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A comparison of ABAcard® Hematrace® and RSID™-Blood tests on dried, diluted bloodstains treated with leucocrystal violet or luminol

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ABSTRACT

Presumptive tests for blood, such as luminol and leucocrystal violet (LCV), are often used by forensic officers when screening for latent (non-visible) or diluted bloodstains at crime scenes. Where positive reactions are observed, a confirmatory test for the presence of blood may be implemented. This study aimed to compare the efficacy of two such confirmatory tests (ABAcard® Hematrace® and RSID™-Blood) on dried, diluted bloodstains that were enhanced using either the Grodsky luminol formulation or LCV. Eighteen replicates per dilution (1:10, 1:100), enhancement, and confirmatory test were performed (144 bloodstains). The RSID™-Blood test produced false negative results for all luminol-enhanced bloodstains, regardless of dilution. This test performed slightly better for bloodstains enhanced with LCV, returning approximately 50% positive results. In contrast, the ABAcard® Hematrace® test performed well, returning positive detections for all luminol-treated bloodstains, and all but two LCV-enhanced stains (both 1:100 dilution). Significant differences were observed between the test results and suggested a potential inhibitory effect on the RSID™-Blood test from the Grodsky luminol formulation and, to a lesser extent, LCV. This research has demonstrated that the RSID™-Blood test is not a reliable confirmatory test faint or latent bloodstains enhanced with luminol or LCV.

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Introduction

It is not uncommon for bloodstains detected at crime scenes to be diluted, particularly where there has been an attempt made to clean the stained area. This may be as simple as the offender washing their bloodied hands in a bathroom sink or attempting to wash bloodstains off a wall or the interior of a vehicle. It is generally recommended that crime scene examiners should first use non-destructive methods such as a visual examination and forensic light sources to screen for the presence of blood[1]. Following visual screening, more destructive methods, such as the use of chemical reagents (e.g., luminol), can then be considered to screen for blood[1,2]. When enhancing impressions in blood (usually on a horizontal surface) leucocrystal violet (LCV) reagent can be used[3]. Both luminol and LCV

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can be classified as peroxidase-based blood enhancement tests[4] and are presumptive due to lack of specificity in testing for human blood, that is, they only indicate the possible presence of blood[5].

LCV reagent is generally used to enhance impression evidence in blood and, as noted by Bodziak[3], when LCV and hydrogen peroxide come into contact with haemoglobin in blood a catalytic reaction occurs that results in a purple colour change. LCV contains the fixative 5-sulphosalicylic acid which allows LCV to be applied in a single step and has been reported to detect diluted blood up to 1:10,000[6]. In contrast, luminol reagent is often used to screen for the presence of latent blood (non-visible) as well as to enhance possible impression evidence in blood. The peroxidase-like reaction of haemoglobin is also used to catalyse the oxidation of luminol resulting in a blue chemiluminescence[5]. The intensity of the emitted light is low and brief in duration; therefore, it is recommended that a darkened room and photography are required to record this chemiluminescence[7,8]. Luminol is highly sensitive and has been reported to detect up to a 1:1,000,000 dilution of blood[9]. There are several commercially available luminol kits, such as Bluestar® Forensic (Bluestar® Forensic, Monte Carlo, Monaco), whose formulations are protected by patent and not listed in the literature. Most luminol solutions (including tablet-based commercial kits) contain hydrogen peroxide as the oxidizing agent activator. However, an alternative formulation, proposed by Grodsky et al[5], comprises a mixture of luminol, sodium carbonate and sodium perborate (the oxidizing agent activator) dissolved in distilled water (hereafter referred to as the Grodsky luminol formulation). This formulation is still considered to be the most commonly used luminol formulation[10] and remains popular because individual components can be purchased in bulk at lower cost than commercial luminol kits whilst maintaining test efficacy. Both LCV and luminol detect blood via the oxidative nature of haemoglobin; however, because of the multitude of oxidizing substances that can also catalyse these reactions, both are considered presumptive tests only for the presence of blood. Therefore, confirmatory testing of putative bloodstains which exhibit a positive reaction to luminol and LCV is necessary. A positive confirmatory blood test will assist in determining if the bloodstain is human in origin and suitable for sampling for DNA analysis. Two commercially available confirmatory tests for the presence of human blood are Rapid Stain Identification of Human Blood (RSID™-Blood) and the ABAcard® Hematrace® test.

