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Research Article

Comparative Evaluation of Presumptive and Confirmatory Methods for the Detection of Blood at Crime Scenes: Sensitivity, Specificity and Substrate Interference

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ABSTRACT:
Bloodstains are among the most important forms of biological evidence encountered during crime-scene investigations. Their detection can provide critical information for crime-scene reconstruction and may subsequently enable human blood identification and DNA profiling. However, bloodstains may be present in very small quantities, may be partially cleaned or degraded, and may occur on substrates that interfere with chemical testing. The present study is proposed to comparatively evaluate commonly used presumptive blood-detection methods, including the Kastle–Meyer phenolphthalein test, leucomalachite green test, and luminol-based chemiluminescence, followed by a human-blood-specific confirmatory immunochromatographic assay. Blood samples at different dilutions will be deposited on representative crime-scene substrates including cotton, paper, glass, metal, wood and tile. The effects of substrate, dilution, aging and selected interfering substances will be evaluated. Analytical performance will be assessed using sensitivity, specificity, positive predictive value, negative predictive value and misclassification rate. Particular emphasis will be placed on the preservation of biological evidence for subsequent DNA analysis. The study is expected to provide a practical evidence-based framework for selecting blood-detection methods at crime scenes and forensic laboratories. The proposed approach may also help identify circumstances in which presumptive tests produce false-positive or false-negative results and establish an appropriate sequential testing strategy.

Keywords: Bloodstain identification; crime scene; Kastle–Meyer; luminol; leucomalachite green; human hemoglobin; immunochromatography; forensic biology; substrate interference; DNA profiling.

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1. INTRODUCTION

Blood is one of the most frequently encountered biological materials in forensic investigations. Bloodstains may occur on clothing, floors, walls, weapons, furniture, vehicles and outdoor surfaces and can provide important evidence regarding the occurrence and reconstruction of violent events. Correct identification of a suspected bloodstain is therefore an important early step in forensic examination.

At a crime scene, visual examination alone is often insufficient because blood may be present in trace quantities, concealed by darkness, diluted, washed or deposited on visually complex substrates. Consequently, presumptive chemical tests have traditionally been employed for rapid screening. Common methods include the Kastle–Meyer phenolphthalein test, leucomalachite green, luminol and related catalytic assays. Comparative studies have shown that luminol can be particularly sensitive for locating dilute or latent bloodstains, although presumptive tests are not inherently specific for human blood.

The chemistry of many presumptive blood tests is based on the peroxidase-like catalytic activity of hemoglobin. In the Kastle–Meyer reaction, the reduced form of phenolphthalein is oxidized to produce a characteristic pink coloration in the presence of an oxidant. Luminol, in contrast, produces chemiluminescence under alkaline oxidative conditions, allowing visualization of blood traces that may not be readily visible to the naked eye. Luminol has consequently been extensively investigated for latent blood detection at crime scenes.

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Despite their usefulness, presumptive tests have limitations. Environmental materials and chemical contaminants can produce false-positive reactions, whereas substrate composition, washing, degradation and chemical treatment can contribute to false-negative results. For example, substrate-associated compounds have been demonstrated to interfere with presumptive and human-hemoglobin testing.

A positive presumptive reaction therefore should not automatically be interpreted as definitive evidence of human blood. Human-specific confirmatory approaches, particularly immunochromatographic assays targeting human hemoglobin, have become important in forensic practice. These assays can provide rapid and relatively specific confirmation of human blood, although degradation, excessive target concentration, detergents, previous chemical treatment and other factors can affect performance.

Recent developments in Raman spectroscopy, ATR-FTIR and chemometric analysis provide additional opportunities for non-destructive bloodstain examination. Raman spectroscopy has demonstrated potential for distinguishing bloodstains from environmental interferences, while ATR-FTIR has been investigated for blood identification, species discrimination and bloodstain aging.

Therefore, the present study proposes a systematic comparative evaluation of conventional presumptive tests and human-blood-specific confirmation under controlled forensic conditions.

2. Aim of the Study

The primary aim is to evaluate the performance of commonly used blood-detection methods for forensic crime-scene examination and to establish an evidence-based sequential strategy for bloodstain screening and confirmation.

2.1 Specific Objectives

1. To evaluate the sensitivity of selected presumptive blood tests at different blood dilutions.
2. To compare the performance of Kastle–Meyer, leucomalachite green and luminol-based testing.
3. To investigate the influence of different substrates on blood detection.
4. To examine the effects of bloodstain aging on test performance.
5. To evaluate selected potential interfering substances.
6. To compare presumptive results with a human-hemoglobin-specific confirmatory method.
7. To determine sensitivity, specificity, PPV, NPV and misclassification rate.
8. To assess the suitability of different testing sequences for preserving evidence for subsequent DNA analysis.
9. To explore the potential of spectroscopic methods as a future non-destructive confirmatory approach.

3. Materials and Methods

3.1 Study Design

A controlled laboratory-based experimental study will be conducted using bloodstains prepared under standardized conditions. The study will simulate common crime-scene substrates and environmental conditions while maintaining appropriate biosafety and institutional ethical requirements.

