Developmental Validation of a Novel Lateral Flow Strip Test for Rapid Identification of Human Blood, Rapid Stain Identification-Blood, RSID™-Blood

Introduction

Blood is the most common body fluid encountered at crime scenes and the ability to reliably, consistently and specifically detect human blood is important for both legal and investigative purposes. An important adjunct to identifying blood (and other body fluid stains) is determining which questioned stains are most likely to yield DNA profiles. Current body fluid tests, including those for human blood, are presumptive, that is they are sufficiently prone to false positives such that no definitive statement as to the origin of the tested stain can be made. Blood detection methods, such as the reduced phenolphthalein and ortho-tolidine tests, are known to give false positives with a variety of common substances including coins, detergents and scrap metal. Other blood detection methods that rely on the immunological detection of hemoglobin are subject to cross-reaction with animal blood (ferret, skunk, and primate) and prone to false negatives due to a pronounced high dose hook effect. High dose hook effect refers to the apparent failure of immunological-based tests in the presence of high analyte concentrations; this failure can be easily interpreted as a false negative result. Presumptive tests are both inefficient, as they are poor predictors of DNA testing outcomes (and DNA testing is expensive) and susceptible to legal and scientific challenges. Here, the developmental validation of a new blood identification test, designed to solve all of these deficiencies, **R**apid **S**tain **Id**entification-Blood (**RSID™-Blood**), is described.

RSID™-Blood is an immunological-based blood detection test designed and conceived by an accredited forensic DNA laboratory. This test is specific for human blood (neither animal nor primate blood cross-reacts with the test) and exhibits no high dose hook effect. The sensitivity of **RSID™-Blood** has been adjusted such that a positive result will generally imply sufficient biological material for DNA-STR analysis.

RSID™-Blood detects a red blood cell membrane antigen, glycophorin A, with two anti-glycophorin A monoclonal antibodies in a lateral flow immuno-chromatographic strip test

format. Formally, the test detects red blood cell membranes, an effective surrogate for human blood. Glycophorin A is abundantly expressed in human red blood cell membranes where its *in vivo* physiological role is to prevent cellular aggregation.

Here we present experimental results demonstrating that **RSID™-Blood** is accurate, reproducible, easy to use, and highly specific for human blood. Importantly, we clearly show that **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, **RSID™-Blood** 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 forensic detection of human blood on questioned stains.

Experiment: RSID™-Blood Limit of detection

Rationale: In order to be useful for forensic purposes, a blood detection test must be able to detect even small amounts of blood without dilution or concentration. In addition, the limit of detection should be calibrated to other important forensic test. *e.g.,* DNA-STR analysis.

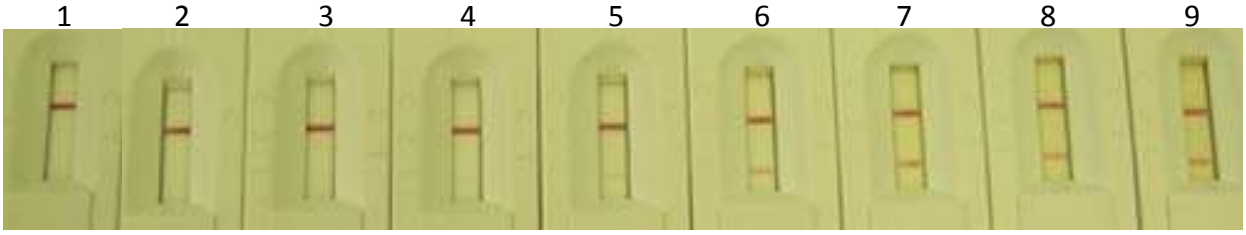
Methods: A known volume of human blood (50 µl) was applied to a sterile cotton swab. The swab was air dried to better simulate a forensic sample and the swab head, including batting, was cut into a 1.5 ml centrifuge tube. One milliliter (1 ml) of **RSID™-Blood** Extraction Buffer was added to the isolated stained swab, the tube was mixed vigorously and incubated at room temperature for 2 hours. Various amounts of blood extract (1, 2, 5, 10, 20, 50, 95, and 100 µl of the original 1 ml extraction) were analyzed with **RSID™-Blood** cassettes. Assuming efficient extraction, this standardized extract will contain 50 nl of human blood per microliter of extract (50 nl human blood/1 µl extract tested) and therefore the analyzed volumes contain 50 nl, 100 nl, 250 nl, 500 nl, 1 µl, 2.5 µl, 4.75 µl, and 5 µl of human blood, respectively (see Table 1). A sham extract (sterile swab extracted in 1 ml of **RSID™-Blood** Extraction Buffer) was produced and analyzed as a negative control. All extract volumes analyzed were adjusted to a total volume of 100 µl with **RSID™-Blood** Running Buffer prior to analysis with **RSID™-Blood**