The ABAcard® Hematrace® (Abacus Diagnostics, West Hills, CA, USA) is an immunochromatographic test that detects the blood of higher primates[11]. The mobile monoclonal antihuman antibody in the ABAcard® Hematrace® test reacts with human haemoglobin, forming a mobile antigen-antibody complex that migrates through the tests' absorbent

Table 1. Number of positive test results, total replicate tests, and 95% confidence intervals for each treatment group.

	Luminol		LCV	
Test	**1:10**	**1:100**	**1:10**	**1:100**
ABAcard® Hematrace®				
Positive tests/total tests	18/18	18/18	17/17	16/18
95% CI	82.4–100%	82.4–100%	81.6–100%	67.2–96.9%
RSID™-Blood				
Positive tests/total tests	0/18	0/18	10/18	8/18
95% CI	0–17.6%	0–17.6%	33.7–75.4%	24.6–66.3%

membrane until it reaches an immobilized polyclonal antihuman haemoglobin antibody[12]. This antibody captures the migrating complex so that an antibody-antigen-antibody is formed and purple dye particles form a purple band in the test area indicating that blood is present. Importantly, ABAcard® Hematrace® is reported to work on bloodstains conditioned with LCV and luminol[12]. The test is highly sensitive; however, known false positives include ferret blood[11] and saliva[13]. Further, the test is reported to suffer from a high dose hook effect[12], which occurs when there is excess free haemoglobin that is not bound to the antibody when it reaches the test result area. The antibodies immobilized at the test strip become saturated with this free haemoglobin, preventing the antigen-antibody complex from binding. Thus, the purple positive test line does not form and the test results appear negative. A solution to this problem is to dilute the extract to 1:10 or 1:100 dilution (to reduce the amount of free haemoglobin present) and re-run the sample on a fresh test card[13].

RSID™-Blood (Independent Forensics, Hillside, IL, USA) is another commercially available confirmatory test for the presence of human blood[14]. It is an immunochromatographic assay that uses two mouse monoclonal antibodies to detect glycophorin-A, a red blood cell membrane-specific protein. Like the ABAcard® Hematrace® test, the sample fluid travels through an absorbent medium and if human blood is present, a red test line is formed. Previous work suggests that this test is specific to human blood only and does not cross-react with other higher primate or ferret blood[14]; however, false negative results have been noted if the substrate sampled was asphalt, sand or a cactus plant[15]. These tests are also not considered to be as sensitive as haemoglobin-based blood detection strips[12].

Nicloux and Bressler[16] compared the Haemoglobin (Hb) and RSID™-Blood tests on a range of blood dilutions (from whole blood to 1:2000) that had been treated with Bluestar® Forensic Magnum, Bluestar® Forensic, Lumiscene and Lumiscene Ultra (Loci Forensics B.V., Nieuw-Vennep, The Netherlands). They showed that the RSID™-Blood test only exhibited positive results with whole blood and the 1:10 dilution, whereas the Hb test gave positive results also with 1:100 dilutions treated with Bluestar® Forensic Magnum, Bluestar® Forensic and Lumiscene. The authors argued this may be due to RSID™-Blood being more specific, detecting glycophorin-A, in comparison to tests which rely on haemoglobin detection that performed better with diluted blood. Turrina et al[17] used RSID™-Blood to test air-dried bloodstains on paper at dilutions of 1:20, 1:200, 1:1000 and 1:2000, following treatment with Bluestar® Forensic. Positive results were obtained from samples diluted to 1:20 and 1:200. Similarly, Stewart et al[18] showed limited reduction in positive blood detection of a blood depletion series treated with Bluestar® Forensic Magnum using RSID™-Blood tests. Taken together, although there appears to be conflicting information regarding the sensitivity of RSID™-Blood, it is generally accepted that it is less sensitive than haemoglobin-based tests. Likewise, the impact on these tests from commercial luminol enhancements appears to vary, though RSID™-Blood appears to be affected more. No studies to date have assessed the impact of LCV or the Grodsky luminol formulation on these tests.

To address this, the present study aims to compare the ability of the ABAcard® Hematrace® test and the RSID™-Blood test to detect dried diluted bloodstains treated with LCV or the Grodsky luminol formulation. Diluted and dried bloodstains more closely mimic the condition of blood found at most crime scenes. Our null hypothesis was that

there would be no significant difference in blood detection between the tests across either enhancement chemistry or dilution. The results of this study will contribute greatly to understanding the operational limits of these confirmatory blood tests and therefore assist forensic officers globally in determining the optimal methods for treatment and interpretation of evidence.