The experimental workflow will consist of:
Blood sample → controlled dilution → deposition on substrate → aging/environmental treatment → presumptive testing → confirmatory human-blood testing → statistical evaluation

3.2 Blood Samples

Appropriately consented and institutionally approved human blood specimens will be used. Samples will be handled as potentially infectious biological material under applicable biosafety procedures.

Where species-comparison experiments are included, appropriately sourced non-human blood samples may be used to evaluate the specificity and cross-reactivity of the analytical methods.

No personally identifying information will be associated with experimental samples.



3.3 Preparation of Bloodstains

Blood will be prepared at predefined dilution levels representing relatively concentrated to highly dilute stains.

For example, an experimental dilution series may include:

- Undiluted blood
- 1:10
- 1:100
- 1:1,000
- 1:10,000

The final dilution range should be established during pilot validation rather than assumed to represent a universal detection limit.

Measured volumes of each preparation will be deposited onto standardized substrate pieces and allowed to dry under controlled laboratory conditions.

3.4 Selection of Crime-Scene Substrates

The following substrates are proposed:

Substrate	**Forensic relevance**
Cotton fabric	Clothing/textile evidence
Paper	Documents and indoor surfaces
Glass	Windows, bottles and vehicles
Stainless steel/metal	Weapons and tools
Wood	Furniture and structural surfaces
Ceramic tile	Floors and walls
Synthetic fabric	Clothing/carpets
Soil	Outdoor crime scenes

The substrate experiment is particularly important because the chemical composition of the surface can alter presumptive-test performance.

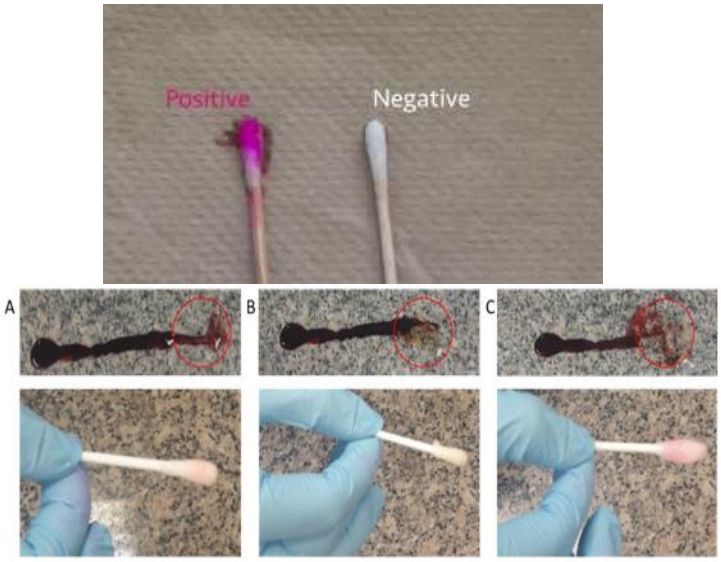
4. Presumptive Blood Tests

4.1 Kastle–Meyer Test

The Kastle–Meyer test will be evaluated as a rapid colorimetric presumptive assay based on the peroxidase-like activity associated with hemoglobin.

A positive reaction will be recorded according to the validated laboratory interpretation criteria and appropriate negative controls.

Because presumptive reactions can be influenced by oxidizing substances and other environmental materials, positive results will not be interpreted as definitive proof of human blood.



4.1.1. Principle and Chemical Basis

The **Kastle–Meyer (KM) test** is a rapid **presumptive colorimetric test** used for the detection of suspected bloodstains.

- **Chemical basis:** The test is based on the oxidation of reduced phenolphthalein (**phenolphthalin**) to phenolphthalein.
- **Catalytic role of heme:** The heme component of hemoglobin exhibits **peroxidase-like catalytic activity**, facilitating the oxidation reaction.
- **Role of hydrogen peroxide:** Hydrogen peroxide acts as the oxidizing agent in the reaction.
- **Color development:** In the presence of suspected blood, phenolphthalin is oxidized to **pink-colored phenolphthalein**, producing a characteristic pink/magenta color.
- **Forensic significance:** Rapid development of the characteristic pink color is interpreted as a **positive presumptive reaction for blood**, not as definitive proof of human blood.

Simplified reaction

Phenolphthalin + H_2O_2 —(heme/peroxidase-like activity)→ Phenolphthalein (pink)

4.1.2. Step-by-Step Procedure

Step 1: Collection of the suspected stain

A clean, slightly moistened cotton swab or suitable absorbent material is gently applied to the suspected stain.

Step 2: Addition of ethanol

A small amount of ethanol may be used according to the laboratory's validated protocol. This can facilitate extraction of components from the stain.

Step 3: Addition of Kastle–Meyer reagent

The **colorless phenolphthalein/phenolphthalin reagent** is applied to the swab.

Important:

No pink color should develop at this stage. A color change before addition of hydrogen peroxide may indicate reagent deterioration or interference.

Step 4: Addition of hydrogen peroxide

Hydrogen peroxide is subsequently added according to the validated laboratory SOP.

Step 5: Interpretation of the reaction

Development of a **rapid pink/magenta color** after the addition of hydrogen peroxide is recorded as a **positive presumptive reaction**.

The laboratory should define the observation time and interpretation criteria in its validated SOP rather than relying on an arbitrary universal time window.