cassettes. The entire 100 µl sample was applied to the sample windows of **RSID™-Blood** cassettes. After 10 minutes, the test line signals were evaluated (see Figure 1).

Table 1.

Cassette#	Volume Analyzed (volume extract / volume blood)
1 (sham)	100 µl /0 µl
2	1 µl/50 nl
3	2 µl/100 nl
4	5 µl/250 nl
5	10 µl/500 nl
6	20µl/1 µl
7	50 µl/2.5 µl
8	95 µl/4.75 µl
9	100 µl/5 µl

Figure 1.



Results: **RSID™-Blood** results were negative when less than 50 nl of human blood was analyzed. **RSID™-Blood** results were positive when 100 nl or more of human blood was analyzed (see Figure 1).

Note: Photograph is *less* sensitive than visual observation of the cassette.

Conclusion: The experimental limit of detection for **RSID™-Blood** is 100 nl of human blood. Stated detection limit is 1 µl human blood and is readily achieved. As designed, limit of detection is within the range of DNA-STR sensitivity.

Experiment: High dose hook effect

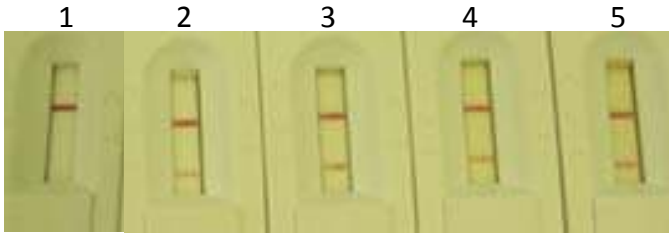
Rationale: Current immunological- based detection strips for human blood rely on antibody-based detection of hemoglobin. These tests exhibit a high dose hook effect (HDHE) that can produce false negative results from forensic samples. This occurs when the analyte, hemoglobin, is in excess as compared to the detection antibody - these tests demonstrate decreased signal intensity to a level where false negatives can be readily observed. **RSID™-Blood** was tested for HDHE and potential false negative results with increasing amount of human blood.

Methods: A known volume of human blood (50 µl) was applied to a sterile cotton swab. The swab was air dried to better simulate a forensic sample and the swab head, including batting, was cut into a 1.5 ml centrifuge tube. One milliliter (1 ml) of **RSID™-Blood** Extraction Buffer was added to the stained swab, the tube was mixed vigorously and incubated for 1 hour at room temperature. Various amounts of blood extract (20, 50, 95, and 100 µl) were analyzed with **RSID™-Blood** (see Table 2). Assuming efficient extraction, testing the designated volumes is equivalent to analyzing 1 µl, 2.5 µl, 4.75 µl, and 5 µl of human blood (see Table 2). A sham extract (sterile swab extracted in 1 ml of **RSID™-Blood** Extraction Buffer) was produced and analyzed as a negative control. All extract volumes analyzed were adjusted to a total volume of 100 µl with **RSID™-Blood** Running Buffer prior to analysis with **RSID™-Blood** cassettes. The entire 100 µl sample was applied to the sample windows of **RSID™-Blood** cassettes. After 10 minutes, the test lines were evaluated (see Figure 2).

Table 2.

Cassette#	Volume Analyzed (volume extract / volume blood)
1 (sham)	100 µl /0 µl
2	20µl/1 µl
3	50 µl/2.5 µl
4	95 µl/4.75 µl
5	100 µl/5 µl

Figure 2.



