect on subsequent analyses**

The luminol test has a great sensitivity and hence the quantities of blood detected can be very small. Advances in the sensitivity in other forensic analyses have made it important to know the test's effect on subsequently used forensic techniques.

### *The luminol test's effect on subsequent chemical and immunochemical analyses*

Spectrophotometry can be used for determining the presence of hemoglobin in a sample. Barbaro et al. [2] conducted a series of spectrophotometrical tests on samples, taken from bloodstains deposited on several different surfaces. The effect of the luminol and fluorescein tests were evaluated. This study showed that in the presence of luminol or fluorescein in a sample the typical absorption peaks of hemoglobin become hard to detect. Barbaro et al. [2] concluded that this was because the reagents used in these tests have absorption that covers the peaks of hemoglobin.

Barbaro et al. [2] also investigated what effect the use of the luminol and fluorescein tests have on the immunochromatographic test kit OC-Hemocard (Alfa Wassermann). Samples taken from luminol treated bloodstains showed a pH-value of 11 and gave false negative results with the test. When the pH-value of the samples was adjusted to 7 the test gave correct positive results. Use of the fluorescein test gave similar effects. The effect on immunodiffusion was also studied and Barbaro et al. [2] concluded that use of the luminol and fluorescein tests don't interfere with the immunochemical methods investigated when pH of taken samples are adjusted to about 7. Similar results have been found in other studies [14].

The luminol test's effect on the subsequent use of presumptive blood tests based on colour changing compounds has been investigated at several occasions. Gross et al. [11] showed

that luminol treatment of bloodstains do not have a remarkable adverse effect on the use of neither the phenolphthalin (Kastle-Meyer) nor the tetramethylbenzidine tests. Other publications describes in a like manner that the luminol test do not interfere with the subsequent use of other particular presumptive blood tests [14].

The use of the luminol test has been found to have an strong adverse effect on subsequent forensic typing of serum protein markers [14].

### *The luminol test's effect on subsequent DNA analyses*

In recent decades great advances have been made in DNA technology. Today, through techniques like PCR and variable number of tandem repeats (VNTR) typing, DNA identification can be carried out on minute quantities of biological evidence. Locating traces of blood by the use of luminol preparations can therefore lead to conclusive DNA results. The extensive forensic use of DNA analyses has produced several investigations on the luminol test's effect on these procedures.

In 1991 Hochmeister et al. [13] published a study dealing with presumptive test reagent's effect on forensic restriction fragment length polymorphism (RFLP) analysis. Bloodstains applied on glass and cotton was treated with different reagents. The study showed that, despite direct exposure to preparations of luminol, DNA extraction produced high molecular weight DNA fragments and that the RFLP procedures still could be performed successfully.

Luminol do not appear to have severe adverse effects on PCR-based STR fluorescent DNA analysis [2][7][9][10][11][13]. Frégeau et al. [9] investigated the effects of exposures to several blood enhancement chemicals (including a luminol preparation) used to visualize bloody fingerprints. A one step organic DNA extraction protocol was used in combination with AmpF/STR Profiler Plus (Applied biosystems) fluorescent STR analysis with capillary electrophoresis. Blood was applied to several different surfaces as

drops and fingerprints. The effects of both short and longterm (up to 54 days) exposures to luminol and the other chemicals after use at one occasion were studied. No inhibitory effect on PCR or interferences with fluorescent-based typing was observed in the luminol treated samples. It was found that, on the different surfaces, the exposure to luminol and the other enhancement chemicals for up to 54 days do not appear to compromise the used STR analysis. However the results showed that longer exposure to the investigated chemicals eventually lead to DNA-degradation and reduced chances of a complete DNA profile compared to untreated bloodstains.