4.1.3. Interpretation of Results

Observation	Interpretation
No color development	Negative presumptive reaction
Rapid pink/magenta color after H_2O_2	Positive presumptive reaction
Color appearing before H_2O_2	Invalid/questionable reaction; reagent/interference should be considered
Slow or weak color development	Equivocal; further examination required

Important: A positive KM test indicates the possible presence of blood-associated peroxidase activity but **does not establish that the material is human blood**.

4.1.4. Controls

For reliable forensic testing, appropriate controls should be incorporated.

Positive control

A known blood sample should produce the expected reaction.

Negative control

A blank swab/reagent control should remain negative.

Reagent control

The reagent should be checked for spontaneous color development before casework interpretation. Controls help distinguish a genuine reaction from reagent deterioration or contamination.

4.1.5. Limitations of the Kastle–Meyer Test

4.1.5.1 Presumptive nature

The Kastle–Meyer test is a **presumptive test**, not a confirmatory test. A positive reaction should therefore be followed by an appropriate human-blood-specific examination and/or downstream DNA analysis where indicated.

4.1.5.2 False-positive reactions

The reaction is based on peroxidase-like/oxidizing activity and therefore is not completely specific for blood. Possible interfering materials include:

- Plant peroxidases
- Certain oxidizing chemicals
- Bleaching agents
- Some metal-containing materials
- Other substances capable of promoting oxidation

Therefore, a positive reaction must be interpreted in the context of the substrate and crime-scene circumstances.

4.1.5.3 False-negative reactions

Weak or negative reactions may occur because of:

- Very low blood concentration
- Extensive washing
- Environmental degradation
- Chemical treatment
- Interference from the substrate
- Improper reagent storage or deterioration

Consequently, a negative result does not necessarily exclude the previous presence of blood.

4.1.6. Forensic Significance

The Kastle–Meyer test is particularly useful during **crime-scene screening** because it is:

- Rapid
- Relatively inexpensive
- Simple to perform
- Suitable for screening suspected stains
- Useful for selecting samples for subsequent laboratory examination

However, because it is presumptive, it should be incorporated into a **sequential forensic workflow**:

Suspected stain
↓
Visual examination & documentation
↓
Kastle–Meyer presumptive test
↓
Human-blood-specific confirmatory examination
↓
DNA extraction and profiling, where appropriate
↓
Forensic interpretation

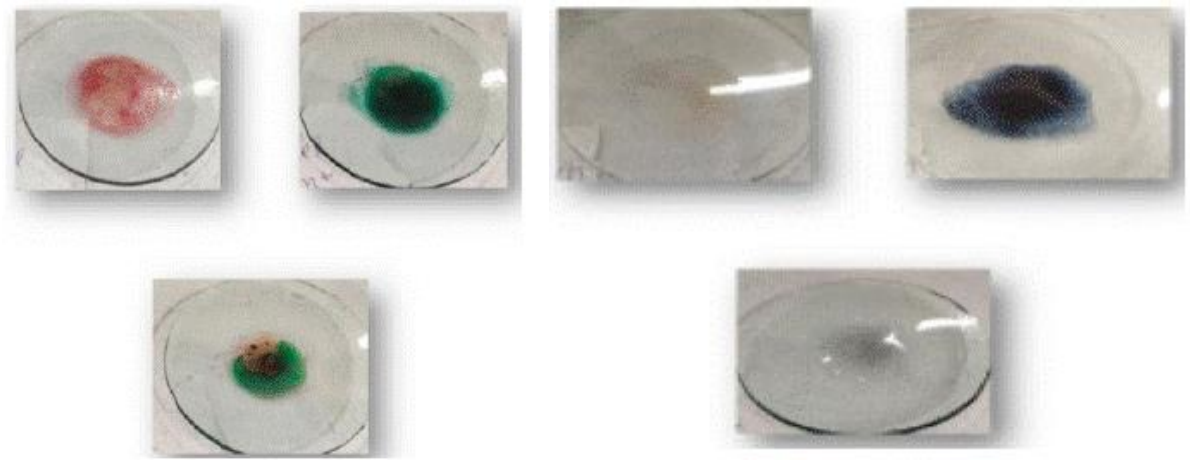
4.1.7. Recommended Research-Paper Table

Table X. Analytical characteristics of the Kastle–Meyer test for presumptive blood detection

Parameter	Kastle–Meyer Test
Test type	Presumptive colorimetric test
Target	Blood-associated heme/peroxidase-like activity
Principal reagent	Phenolphthalein/phenolphthalin system
Oxidizing agent	Hydrogen peroxide
Positive indication	Rapid pink/magenta color
Main advantage	Rapid and sensitive screening
Major limitation	Limited specificity
Human-specific?	No
Potential interferences	Plant peroxidases, oxidants, certain metals/chemicals
Crime-scene application	Screening of suspected bloodstains
Follow-up	Human-blood confirmation and/or DNA analysis

4.2 Leucomalachite Green Test

Leucomalachite green will be evaluated as another catalytic colorimetric presumptive method. Results will be recorded as positive, negative or equivocal according to predefined interpretation criteria. The performance of this method will be compared with Kastle–Meyer under identical dilution and substrate conditions.



