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Short communication

Developmental validation of a novel lateral flow strip test for rapid identification of human blood (Rapid Stain Identification™-Blood)

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Abstract

Human blood is the body fluid most commonly encountered at crime scenes, and blood detection may aid investigators in reconstructing what occurred during a crime. In addition, blood detection can help determine which items of evidence should be processed for DNA-STR testing. Unfortunately, many common substances can cause red-brown stains that resemble blood. Furthermore, many current human blood detection methods are presumptive and prone to false positive results. Here, the developmental validation of a new blood identification test, Rapid Stain Identification™-Blood (RSID™-Blood), is described. RSID™-Blood utilizes two anti-glycophorin A (red blood cell membrane specific protein) monoclonal antibodies in a lateral flow strip test format to detect human blood. We present evidence demonstrating that this test is accurate, reproducible, easy to use, and highly specific for human blood. Importantly, RSID™-Blood does not cross-react with ferret, skunk, or primate blood and exhibits no high-dose hook effect. Also, we describe studies on the sensitivity, body fluid specificity, and species specificity of RSID™-Blood. In addition, we show that the test can detect blood from a variety of forensic exhibits prior to processing for DNA-STR analysis. In conclusion, we suggest that RSID™-Blood is effective and useful for the detection of human blood on forensic exhibits, and offers improved blood detection when compared to other currently used methods.
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Keywords: Human blood detection; Lateral flow strip test; Stain identification; DNA-STR analysis

1. Introduction

Most human blood detection tests that are currently available to the forensic community are presumptive, i.e., results from these tests are consistent with the presence of human blood, but require further testing for confirmation of the initial presumption [1–4]. Specifically, many blood detection tests rely on the iron chelated to heme present in blood, and its ability to act as a reducing agent (eg., reduced phenolphthalein and *ortho*-tolidine) [1–4]. In addition to reacting with animal blood, these tests are known to give false positives with a variety of common substances including iron and copper containing items such as coins and rust [1–4].

Other blood detection methods rely on hemoglobin protein detection. As stated in the product inserts of hemoglobin based lateral flow strip tests, these tests exhibit cross-reaction with animal blood (ferret, skunk, and primate) [5–9]. Furthermore, the hemoglobin-based tests, while sensitive, are susceptible to false negatives due to the high-dose hook effect [5,6,10]. A high-dose hook effect refers to weak positive or false negative results seen with lateral flow strip tests when high levels of target are present in the tested sample [10].

The weaknesses of these widely used blood detection methods can result in legal and scientific challenge, and they therefore illustrate the need for superior blood detection tests that are specific for human blood and not susceptible to high dose induced false negatives. Here we present the developmental validation of a new lateral flow strip test for the detection of human blood that exhibits neither animal blood cross-reactivity nor high-dose hook effect induced false negatives.

2. Materials and methods

2.1. RSID™-Blood kit components

Each RSID™-Blood kit contains 25 RSID™-Blood lateral flow strip tests, 25 ml of RSID™-Blood Extraction Buffer, 10 ml of RSID™-Blood Running Buffer, an RSID™-Blood

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Technical Information Sheet, and an RSIDTM-Blood Protocol Sheet. RSIDTM-Blood Extraction Buffer has been formulated to promote efficient extraction of the glycophorin A antigen from evidence samples, while RSIDTM-Blood Running Buffer promotes consistent test flow rates and eliminates background signal. Also, the RSIDTM-Blood buffers are compatible with downstream DNA analysis procedures, allowing users to employ single tube stain identification and DNA extraction approaches that optimize evidence sample conservation.

2.2. *RSIDTM-Blood kit procedure (Fig. 1)*

Stains may be sampled by swabbing or cutting. Next, the swab head or cutting is incubated in RSIDTM-Blood Extraction Buffer for 1 h. An aliquot of the extract (up to 20 μl) is brought up to 100 μl with RSIDTM-Blood Running Buffer and added to the sample window (S) of an RSIDTM-Blood cassette. Test results are read 10 min after sample addition. One line at the control position (C) indicates that the test performed properly, but no human blood was detected. Two lines, one at the control position (C) and one at the test position (T), indicate that the test performed correctly and human blood was detected.