Results: **RSID™-Blood** clearly produces a positive results at all volumes tested.

Conclusion: **RSID™-Blood** does not exhibit a high dose hook effect at any range of human blood tested.

Experiment: Animal blood cross reactivity

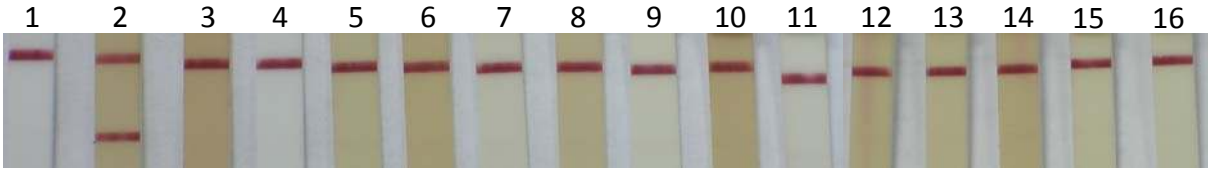
Rationale: Current hemoglobin-based strip tests used for blood detection exhibit cross reaction with animal (ferret, skunk, and primate) blood, which can lead to false positive results for human blood detection. Here we test **RSID™-Blood** for species specificity.

Methods: Standard extracts of animal blood (50 µl of animal blood applied to a sterile cotton swab, air dried and extracted in 1 ml of **RSID™-Blood** Extraction Buffer) were prepared and tested (see Table 3 for list of animals tested). From these extractions, 100 µl of the animal blood extracts were analyzed with **RSID™-Blood** cassettes. Assuming efficient extraction, each extract will contain 50 nl of animal blood per microliter of extract; the entire 100 µl sample, equivalent to testing 5 µl of blood, was applied to the sample windows of **RSID™-Blood** cassettes. After 10 minutes, the test line signals were evaluated (see Figure 3). The volumes chosen were deemed more than sufficient to reveal potential cross-reaction.

Table 3.

Strip#	Animal
1 (-)	n/a
2 (+)	human
3	ferret
4	skunk
5	dog
6	cat
7	cow
8	pig
9	chicken
10	horse
11	goat
12	orangutan
13	gorilla
14	spider monkey
15	pygmy chimp
16	baboon

Figure 3.



Results: **RSID™-Blood** results were negative with all of the animal blood samples tested (see Figure 3). **Note: strips have been removed from cassettes to provide a clearer photographic record.**

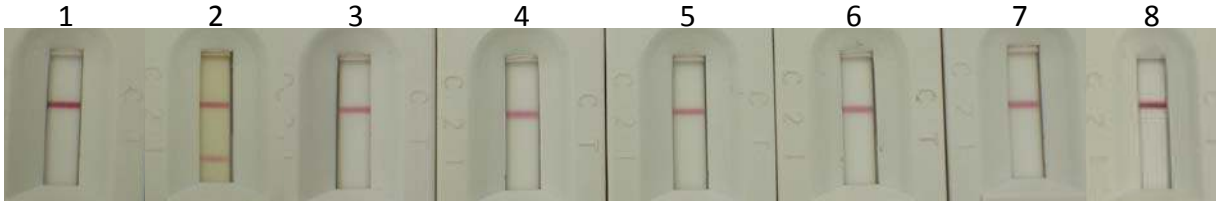
Conclusion: **RSID™-Blood** is specific for human blood and does not cross react with any animal species tested including primate and ferret**. RSID™-Blood is a confirmatory test for the presence of human blood.**

Experiment: Human body fluid cross reactivity

Rationale: To further test the specificity of **RSID™-Blood** against a wide selection of human body fluids.

Methods: Standard extracts from human body fluids (50 µl of indicated body fluid) were prepared from swabs and tested. The indicated body fluid (blood, urine, saliva, semen, breast milk, amniotic fluid) was extracted in 1 ml **RSID™-Blood** Extraction Buffer for 1 hour at room temperature. Negative and positive controls, 20 µl of sham extract and human blood samples were included. A full 100 µl of each body fluid extract was tested, equivalent to 5 µl of each body fluid, with **RSID™-Blood** cassettes. After 10 minutes, the test line signals were evaluated (see Figure 4).