Babaro et al. [2] investigated the luminol test's effect on the ability to extract DNA using methods based on the DNA IQ system (Promega), chelex (Biorad) or phenol chloroform. The test's effect on STR analysis using the AmpF/STR Profiler Plus (Applied biosystems) and AmpF/STR Identifiler (Applied biosystems) kits was also studied. Bloodstains were applied on different substrates and treated (no prolong exposure time though) with preparations of either luminol or fluorescein. No degradation of DNA correlated to the use of neither luminol nor fluorescein. Babaro et al. [2] concluded that neither of these tests does interfere with the ability to obtain accurate DNA profiles. Garofano et al. [10] came to a similar conclusion when bloodstains of different dilutions applied on different substrates where treated with luminol or fluorescein. In this study DNA extraction where carried out with the ChargeSwitch forensic DNA Purification kit (Invitrogen) and amplification was carried out by using the AmpF/STR Identifiler DNA amplification kit (Applied biosystems). Complete DNA profiles were obtained at relatively high blood dilutions (1:1000) after previous treatment with either of the tests.

## Conclusions

Preparations of luminol can successfully be used for screening large areas for blood.

Minute amounts of blood can be visualized through the CL. Many factors influence the potential to detect blood. Within reasonable limitations the bloodstains age and preheating give stronger and more lasting CL. Several compounds and substrates can trigger CL that can be mistaken for that of blood. When bleaches are used to clean bloodstained areas they interfere with the luminol test. To diminish this problem a crime scene can be allowed to air for one to two days.

In several scientific studies it has been shown that use of preparations of luminol does not appear to have a severe inhibitory effect on neither PCR nor fluorescent STR typing. However there is a risk of DNA-degradation that increase with prolonged exposure and the test implicates a risk of contaminating the detected blood when the solutions are sprayed upon it. It should though be kept in mind that the luminol test for blood often visualizes blood that otherwise would not have been found at all by other reasonable means.

## Acknowledgement

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## Pressrelease

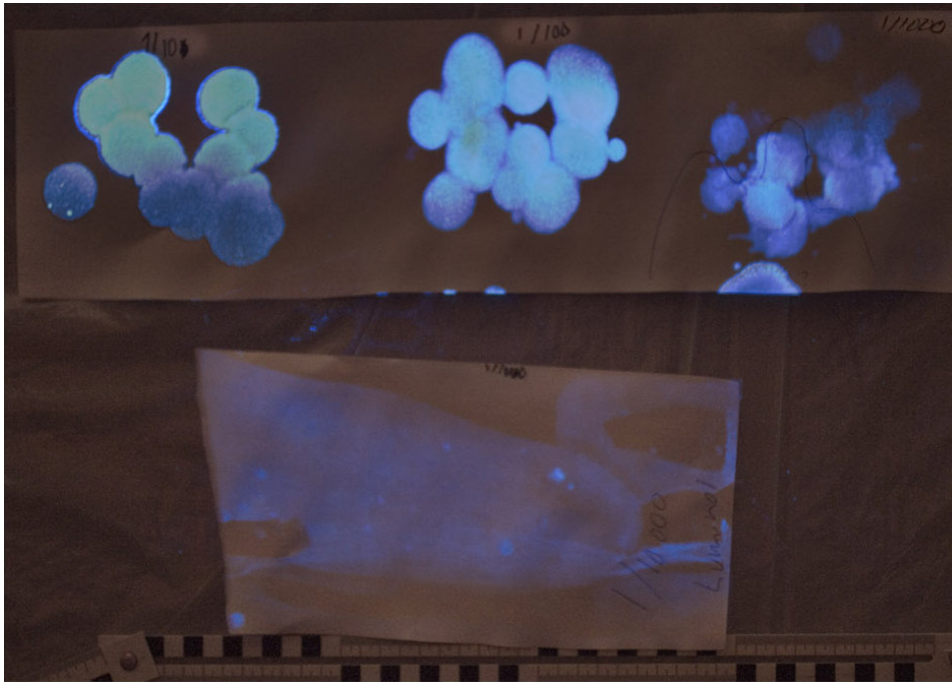
Chemiluminescent visualisering av blod, genom att en luminollösning sprayas ut på brottsplats, är ett känsligt och kraftfullt hjälpmedel i kriminaltekniska utredningar efter våldsbrott. Metoden har dock begränsad selektivitet för blod. Material som rotfrukter, metaller och blekmedel ger också positiva utslag. Med dagens DNA-teknik kan de ofta små mängderna blod som hittas genom luminol analyseras för att identifiera gärningsman eller brottsoffer.