2.3. *Configuration of RSIDTM-Blood lateral flow test*

RSIDTM-Blood is an immunochromatographic assay that uses two monoclonal antibodies to detect glycophorin A, a red blood cell membrane specific protein [11]. Importantly, each antibody recognizes a distinct glycophorin A epitope [12]. The test strip consists of overlapping components arranged such that the tested fluid is transported from the conjugate pad to the membrane and is finally retained on the wick. The conjugate pad and membrane are treated, prior to assembly into a plastic housing, such that the user need only add his/her extract in diluent buffer (provided) to initiate the test. Once the tested sample is added to the sample window, the running buffer and sample diffuse through the conjugate pad, which has pre-dispersed colloidal gold conjugated to anti-human glycophorin A monoclonal antibodies. The diluent redissolves the colloidal gold labeled anti-glycophorin A antibodies which will bind glycophorin A if it is present in the sample. Glycophorin A-colloidal gold antibody complexes are transported by bulk fluid flow to the membrane phase of the test strip. The immobilized anti-glycophorin A antibodies on the test line capture the glycophorin A-antibody-gold complexes, producing a red line at the test position. If no human glycophorin A is present in the sample, then gold-conjugated antibody–antigen complexes do not form, and colloidal gold will not be accumulated at the test line. The anti-mouse IgG on the control line captures any mouse antibodies flowing past the test line, producing a red line at the control position. This demonstrates that the sample fluid was transported through the length of the test, and that the components of the strip test functioned properly.

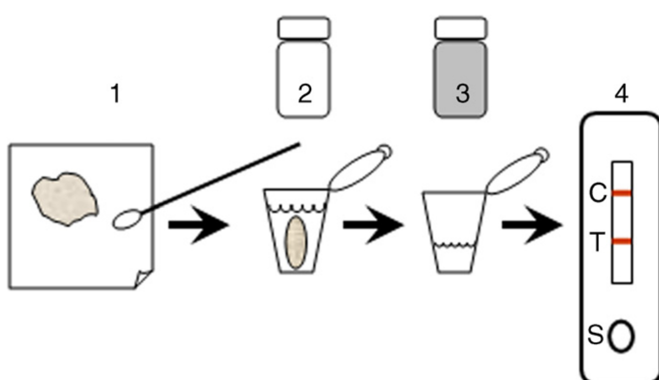

Fig. 1. RSIDTM-Blood method diagram. 1, Stain is sampled by sponging with moistened cotton swab. If feasible, the stain can be sampled by removal of a cutting. 2, Swab head or cutting is incubated in provided RSIDTM-Blood Extraction Buffer for 1 h. 3, An aliquot of extract is removed and the volume is increased to 100 μl with provided RSIDTM-Blood Running Buffer. 4, The entire 100 μl analyte volume is added to the sample window (S) of RSIDTM-Blood cassette. Test results are read 10 min after sample addition. One band at the control position (C) indicates that the test performed properly, but no human blood was detected. The presence of two bands, at the control (C) and test (T) positions, indicates that the test performed properly, and human blood was detected.

2.4. *Quantification of RSIDTM-Blood lateral flow test results*

In order to minimize operator variance and provide quantitative data for validation, strip test results were quantified by using an intensity score sheet. The intensity of the test line was compared to a score sheet with a series of 10 graded pink to red lines, from faint to strong (1–10), where the displayed line is given an intensity score. The operator compared the test line of the strip test to the score sheet and recorded the observed intensity. This quantification was employed for the purposes of RSIDTM-Blood development and validation, but the test was not designed to be a quantitative test. Therefore, RSIDTM-Blood should be used as a positive/negative test in accordance with the manufacturer's suggested protocol.

2.5. *Positive, negative, and experimental sample preparation*

Positive controls for RSIDTM-Blood were produced from 50 μl of human blood air-dried onto a sterile cotton swab. The blood swab was then extracted in 1 ml of RSIDTM-Blood Extraction Buffer for 1 h at room temperature. A 5 μl portion of this extract was added to 95 μl of RSIDTM-Blood Running Buffer and the entire 100 μl was added to the sample window of an RSIDTM-Blood cassette in order to generate a positive control. Animal blood extracts were produced from 50 μl of animal blood deposited on sterile cotton swabs that were air dried and extracted in the same manner described above. Human body fluid extracts were produced from 50 μl of body fluid deposited on sterile cotton swabs that were air dried and extracted in the same manner described above. Extraction and analysis of a clean, sterile cotton swab was performed in the same manner in order to generate negative controls. Experimental stains present on fabric were sampled by removing a 20 mm^2 punch or cutting that was then extracted in 100 μl of RSIDTM-Blood Extraction Buffer. A 20 μl portion of this

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extract was added to 80 μl of RSID™-Blood Running Buffer and the entire 100 μl was added to the sample window of an RSID™-Blood cassette in order to test the extract for the presence of human blood.