4.2.1. Principle and Chemical Basis

The **Leucomalachite Green (LMG) test** is a rapid **presumptive colorimetric test** used in forensic science for the detection of suspected visible or latent bloodstains.

- **Reagent system:** The test generally employs **leucomalachite green**, an oxidizing system such as **sodium perborate**, and an acidic medium such as **glacial acetic acid**, according to the validated laboratory protocol.
- **Catalytic role of heme:** The heme component of hemoglobin exhibits **peroxidase-like catalytic activity** and facilitates the oxidation reaction.
- **Color development:** Reduced/colorless leucomalachite green is oxidized to its colored form, producing a characteristic **green to blue-green coloration**.
- **Forensic interpretation:** Rapid development of an intense green/blue-green color is interpreted as a **positive presumptive reaction for blood-associated heme activity**.

Simplified reaction

Leucomalachite green (reduced/colorless form) + oxidant —(heme/peroxidase-like activity)→ Colored leucomalachite green

4.2.2. Step-by-Step Procedure

Step 1: Collection of the suspected stain

A clean swab or suitable absorbent material is used to collect a small portion of the suspected stain.

Step 2: Preparation of the test system
The LMG reagent and oxidizing system are prepared and maintained according to the laboratory's validated SOP.

Step 3: Application of LMG reagent
The reagent is applied to the suspected sample.

Step 4: Addition of oxidizing reagent
The appropriate oxidizing component is added according to the validated laboratory procedure.

Step 5: Observation
Development of a **rapid, intense green or blue-green coloration** is recorded as a positive presumptive reaction.

The exact observation period and interpretation criteria should be established through laboratory validation rather than relying on a universal reaction time.

4.2.3. Interpretation of Results

Observation	Interpretation
No color development	Negative presumptive reaction
Rapid green/blue-green color	Positive presumptive reaction
Unexpected color before complete reagent sequence	Questionable/invalid reaction; reagent or interference should be considered
Weak or delayed color	Equivocal; further testing required

Important: A positive LMG reaction does **not confirm human blood**. It indicates a presumptive reaction associated with heme/peroxidase-like activity.

4.2.4. Advantages of the LMG Test
4.1 Rapid screening
LMG provides a relatively rapid visual response and can therefore be useful for screening suspected stains.

4.2 High analytical sensitivity
The method can detect relatively small quantities of blood-associated material and may be useful for diluted or partially visible stains.

4.3 Simple visual interpretation
The development of a characteristic green/blue-green color allows straightforward preliminary interpretation.

4.4 Crime-scene applicability
Because of its relatively simple methodology, LMG can be considered as a presumptive screening method within an appropriate forensic workflow.

4.2.5. Limitations of the LMG Test
4.2.5.1 Presumptive nature
LMG is **not a confirmatory test for human blood**. A positive reaction should therefore be followed by an appropriate confirmatory examination.

4.2.5.2 False-positive reactions
Because the reaction depends on oxidation/peroxidase-like activity, substances other than blood may produce a positive reaction. Potential interfering materials include:

- Plant peroxidases
- Certain vegetables and plant materials
- Strong chemical oxidants
- Bleaching agents
- Other substances capable of promoting oxidation

Therefore, positive results should be interpreted cautiously.

4.2.5.3 False-negative reactions
Some substances can inhibit oxidation and potentially suppress the expected color reaction. **Ascorbic acid (vitamin C)** is an important example of a reducing agent that may interfere with oxidative presumptive tests.

Other factors that may contribute to weak or negative reactions include:

- Extensive washing
- Environmental degradation
- Very low blood concentration
- Chemical treatment
- Substrate effects
- Reagent deterioration

4.2.5.4 Effect on subsequent DNA analysis
Because forensic bloodstains may subsequently be subjected to DNA profiling, the compatibility of a presumptive reagent with downstream DNA analysis should be considered

during method selection and validated under the laboratory's conditions.

For casework, unnecessary consumption or chemical treatment of the entire biological sample should be avoided whenever sufficient material can be retained for subsequent examination.

4.2.6. Quality-Control Requirements
Reliable forensic interpretation requires appropriate controls.

Positive control
A known blood sample should produce the expected LMG reaction.

Negative control
A blank/sample-free control should remain negative.

Reagent control
The reagent system should be checked for unexpected background coloration or deterioration. These controls help distinguish genuine reactions from reagent-related or environmental effects.

4.2.7. Forensic Significance
LMG can be incorporated into a **sequential bloodstain examination strategy**:

Suspected bloodstain
↓
Visual examination & documentation
↓
LMG presumptive test
↓
Human-blood-specific confirmatory examination
↓
DNA extraction and profiling, where appropriate
↓
Forensic interpretation

This approach prevents a presumptive LMG reaction from being incorrectly reported as definitive evidence of human blood.