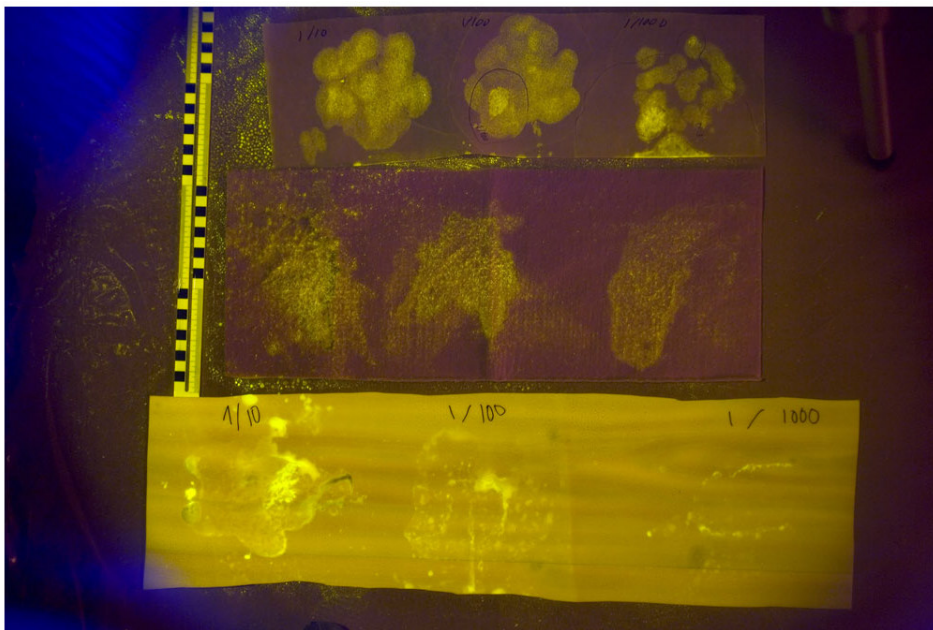
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### Appendix A: Visualization of blood using preparations of luminol and fluorescein



**Fig:** Visualization of bovine blood using a preparation of luminol (Krim. Teknisk Materiel AB). The upper half shows blood on plastic carpet diluted (from left to right) 1:10, 1:100 and 1:1000. The lower half shows blood diluted 1:10 000 on filter paper.



**Fig:** Visualization of bovine blood using a preparation of fluorescein (KPS Technologies). The upper surface shows blood on filter paper diluted (from left to right) 1:10, 1:100 and 1:1000. In the middle blood diluted (from left to right) 1:10, 1:100 and 1:1000 on absorptive cloth are shown. The lower part shows blood on plastic carpet diluted (from left to right) 1:10, 1:100 and 1:1000. The surfaces were illuminated with light with a wavelength of 450 nm and the photo was taken with an orange camera lens.

## Appendix B: Examples of false positive reactions



**Fig:** A forensic preparation of luminol where applied on samples of different household vegetables, chemicals and materials. The tests were performed merely to illustrate that false positive reactions are possible. All samples were prepared shortly before use. The amount of material on each filter paper varied. The quantity of luminol solution applied varied also considerably between the filter papers.

The samples in the upper picture from left to right are: parsnip, beetroot, turnip, horseradish, a cleaning agent (Kraft grovrent) without sodium hypochlorite and a cleaning agent (Klorin) with sodium hypochlorite

The samples in the lower picture from left to right and up to down are: iron (rust), liquid soap, varnish for parquet, enamel paint, varnish, daphnia, garden onion, potato, orange, carrot, orange juice, carpet with sisal fibers and two steel screws

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Training for the Crime Scene Professional

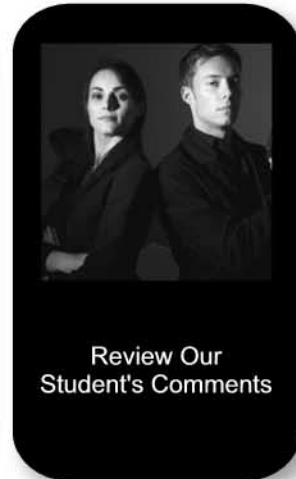
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