2.6. Summary of samples tested

The following list includes all samples analyzed and reported in this manuscript: human blood swabs from 57 different individuals; human body fluid extracts from saliva, semen, urine, breast milk, and amniotic fluid; animal blood from ferret, skunk, dog, cat, cow, horse, chicken, pig, goat, turtle, elk, mule deer, tiger, owl, opossum, alpaca, orangutan, gorilla, bonobo (pygmy chimpanzee), spider monkey, and baboon; dried human blood stains from a cotton sheet, cotton gauze, and a non-porous surface; mixed body fluid (blood, semen, saliva, and urine) stained cotton sheet; human blood stains on cotton fabric subsequently rubbed with ammonia based floor cleaner, anti-bacterial aerosol spray, anti-bacterial hand soap, rubbing alcohol, laundry soap, bleach, and hydrogen peroxide.

3. Results and discussion

To be useful for forensic purposes, a blood detection test must be able to detect exceedingly small amounts of blood, must be exquisitely specific for human blood, must not necessitate consumption of large amounts of evidence, and must be easily integrated into existing laboratory protocols including DNA-STR analyses. Experiments demonstrating how RSID™-Blood meets and exceeds these requisite specifications are described below.

3.1. Sensitivity

Decreasing amounts of blood were analyzed in order to determine the lower limit of detection of RSID™-Blood (Fig. 2A). Assuming 100% extraction efficiency, the following volumes of human blood were analyzed: 0.0, 0.01, 0.05, 0.25, 1.0, and 5.0 μl (Fig. 2A, strips 1–6, respectively). RSID™-Blood results were negative on strips 1 and 2, weakly positive (barely visible intensity score of 1) on strip 3, and clearly positive on strips 4–6 (Fig. 2A). Although the test line is weak on strip 3, this result shows that the limit of detection for RSID™-Blood is approximately 0.05 μl of whole blood. It is also important to note that analysis of extremely small amounts of blood (approximately 0.25 μl, strip 4, intensity score of 5) results in an easily visible band at the test line. While RSID™-Blood is not as sensitive as the hemoglobin-based blood detection strip tests which exhibit detection limits of ~50 picoliters, the sensitivity of RSID™-Blood has been adjusted so that a positive RSID™-Blood result correlates with the likelihood of obtaining a DNA-STR profile [5–7]. Therefore, RSID™-Blood results provide greater power of discrimination when triaging samples prior to DNA based analyses.

3.2. High-dose hook effect

Hemoglobin-based strip tests used for blood detection exhibit a significant high-dose hook effect that can produce false negative results when forensic samples are analyzed. In fact, the presence of as little as 1.0 μl of blood induces a high-dose hook effect on the hemoglobin-based strip tests [5,6]. Therefore, if the operator inadvertently overloads the cassette, misleading test results may occur. In order to determine if RSID™-Blood exhibits a high-dose hook effect, increasing amounts of human blood were analyzed. Assuming 100 % extraction efficiency, the following volumes of human blood were analyzed: 5.0, 10.0, and 25.0 μl (Fig. 2B, strips 1–3, respectively). RSID™-Blood results were positive with all volumes of human blood tested. Even when the amount of blood in the extract is sufficient to discolor the strip, a positive signal at the test line is clearly visible (strip 3, Fig. 2B). These results demonstrate that RSID™-Blood does not exhibit a significant high-dose hook effect. Therefore, false negative results will not be observed when large amounts of blood are present in the tested sample. Importantly, for practical considerations, this simplifies RSID™-Blood usage because samples do not need to be excessively diluted prior to analysis.

3.3. Specificity

In order to determine whether RSID™-Blood exhibits the specificity required for forensic applications, extracts from other human body fluids and animal blood were analyzed (Fig. 3). First, human body fluid extracts from saliva, semen, urine (Fig. 3A), breast milk, and amniotic fluid (data not shown) were analyzed. Assuming 100% extraction efficiency, 5.0 μl of each body fluid was analyzed. None of the human body fluid extracts tested caused a positive result (Fig. 3A). Next, blood from a wide variety of domestic, wild, and exotic animal species was tested (Fig. 3B). The tested species include ferret,