4.2.8. Recommended Research-Paper Table

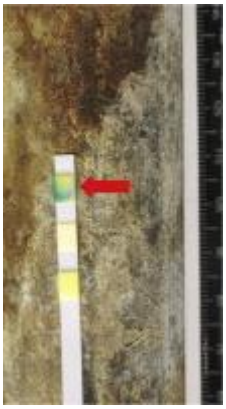
Table X. Analytical characteristics of the Leucomalachite Green test for presumptive blood detection

Parameter	Leucomalachite Green (LMG) Test
Test type	Presumptive colorimetric test
Target	Heme/peroxidase-like catalytic activity
Principal reagent	Leucomalachite green
Oxidizing system	Sodium perborate or validated oxidant system
Reaction medium	Acidic medium
Positive indication	Green/blue-green color
Major advantage	Rapid and sensitive screening
Human-specific?	No
Potential false positives	Plant peroxidases, oxidants, bleach and related materials
Potential false negatives	Reducing agents such as ascorbic acid, degradation and substrate effects
Crime-scene application	Screening of suspected bloodstains
Confirmatory requirement	Yes
Downstream analysis	Human-blood confirmation and/or DNA profiling

4.3 Luminol-Based Detection
Luminol-based chemiluminescence will be evaluated for visualization of latent or highly diluted bloodstains.

The method is particularly relevant to crime scenes where blood may be difficult to visualize directly. Previous comparative work has found luminol to be highly sensitive among commonly used presumptive methods, particularly for diluted stains.

However, the possibility of interference and the consequences of chemical treatment for subsequent forensic analyses must be considered.



4.3.1. Principle and Chemical Basis

The **luminol test** is a highly sensitive **presumptive chemiluminescent method** used in forensic science for the visualization of suspected latent or diluted bloodstains.

- **Chemiluminescent principle:** Luminol undergoes an oxidation reaction in an alkaline medium in the presence of an appropriate oxidizing system.
- **Catalytic role of heme:** The iron-containing **heme group of hemoglobin** exhibits peroxidase-like catalytic activity and facilitates the oxidative reaction.
- **Excited-state formation:** Oxidation of luminol produces an electronically excited reaction product.
- **Light emission:** As the excited product returns to a lower-energy state, energy is released as visible light, producing the characteristic **blue chemiluminescence**, with an emission maximum commonly reported near **425 nm**.
- **Forensic significance:** The emitted blue light can reveal blood traces that are not readily visible under normal illumination.

Simplified reaction

Luminol + oxidizing system —(heme/metal-catalyzed oxidation)→ excited reaction product → blue chemiluminescence (~425 nm)

4.3.2. How the Luminol Test Works

Step 1: Preparation of the reagent system

Luminol is prepared in an **alkaline medium**, together with an appropriate oxidizing/activating system according to the validated laboratory protocol.

Step 2: Application to the suspected area

The luminol reagent is applied or sprayed over the area suspected of containing a latent bloodstain.

Step 3: Catalytic oxidation

If blood-associated heme is present, the iron-containing heme component facilitates the oxidation of luminol.

Step 4: Chemiluminescence

The oxidation process generates an excited-state product that releases energy as visible blue light.

Step 5: Documentation

Because the emission can be relatively short-lived, the reaction should be documented using appropriate **low-light photography/video and validated crime-scene documentation procedures**.

4.3.3. Interpretation of Results

Observation	Interpretation
No visible chemiluminescence	Negative presumptive observation
Rapid blue chemiluminescence	Positive presumptive reaction
Weak/ambiguous luminescence	Equivocal; further examination required
Luminescence in an unexpected pattern	Consider substrate/environmental interference

Important: A blue luminol reaction is **not definitive proof of human blood**. It indicates a presumptive chemiluminescent reaction that requires further forensic evaluation.

4.3.4. Key Advantages

4.3.4.1 Very high sensitivity

Luminol is particularly useful for detecting **highly diluted or latent blood traces** that may be difficult or impossible to observe visually.

4.3.4.2 Detection of cleaned or diluted stains

It can assist investigators in locating areas where blood may have been **washed, diluted or otherwise rendered visually inconspicuous**.

4.3.4.3 Large-area screening

Unlike a swab-based test that samples a small location, luminol can be used to examine relatively large areas for possible bloodstain patterns.

4.3.4.4 Crime-scene visualization

The characteristic blue chemiluminescence can help investigators identify previously unrecognized areas requiring detailed examination and sampling.

4.3.5. Limitations

4.3.5.1 Presumptive nature

Luminol is a **presumptive test** and cannot establish that the detected material is human blood.
Therefore:

Positive luminol reaction ≠ confirmed human blood

A subsequent human-blood-specific examination is required where appropriate.

4.3.5.2 False-positive reactions

Luminol chemiluminescence is not completely specific to blood. Various substances possessing suitable catalytic or oxidative properties may produce luminescence.

Potential interferents include:

- Certain metals
- Oxidizing chemicals
- Bleaching agents
- Some plant materials
- Other substances capable of catalyzing or participating in oxidation reactions

Consequently, the location and pattern of luminescence must be interpreted in conjunction with scene circumstances and appropriate controls.

4.3.5.3 Short duration of luminescence

The emitted light is transient. Therefore, proper documentation and timely observation are important.

4.3.5.4 Dilution of biological material

Application of an aqueous luminol formulation may potentially dilute biological material. Consequently, subsequent sample collection and DNA analysis should be carefully planned.

4.3.5.5 Potential effect on downstream analysis

Where DNA profiling is anticipated, the testing sequence should be validated to ensure that the chosen reagent and application procedure do not compromise subsequent biological examination.