Materials and methods

An initial pilot study was undertaken to determine which dilution rates were viable for the main study. Human blood drawn from the lead author was diluted to 1:100 and 1:1000 with distilled water and six replicates of 50 µL of each dilution was placed into clean petri dishes. After air drying for approximately four hours, the bloodstains were then tested with RSID^{TM}-Blood as per the manufacturer's instructions. No positive results were observed for the 1:1000 dilution, whereas all six replicates of the 1:100 dilution returned positive results. Based on these results 1:10 and 1:100 dilution rates were used in the main experiment.

For the main study, petri dishes were cleaned by soaking in 5% Trigene II solution (Blackwoods, Hamilton, QLD, Australia) for five minutes and rinsed with distilled water. The surface of each petri-dish was then wiped down with 70% ethanol (Banksia Scientific, Bulimba, QLD, Australia). Petri dishes were used as a proxy for non-porous surfaces that are commonly encountered at crime scenes, and because they were easily cleaned and provided a consistent surface that reduced potential substrate variability. Blood was drawn from the lead author by a trained phlebotomist; no anti-coagulants were added, and blood was diluted to 1:10 and 1:100 with distilled water for subsequent experiments. This research was conducted under Griffith University Human Research Ethics Committee oversight and was considered out-of-scope for formal approval.

The Grodsky luminol formulation was prepared by dissolving 0.5 g of luminol (Labtek, Brendale, QLD, Australia) with 25 g of sodium carbonate (Banksia Scientific) in 250 mL distilled water, and 3.5 g of sodium perborate (Banksia Scientific) in 250 mL of distilled water. The two solutions were then combined immediately prior to use. The luminol reagent was checked using positive and negative controls (a blank sterile swab for the negative control and a premade blood swab as positive control). The LCV reagent was prepared by dissolving 9 g of sulpho-salicylic acid (Sigma Aldrich, Castle Hill, NSW, Australia) in 450 mL of distilled water (the fixative). The fixative was combined with 45 mL of 30% hydrogen peroxide (Banksia Scientific) and mixed until dissolved using a magnetic stirrer. Then 0.6 g of LCV (Sigma Aldrich) was added to the solution and stirred until dissolved. The LCV reagent was checked using positive and negative controls as described for luminol.

Fifty microlitres of 1:10 diluted blood was deposited using a micropipette into each of 36 petri dishes and 50 µL of 1:100 diluted blood deposited into each of a further 36 petri dishes. The bloodstains were air dried at room temperature for approximately 18 hours. Bloodstain volume was consistent for all samples and bloodstain size appeared the same but was not measured. Eighteen bloodstains of each dilution were treated with either 50 µL of Grodsky luminol reagent or 50 µL of LCV reagent using a micropipette. The chemically treated bloodstains were allowed to air dry for approximately 20 minutes. The bloodstains were then sub-sampled with two sterile rayon swabs (Lovell Surgical Supplies,

Melbourne, Australia), with one tested using ABAcard® Hematrace® and the other with the RSID™-Blood test, both according to the manufacturer's instructions. The following controls were included: no blood, no enhancements; no blood+enhancements; 1:10 blood, no enhancements; 1:100 blood, no enhancements.

Statistical analysis

The results for each RSID™-Blood test and ABAcard® Hematrace® test were recorded as either positive or negative. The Wilson's method[19] was used to calculate 95% confidence intervals (CI) as implemented in Epitool[20]. Confidence intervals were constructed for each of the eight treatment groups (2 confirmatory tests x 2 enhancements x 2 dilutions) to infer the probability of detecting a true positive. Overlapping confidence intervals between treatment groups indicated no significant difference ($p > 0.05$) between the treatment groups, whereas non-overlapping intervals suggest statistically significant difference ($p < 0.05$) between the treatment groups. In addition, a chi-square test of independence was performed on the total data set to test for significant differences among treatment groups. Standard residuals of the observed/expected values were calculated for each treatment group to determine which treatment had the greatest effect on the test of independence. Individual chi-square goodness of fit tests were then performed on each treatment to determine whether any deviated significantly from an expectation of 99% positive results (1 false negative in 100 tests). We consider this to be a reasonable, if conservative, expectation for the efficacy of these tests. All chi-square tests were conducted in R[21].