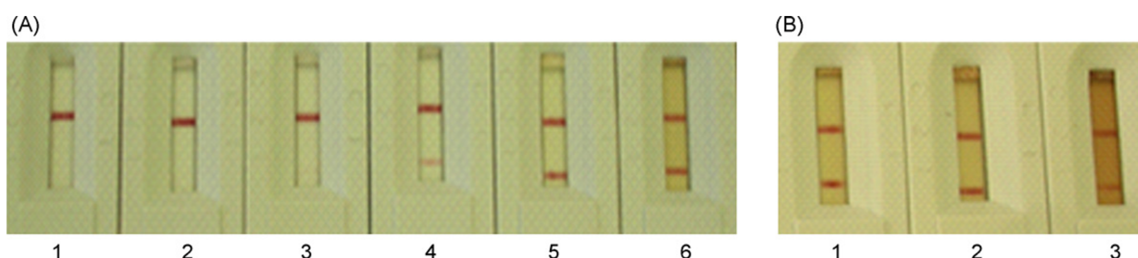

Fig. 2. RSID™-Blood detects as little as 0.05 μl of human blood. (A) Analysis of human blood in the volumes of 0.0, 0.01, 0.05, 0.25, 1.0, and 5.0 μl (strips 1–6, respectively). (B) Analysis of human blood in the volumes of 5.0, 10.0, and 25.0 μl (strips 1–3, respectively).

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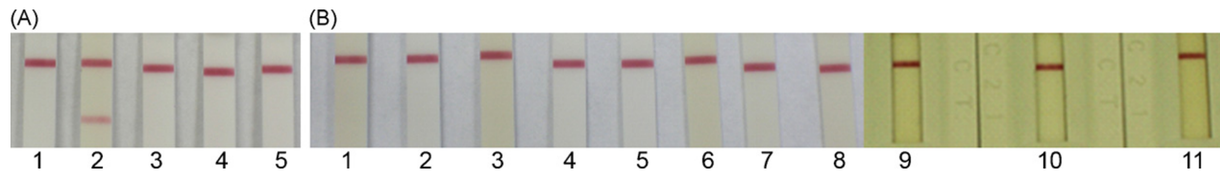

Fig. 3. RSID^{TM}-Blood does not cross-react with other body fluids or animal blood. (A) Analysis of 5.0 μl of human saliva, semen, and urine (strips 3–5, respectively). Blank and human blood extracts were analyzed as negative and positive controls (strips 1 and 2, respectively). Strips were removed from cassettes for improved photographic clarity. (B) Analysis of 5.0 μl of animal blood from ferret, skunk, dog, cat, cow, horse, chicken, pig, orangutan, gorilla, and bonobo (pygmy chimpanzee) (strips 1–11, respectively). Strips 1–8 were removed from cassettes for improved photographic clarity.

skunk, dog, cat, cow, horse, chicken, pig, orangutan, gorilla, and bonobo (pygmy chimpanzee) (Fig. 3B, strips 1–11, respectively). Animal blood was also tested from goat, turtle, elk, mule deer, tiger, owl, opossum, alpaca, spider monkey, and baboon (data not shown). Assuming 100% extraction efficiency, 5.0 μl of animal blood was tested from each species. RSID^{TM}-Blood results were negative with all of the animal blood samples tested (Fig. 3B). These experiments show that RSID^{TM}-Blood is not prone to false positives caused by the presence of other human body fluids or animal blood.

The animal blood experiments are important because the major weakness of hemoglobin-based strip tests is their cross-reaction with animal blood (eg., ferret, skunk, and primate) [5–9]. This cross-reactivity can lead to false positive results, and mandates that hemoglobin based strip test positive results be reported as presumptive for human blood. The finding that RSID^{TM}-Blood does not cross-react with ferret, skunk or primate blood demonstrates that the glycophorin A antibodies provide specificity superior to the hemoglobin antibodies, and that RSID^{TM}-Blood exhibits an absolute specificity for human blood.

3.4. *Detection of blood from sample evidence*

The sensitivity and specificity experiments presented above were performed with lab created control samples. However, to be useful for forensic applications, RSID^{TM}-Blood must be able to detect blood from a wide variety of mixtures, stains, and surfaces. Therefore, RSID^{TM}-Blood was tested for its ability to detect blood from a variety of samples. First, dried blood stains from a cotton sheet, cotton gauze, and a non-porous surface (blood drop on laminate countertop sampled by sponging with moistened cotton swab) as well as a mixed body fluid (blood, semen, saliva, and urine) stained cotton sheet were tested (Fig. 4A). Stains were sampled, extracted and analyzed as described above (see Section 2). All of these stains produced strong positive results when analyzed with RSID^{TM}-Blood (Fig. 4A). These results show that RSID^{TM}-Blood is able to detect blood from forensic evidence samples. Also, the result seen on strip 3 shows that the presence of other human body fluids in a mixture stain does not interfere with the ability of RSID^{TM}-Blood to detect human blood. This is important because the stains encountered at crime scenes are often body fluid mixtures.