4.3.6. Forensic Applications

Crime-scene investigation

Luminol is primarily valuable for:

- Locating latent bloodstains
- Detecting diluted blood
- Identifying possible cleaned blood areas
- Screening floors, walls and other large surfaces
- Guiding subsequent evidence collection
- Assisting reconstruction of suspected bloodshed events

4.3.7. Other Scientific Applications of Luminol

Although the present study focuses on forensic blood detection, luminol-based chemiluminescence has broader analytical applications.

Biochemical analysis

Chemiluminescent luminol systems are used in analytical and biochemical assays, including detection systems associated with enzyme-mediated reactions.

Immunoassays

Chemiluminescence can be incorporated into highly sensitive analytical detection platforms.

Analytical chemistry

Luminol chemistry has also been investigated for the detection of various catalytic species and oxidative processes.

For a forensic bloodstain paper, however, these applications should remain secondary and the manuscript should focus primarily on **crime-scene blood detection and forensic validation**.

4.3.8. Quality-Control Considerations

For forensic application, appropriate controls should be incorporated.

Positive control

A known blood sample should produce the expected chemiluminescent response.

Negative control

A clean substrate should not produce the characteristic response under the validated conditions.

Substrate control

Unstained portions of the same substrate should be examined where appropriate to identify substrate-associated background reactions.

These controls are particularly important because luminol is highly sensitive and is not completely specific for blood.

4.3.9. Recommended Crime-Scene Workflow

Suspected/hidden bloodstain
↓
Visual examination & photography
↓
Luminol-based presumptive screening
↓
Documentation of chemiluminescent pattern
↓
Targeted collection of biological material
↓
Human-blood-specific examination
↓
DNA extraction and profiling, where appropriate
↓
Forensic interpretation

4.3.10. Recommended Research-Paper Table

Table X. Analytical and forensic characteristics of luminol-based blood detection

Parameter	Luminol Test
Test type	Presumptive chemiluminescent test
Principal analyte	Blood-associated heme catalytic activity
Reaction medium	Alkaline
Oxidizing system	Validated peroxide/oxidant system
Catalytic component	Heme/iron-associated activity
Observable response	Blue chemiluminescence
Approximate emission maximum	~425 nm
Major advantage	Very high sensitivity for latent/diluted stains
Human-specific?	No
Major limitation	Potential chemical/environmental interference
Crime-scene application	Visualization and localization of suspected blood
Documentation	Low-light photography/video
Confirmatory requirement	Yes
Downstream examination	Human blood confirmation and/or DNA profiling

5. Confirmatory Human Blood Testing

Samples demonstrating presumptive reactions will be evaluated using an appropriate validated human-hemoglobin-specific immunochromatographic assay.

The confirmatory assay will be interpreted according to the manufacturer's validated laboratory procedure and appropriate positive and negative controls.

Immunochromatographic assays targeting human hemoglobin are widely used because of their rapidity and high specificity. However, false-negative results can occur because of target degradation, excessive antigen concentration,

detergents, washing and interference from chemicals used during previous testing.

5.1. Purpose

Presumptive tests such as **Kastle–Meyer, Leucomalachite Green (LMG), and luminol** indicate the possible presence of blood but do not establish that the stain is **human blood**. Therefore, a positive presumptive reaction should be followed, where appropriate, by a **human-blood-specific confirmatory examination**.

5.2. Human Hemoglobin-Specific Testing

One commonly used approach is an **immunochromatographic assay targeting human hemoglobin (Hb)**.

- **Principle:** The test uses antibodies that specifically recognize human hemoglobin or a selected human hemoglobin epitope.
-

- **Sample extraction:** A portion of the suspected stain is extracted using the validated laboratory extraction procedure.
- **Antigen–antibody interaction:** If human hemoglobin is present, it binds to the specific antibodies incorporated into the test system.
- **Signal development:** Formation of an antigen–antibody complex produces a visible test line in the immunochromatographic device.
- **Interpretation:** Development of the appropriate test-line pattern is interpreted according to the manufacturer's validated criteria as **positive for human hemoglobin**.

Simplified principle

Suspected bloodstain → extraction → human Hb + specific antibody → antigen–antibody complex → visible test signal

5.3. Interpretation

Observation	Interpretation
Valid control line + test line	Positive for human hemoglobin
Valid control line only	Negative for human hemoglobin
No control line	Invalid test; repeat according to SOP
Weak/equivocal test line	Requires repeat/additional examination

A positive human-hemoglobin result provides substantially stronger evidence for **human blood** than a presumptive chemical reaction, but the result should still be interpreted within the laboratory's validated scope and case context.

5.4. Advantages

- **Human-specific:** Designed to distinguish human hemoglobin from many non-human biological materials.
- **Rapid:** Results can generally be obtained much faster than traditional laboratory methods.
- **Simple:** Requires comparatively limited instrumentation.
- **Useful for forensic stains:** Applicable to small or aged bloodstain samples when sufficient target material remains.
- **Complementary to DNA analysis:** Human-blood confirmation and DNA profiling answer different forensic questions and can therefore be used sequentially.

5.5. Limitations

5.5.1 Degradation

Old, environmentally exposed or chemically treated bloodstains may contain insufficient intact hemoglobin for reliable detection.