Results

All positive and negative controls adopted for this study worked correctly with no false positives or false negatives observed. For one replicate of the 1:10 dilution for LCV tested with ABAcard® Hematrace®, considerable debris remained in the test well rendering this replicate invalid; thus, only 17 replicates were available for this treatment group. All ABAcard® Hematrace® tests of luminol-treated blood returned positive results for both dilutions giving an 82.4–100% probability of detecting a true positive result (Table 1). Only two out of 18 ABAcard® Hematrace® tests of the 1:100 dilution treated with LCV returned a false negative (95% CI: 67.2–96.9%). In contrast, no RSID™-Blood tests returned positive results for bloodstains treated with luminol for either of the dilutions (95% CI: 0–17.6%). Slightly better results were observed for LCV-treated bloodstains with the RSID™-Blood test: for the 1:10 dilution, ten of 18 replicates were positive (95% CI: 33.7–75.4%), whereas for the 1:100 dilution, eight of 18 replicates were positive (95% CI: 24.6–66.3%). Overlap of CIs suggested that there were no significant differences in sensitivity among ABAcard® Hematrace® tests associated with dilution or blood enhancement (Figure 1). However, CIs for RSID™-Blood tests suggested significant differences between blood enhancements. In general, CIs across the two tests did not overlap, with the exception of those for ABAcard® Hematrace® tests of luminol-enhanced 1:100 bloodstains and RSID™-Blood tests for LCV-treated 1:10 bloodstains.

Chi-square tests provided additional support to this interpretation of the results. The global test of independence between ABAcard® Hematrace® and RSID™-Blood test

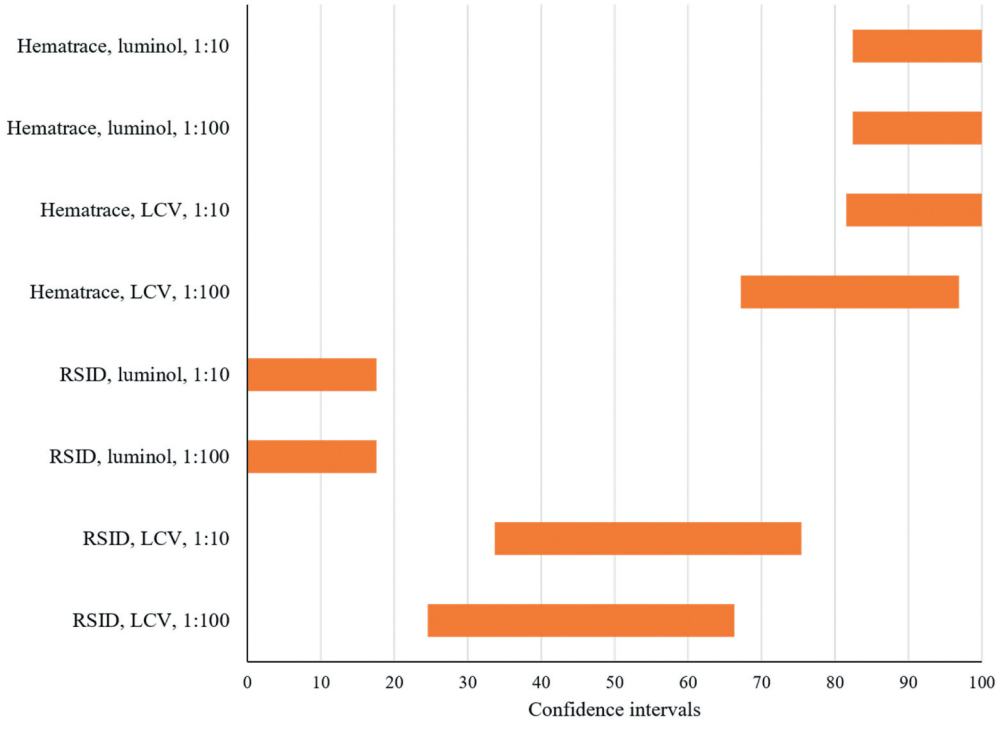

Figure 1. Diagrammatic representation of 95% confidence intervals for the eight treatment groups which represent the probability of obtaining a positive result, as per Table 1. Test names on the *y*-axis are abbreviated as follows: Hematrace = ABAcard® Hematrace®; RSID = RSID™-Blood.

results suggested their sensitivities were significantly different ($\chi = 80.92$, $p < 0.05$). Similarly, interrogation of standard residuals for results grouped by confirmatory tests suggested that it was the RSID™-Blood test results that drove this difference (Table 2). When results were separated into blood enhancement treatments, ABAcard® Hematrace® results were not significant for luminol and only marginally significant for LCV ($p = 0.005$), whereas RSID™-Blood results significantly differed from the expected ratio for both enhancements. Breaking the data down further into the eight treatment groups, all RSID™-Blood test groups were significantly different from expectations, whereas only the ABAcard® Hematrace® results of LCV-treated 1:100 dilution bloodstains were statistically significant.