As an additional and more challenging test, RSID^{TM}-Blood was assayed for its ability to detect blood from stains that had been treated with various household cleaning solutions (Fig. 4B). Human blood was dropped onto cotton fabric and various cleaning solutions were added to the stains. The cleaning solutions that were tested include ammonia based floor cleaner, anti-bacterial aerosol spray, anti-bacterial hand soap, rubbing alcohol, laundry soap, bleach, and hydrogen peroxide. The stains were sampled, extracted and analyzed as described in Section 2. Blood was not detected from the drop treated with laundry detergent (Fig. 4B, strip 5). Also, the bleach treated stain produced a very weak (intensity score 2) positive result (Fig. 4B, strip 6). The weak control and absent test line on strip 5 suggests that the laundry soap extract contains large amounts of detergent that inhibits the antibody interactions and interferes with RSID^{TM}-Blood function. Visual inspection of the extract analyzed on strip 6 (bleach) revealed that the extract was not discolored to the same extent of the other extracts (data not shown). This suggests that bleach treatment locks the stain into the fabric and decreases the extraction efficiency leading to the weak positive result. The other cleaning solutions caused test line intensities to vary (graded 4–9) but all resulted in

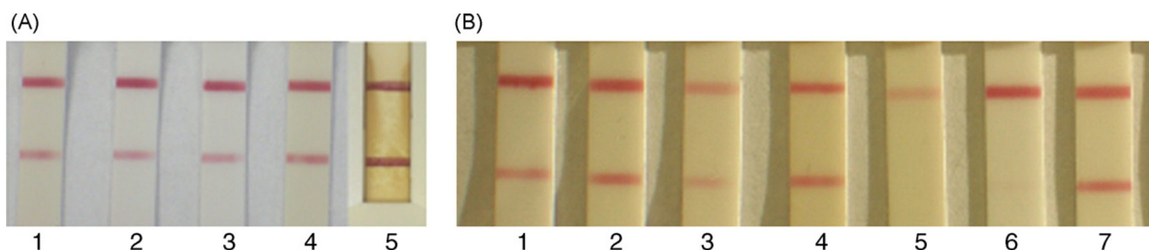

Fig. 4. RSID^{TM}-Blood detects human blood from forensic evidence. (A) Analysis of dried blood stains from a cotton sheet, cotton gauze, a non-porous surface, and a mixed body fluid (blood, semen, saliva, and urine) stained cotton sheet were tested (strips 2–5, respectively). Human blood extract was analyzed as a positive control (strip 1). Strips 1–4 were removed from cassettes for improved photographic clarity. (B) Analysis of blood stains that were treated with ammonia based floor cleaner, anti-bacterial aerosol spray, anti-bacterial hand soap, rubbing alcohol, laundry soap, bleach, and hydrogen peroxide (strips 1–7, respectively). Strips were removed from cassettes for improved photographic clarity.

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clearly positive test results (Fig. 4B, strips 1–4 and 7). These results show that RSID™-Blood is versatile in its ability to detect human blood from stains that have been treated with many common household cleaners. However, large amounts of detergent present in the extract may interfere with test performance, while bleach treated stains may prove difficult to extract. The weak control line on strip 5 illustrates that, due to the variable nature of forensic evidence, the presence of certain substances in analyzed extracts may interfere with RSID™-Blood performance. The importance of the dual stripe approach emphasizes the fact that a clear band must be present at the control line for the test results to be considered valid.

Finally, since the majority of the validation studies were performed with blood from three different individuals, it was important to show that RSID™-Blood is able to detect human blood from multiple individuals. Therefore, blood from a human body fluid stain library, collected with Institutional Review Board approval, was tested. Blood from 57 different individuals was tested. All of the 57 different blood samples tested positive, showing that RSID™-Blood is able to detect blood from multiple individuals (data not shown).

4. Conclusion

Here we have shown that RSID™-Blood meets or exceeds the standards needed to become a valuable tool for human blood detection from forensic evidence. RSID™-Blood is exquisitely sensitive and able to detect as little as 0.05 μl of human blood. Considering that the volume of an average sized drop is approximately 50 μl, this test is able to detect 1/1000th of a blood drop. Also, RSID™-Blood is able to detect human blood from a wide variety of forensic evidence stains and cleaned stains. The high-dose hook effect that is responsible for false negative results with hemoglobin-based blood detection tests is not observed with RSID™-Blood. Furthermore, animal blood cross-reactivity, which is directly responsible for the presumptive nature of hemoglobin-based tests, is not observed with RSID™-Blood. These findings suggest that RSID™-Blood is a more reliable human blood detection test than hemoglobin-based strip tests or chemical reduction tests. Additionally, the fact that RSID™-Blood has been tested with a wide variety of body fluids, animal bloods, and other red-brown stains, without a single instance of cross-reactivity, suggests that RSID™-Blood can be used as a confirmatory test for human blood.

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