5.5.2 Chemical interference

Previous exposure to cleaning agents, detergents or other chemicals may affect extraction or antibody–antigen interactions.

5.5.3 Hook/prozone effect

Very high concentrations of target antigen can occasionally produce an atypical or reduced test response in some immunoassay systems. Appropriate dilution/validation procedures can help identify this problem.

5.5.4 Sample limitation

Forensic evidence may be available only in very small quantities. Therefore, sufficient material should be retained for **DNA profiling and other required examinations**.

5.5.5 Not individual identification

Human hemoglobin testing establishes the **human origin of the blood-associated material**, but it **does not identify the individual** from whom the blood originated.

5.6. Role of DNA Profiling

Once human blood has been established, the remaining biological material may be considered for **DNA extraction and STR profiling**, subject to sample quantity, quality and laboratory SOPs.

The analytical questions are different:
Presumptive test → *Is a blood-like reaction present?*
Human Hb test → *Is human hemoglobin detected?*
DNA profiling → *Whose biological DNA profile is present?*
Thus, these methods should not be considered interchangeable.

5.7. Recommended Forensic Workflow

Suspected bloodstain↓
Visual examination↓
Presumptive test
Kastle–Meyer / LMG / Luminol
Human hemoglobin-specific confirmation↓
DNA extraction↓
STR profiling↓
Comparison with reference profile↓
Forensic interpretation

5.8. Recommended Research-Paper Table

Table X. Comparison of presumptive and confirmatory blood testing

Parameter	Presumptive Test	Human Hb-Specific Test	DNA Profiling
Primary purpose	Screening	Human blood identification	Individualization
Example	KM, LMG, luminol	Human Hb immunochromatographic assay	STR analysis
Human-specific	No	Yes, within validated assay limitations	Human DNA
Speed	Rapid	Rapid	Longer
Instrumentation	Minimal	Minimal	Molecular laboratory required
Main output	Presumptive reaction	Human Hb detected/not detected	DNA profile
Individual identification	No	No	Yes, subject to interpretation
Role in workflow	Initial screening	Confirmation	Individual comparison

6. Interference Study

Potential interfering substances relevant to crime scenes will be investigated.

Proposed interference groups

- Household bleach
- Detergent
- Rust/iron-containing material
- Plant-derived material
- Soil
- Cleaning agents
- Food-related biological material
- Animal blood

The objective will be to determine whether these materials generate false-positive reactions or suppress genuine blood reactions.

Substrate-associated interference is particularly important because previous studies have demonstrated false-negative blood-test results caused by components of the substrate itself.

7. Aging Study

Bloodstains will be examined at predefined post-deposition intervals, for example:
Fresh → 1 day → 7 days → 14 days → 30 days → extended aging

The exact intervals should be finalized according to the laboratory's experimental capacity.
The objective will be to determine whether aging changes the response intensity of presumptive and confirmatory tests.

Importantly, the study should not claim that a particular test can determine the exact age of a bloodstain unless a validated aging model demonstrates such capability.

8. Statistical Analysis

The following diagnostic parameters will be calculated.

Sensitivity

Sensitivity=TPTP+FN×100Sensitivity=\frac{TP}{TP+FN}\times100

Specificity

Specificity=TNTN+FP×100Specificity=\frac{TN}{TN+FP}\times100

Positive Predictive Value

PPV=TPTP+FP×100PPV=\frac{TP}{TP+FP}\times100

Negative Predictive Value

NPV=TNTN+FN×100NPV=\frac{TN}{TN+FN}\times100

where:

- **TP** = true positive
- **TN** = true negative
- **FP** = false positive
- **FN** = false negative

Overall misclassification rate will also be calculated.

For comparisons among multiple tests, appropriate statistical approaches may include chi-square/Fisher's exact test for categorical outcomes and logistic regression or generalized linear models for substrate, dilution and aging effects.

For spectroscopic extensions, PCA, PLS-DA, SVM or Random Forest models may be evaluated with independent validation datasets.

9. Proposed Results Presentation

Actual experimental values should be inserted after laboratory experimentation.

Table 1. Detection performance of presumptive tests at different blood dilutions

Dilution	Kastle–Meyer	Leucomalachite Green	Luminol	Human Hb confirmation
Undiluted	Experimental	Experimental	Experimental	Experimental
1:10	Experimental	Experimental	Experimental	Experimental
1:100	Experimental	Experimental	Experimental	Experimental
1:1,000	Experimental	Experimental	Experimental	Experimental
1:10,000	Experimental	Experimental	Experimental	Experimental

Table 2. Effect of substrate

Substrate	KM	LMG	Luminol	Human Hb test
Cotton	—	—	—	—
Paper	—	—	—	—
Glass	—	—	—	—
Metal	—	—	—	—
Wood	—	—	—	—
Tile	—	—	—	—
Soil	—	—	—	—

Table 3. Diagnostic performance

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Misclassification (%)
Kastle–Meyer	To be determined	To be determined	To be determined	To be determined	To be determined

Leucomalachite green	To be determined	To be determined	To be determined	To be determined	To be determined
Luminol	To be determined	To be determined	To be determined	To be determined	To be determined
Human Hb assay	To be determined	To be determined	To be determined	To be determined	To be determined

10. Discussion

The proposed study is designed to demonstrate that blood detection at a crime scene should be regarded as a sequential analytical process rather than as a single-test procedure.