Discussion

Overall, the ABAcard® Hematrace® test performed relatively well across the blood enhancement and dilution regimes tested here. Only two out of 71 tests returned false negatives (both from 1:100 diluted bloodstains treated with LCV), which implies the sensitivity of this test significantly decreases for diluted blood enhanced with LCV. In contrast, the RSID™-Blood test performed quite poorly, producing false negative results for all bloodstains treated with luminol, and for almost half of the LCV-enhanced bloodstains regardless of dilution. This result was surprising and has significant implications for

Table 2. Summarized test results and standard residuals based on an expected 99% positive test rate. Values in bold returned significant chi-square tests; *p < 0.05, **p < 0.001.

Test	Positive	Negative	Expected positive	Expected negative	Positive standard residual	Negative standard residual
Full dataset						
ABAcard® Hematrace®	69	2	70.29	0.71	−0.1539	1.531
RSID™-Blood	18	54	71.28	0.72	−6.3107	**62.791****
Enhancement only						
ABAcard® Hematrace®, luminol	36	0	35.64	0.36	0.0603	−0.600
ABAcard® Hematrace®, LCV	33	2	34.65	0.35	−0.2803	**2.789***
RSID™-Blood, luminol	0	36	35.64	0.36	−5.9699	**59.4****
RSID™-Blood, LCV	18	18	35.64	0.36	−2.9548	**29.4****
Dilution & enhancement						
ABAcard® Hematrace®, luminol, 1:10	18	0	17.82	0.18	0.0426	−0.424
ABAcard® Hematrace®, luminol, 1:100	18	0	17.82	0.18	0.0426	−0.424
ABAcard® Hematrace®, LCV, 1:10	17	0	16.83	0.17	0.0414	−0.412
ABAcard® Hematrace®, LCV, 1:100	16	2	17.82	0.18	−0.4311	**4.289****
RSID™-Blood, luminol, 1:10	0	18	17.82	0.18	−4.2214	**42.002****
RSID™-Blood, luminol, 1:100	0	18	17.82	0.18	−4.2214	**42.002****
RSID™-Blood, LCV, 1:10	10	8	16.83	0.17	−1.6649	**18.991****
RSID™-Blood, LCV, 1:100	8	10	17.82	0.18	−2.3263	**23.146****

operational use of this confirmatory test on enhanced bloodstains, particularly those treated with the Grodsky luminol formulation.

Previous studies have reported positive results from RSID™-Blood tests of diluted blood treated with commercial luminol kits between dilutions of 1:10 and 1:200[14–16]. However, no studies exist concerning RSID™-Blood returning false negatives from bloodstains treated with the Grodsky luminol formulation. It is possible that this particular formulation of luminol has interfered with RSID™-Blood; however, the mechanism is unclear. The luminol formulation used in this study utilized sodium perborate as the activator (oxidizer), while the commercial luminol products used in the other studies have a hydrogen peroxide activator. Therefore, it is possible the sodium perborate in the Grodsky formulation interfered with the immunoassay.

False positive or false negative results are known to occur due to antibody interferences in other immunoassays[22]. This can occur when a sample induces changes in pH and ionic strength of the reaction mixture, or from pre-analytical variables affecting the sample analyte concentration by physically masking the antibody label. Stolk and Scheijen[23] reported interference of immunoassays to detect MDMA, cocaine and cannabinoids in urine specimens that had been adulterated with denture cleaning tablets containing 1.5% sodium perborate. They surmised that the mechanism of interference may be the oxidation of the drug/metabolite by sodium perborate and an increase in the salt concentration. Furthermore, a validation study of the Hexagon OBTI (Sirchie, Youngsville, USA) blood immunoassay that investigated the impacts of various luminol formulations on the test's sensitivity identified that formulations containing sodium perborate required adjustment of their pH to <12 to produce positive results for any blood dilutions[24]. The preparation of the Grodsky luminol formulation used here does not include pH adjustment and thus this may be the cause of the observed interference with the RSID™-Blood assay. Alternatively, it has been reported that the levels of the specific protein targeted by RSID™-Blood, glycophorin-A, can vary individually and this may result in a low intensity of discolouration at the Test (T) site[14]. However, it is unlikely this contributed to the poor results observed for luminol-treated bloodstains here, as the positive controls worked as expected and better results were seen in the LCV treated bloodstains. Thus, we contend that the interaction between sodium perborate, the pH of the Grodsky luminol solution, and haemoglobin in the bloodstains is interfering with the glycophorin-A immunoassay in some way. Further study is recommended to investigate why this luminol formulation resulted in false negative results for all RSID™-Blood tests in comparison to other formulas of luminol.