Presumptive tests are valuable because they are rapid, relatively inexpensive and suitable for screening large areas. Among commonly evaluated methods, luminol has demonstrated particularly high sensitivity for dilute and latent stains.

Nevertheless, high sensitivity does not necessarily imply high specificity. A presumptive positive reaction should therefore be regarded as an indication that further examination is required rather than definitive evidence of human blood.

This distinction is particularly important in forensic casework because false-positive reactions may result from environmental materials, oxidizing substances and biological materials. Conversely, false-negative results can occur because of substrate effects, washing, chemical treatment or degradation.

Human-hemoglobin immunochromatographic assays provide an important second stage because they are designed to address the question of whether the detected material is human blood. Recent forensic literature describes these assays as rapid and highly specific, while also emphasizing limitations associated with degradation, chemical interference and destructive sampling.

The sequence of testing is therefore critical. Excessive application of chemical reagents before collection of biological evidence may complicate subsequent analyses. The ultimate forensic objective is frequently DNA profiling; consequently, blood identification should be performed using a strategy that preserves sufficient material for downstream genetic analysis.

Advanced spectroscopic methods represent a promising complementary direction. Raman spectroscopy has demonstrated potential for differentiating bloodstains from environmental interferences, while ATR-FTIR approaches are being investigated for blood identification, species discrimination and aging.

A future extension of the present work could therefore combine conventional presumptive tests with Raman or ATR-FTIR spectroscopy and chemometric classification. Such a strategy could reduce dependence on subjective visual interpretation and potentially provide a more objective, non-destructive analytical platform.

11. Proposed Crime-Scene Testing Algorithm

Suspected stain
↓
Visual examination and photographic documentation
↓
Presumptive blood screening
↓
Positive / suspicious reaction
↓
Collection and preservation of biological evidence
↓
Human hemoglobin-specific confirmatory test
↓
DNA extraction and STR profiling, where appropriate
↓
Forensic interpretation and reporting

This sequential strategy recognizes that presumptive testing and human-blood confirmation answer different analytical questions.

12. Limitations

The proposed study has several limitations.

1. Laboratory-prepared bloodstains may not completely reproduce the complexity of actual crime scenes.
2. Substrate composition can vary considerably between real-world materials.
3. Environmental exposure can alter blood chemistry.
4. Presumptive tests do not establish human origin.
5. Confirmatory immunochromatographic assays can be affected by degradation and chemical interference.
6. Results obtained under controlled laboratory conditions should not automatically be generalized to every casework situation.
7. Spectroscopic approaches require extensive validation before routine forensic implementation.

13. Conclusion

The detection of blood at a crime scene is a multistage forensic process requiring appropriate integration of screening, confirmation and downstream biological analysis. Kastle–Meyer, leucomalachite green and luminol-based methods remain useful presumptive approaches, particularly for rapid screening and visualization of suspected bloodstains. However, their results must be interpreted cautiously because environmental and substrate-related interferences can affect analytical performance.

Human-hemoglobin-specific immunochromatographic testing provides an important confirmatory step, while preservation of the biological sample for subsequent DNA analysis should remain a central consideration. Modern spectroscopic methods, particularly Raman spectroscopy and ATR-FTIR coupled with chemometric analysis, offer promising opportunities for developing rapid and minimally destructive forensic bloodstain examination.

The proposed comparative validation study can therefore provide practical data for selecting appropriate blood-detection methods according to stain concentration, substrate, aging and environmental interference. Such evidence-based validation is essential for improving the reliability and scientific defensibility of bloodstain examination in forensic casework.

14. REFERENCES

1. Webb JL, Creamer JI, Quickenden TI. A comparison of the presumptive luminol test for blood with four non-chemiluminescent forensic techniques. *Luminescence*. 2006;21:214–220. DOI: 10.1002/bio.908.
2. Barni F, Lewis SW, Berti A, Miskelly GM, Lago G. Forensic application of the luminol reaction as a presumptive test for latent blood detection. *Talanta*. 2007;72:896–913. DOI: 10.1016/j.talanta.2006.12.045.
3. Castelló A, Francès F, Verdú F. Bloodstains on leather: Examination of false negatives in presumptive test and human hemoglobin test. *Journal of Forensic Sciences*. 2017;62:1308–1313. DOI: 10.1111/1556-4029.13407.
4. Rosenblatt R, Halámková L, Doty KC, de Oliveira EAC Jr, Lednev IK. Raman spectroscopy for forensic bloodstain identification: Method validation vs. environmental interferences. *Forensic Chemistry*. 2019;16:100175. DOI: 10.1016/j.forc.2019.100175.
5. Comparative evaluation of presumptive tests for detection of blood on dark materials. *Journal of Forensic Sciences*. 2019. The study reported strong performance of luminol for diluted blood on several dark substrates.
6. Immunochromatographic Detection of Human Blood: A Forensic Review. *Separations*. 2024;11:66.
7. Recent advances in ATR-FTIR spectroscopy for forensic bloodstain analysis, including blood identification, species discrimination and aging. *Microchemical Journal*. 2025;112655. DOI: 10.1016/j.microc.2025.112655.