In contrast to the poor RSID™-Blood test results on luminol-enhanced bloodstains, these tests showed moderate success on LCV-enhanced bloodstains. The oxidizing agent in LCV is hydrogen peroxide rather than sodium perborate, meaning the reduced success rate compared with unenhanced blood cannot be explained by the same mechanism. Some previous work on body fluid identification which involved detection of glycophorin-A suggested that LCV can have an adverse effect on detection[25]. This study reported LCV is highly acidic and its application results in high concentrations of hydrogen peroxide, sodium acetate and sulpho-salicylic acid on the substrate surface. The authors concluded that interference of LCV on the detection of glycophorin-A may be due to how the LCV is applied, sample variability, and the reactivity of LCV itself. It remains possible that LCV has

a similar inhibitory effect on RSID™-Blood's ability to detect glycophorin-A; however, this appears to be less strong than the Grodsky luminol formulation.

The main aim of this study was to compare the efficacy of ABAcard® Hematrace® and RSID™-Blood confirmatory tests for use on diluted, enhanced bloodstains at crime scenes. When used on visible, undiluted crime scene stains RSID™-Blood is known to perform satisfactorily, but it is not as easy or practical to use at the scene as the ABAcard® Hematrace® test. The RSID™-Blood universal extraction buffer needs to be measured and placed in a sample tube. A sterile swab is then used to sub-sample the questioned stain, and this swab is then placed in the extraction buffer for one to two hours. After this time the extraction buffer needs to be pipetted into the test well of the RSID™-Blood card. Test results can take up to 10 minutes. The pipette, sample tube for the extraction buffer and swab are not provided unless the more expensive field kit is purchased. In comparison, the ABAcard® Hematrace® test comes with individual plastic bottles of premeasured single-use extraction buffer, sterile swab and a pipette making it more suitable for crime scene use. The sub-sampled stain only needs to remain in the extraction buffer for 10 minutes. Once the extraction buffer is placed into the sample well of the ABAcard® Hematrace® test card, results can take up to 5 minutes. Furthermore, the ABAcard® Hematrace® test has a reported sensitivity of approximately 1:32,000 compared with the 1:100 to 1:200 range for RSID™-Blood. Potential high dose hook effects can easily be rectified by diluting the extraction buffer and re-running the sample, and the reactivity to higher primate (other than human) or ferret blood is not a practical issue at crime scenes.

Given the practical features of these tests, and the more frequent use of luminol compared with LCV for locating or enhancing bloodstains in Queensland, we argue that the RSID™-Blood test is not a viable option for confirming the presence of enhanced diluted or latent blood at crime scenes. This is a serious operational concern: false negative results could have dire consequences for investigations, potentially resulting in bloodstains not being detected and thus critical evidence being overlooked. Further research is required to understand the cause of apparent inhibition of these tests from bloodstain enhancement chemicals, and what additional treatments may ameliorate this effect. Such research would benefit from investigating the influence of delivery mechanism for enhancement reagents (pipetting/spraying). We used a micropipette to control the delivered volume and reduce variability; however, crime scene examiners commonly spray luminol/LCV onto surfaces resulting in more uneven application. The impact of this on subsequent confirmatory blood tests should also be explored in future research.

Conclusions

The RSID™-Blood test produced false negative results for all test bloodstains (1:10 and 1:100 dilution) treated with the Grodsky luminol formulation. This represents the first time this has been reported in the literature and suggests that the Grodsky luminol formulation has an unknown inhibitory effect on the efficacy of the RSID™-Blood test. The implications of this previously unknown inhibitory effect for crime scene examination include the false negative classification of blood, and prevention of a bloodstain being sampled for DNA analysis. The ability of the RSID™-Blood test to confirm the presence of human blood from bloodstains of the same dilutions treated with LCV was better, but not ideal,

and the ABAcard® Hematrace® test outperformed the RSID™-Blood test on all counts. In conclusion, at crime scenes where bloodstains are likely to be dilute (e.g., as a result of clean up), or luminol or LCV have been used on faint or non-visible (latent) bloodstains, we recommend that the RSID™-Blood test is not a viable confirmatory test.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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