Figure 4.



Cassette#	Extract
1	Sham (- control)
2	Blood (+ control)
3	Urine
4	Saliva
5	Semen
6	Breast Milk
7	Amniotic Fluid
8	Vaginal Fluid

Results: **RSID™-Blood** is specific for blood: no other body fluid gave any detectable signal in this test (see Figure 4).

Conclusions: **RSID™-Blood** is confirmatory for human blood.

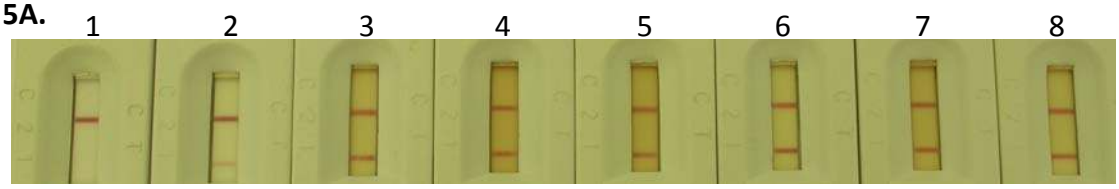
Experiment: Detection of blood from multiple individuals

Rationale: To further test the specificity and reliability of **RSID™-Blood**. Samples from a library of human body fluids collected from volunteers under IRB supervision were used to test the ability of **RSID™-Blood** to detect blood from diverse individuals.

Methods: Fresh blood from 6 different individuals was deposited onto FTA cards and air dried. Circular punches (5 mm diameter) were then removed from the FTA card blood stains and extracted in 200 µl **RSID™-Blood** Extraction Buffer for 2 hours at room temperature. 20 µl of each FTA extract was added to 80 µl **RSID™-Blood** Running Buffer and loaded onto **RSID™-Blood** cassettes. 20 µl of sham extract and positive control blood extract were included for comparison (see Figure 5A).

In parallel, samples from a second body fluid library (blood deposited onto swabs) were extracted and tested with **RSID™-Blood.** These blood samples were of variable volumes and were stored for variable times before extraction. All body fluid library swab samples were extracted in 200 µl **RSID™-Blood** Extraction Buffer for 2 hours at room temperature. 20 µl of each swab extract was added to 80 µl **RSID™-Blood** Running Buffer, sham extract (negative control from clean swab extract), and 20 µl blood extract (positive control, equivalent to 1 µl blood) were analyzed side-by-side for comparison (see Figure 5B). After 10 minutes, the test line signals were evaluated.

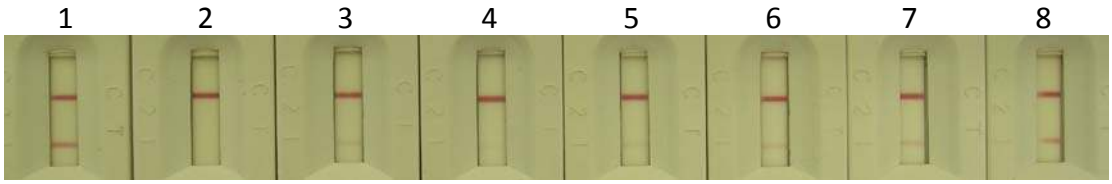
Figure 5A.



Cassette#	Extract
1	100 µl Sham (- control)
2	20 µl Blood (+ control)
3	FTA 1
4	FTA 2
5	FTA 3
6	FTA 4
7	FTA 5
8	FTA 6

FTA Card Results: All extracts gave strong positive results at the test line (see Figure 5A). **Note: photograph is less sensitive than visual observation.**

Figure 5B.



Cassette#	Extract
1	20 µl Blood (+ control)
2	100 µl Sham (- control)
3	Swab #1
4	Swab #2
5	Swab #3
6	Swab #4
7	Swab #5
8	Swab #6

Swab Results: Extracts from all six individuals gave positive results at the test line (see Figure 5B). The intensity variation was predicted to be due to sample variability (both volumes and age of the samples were not controlled).

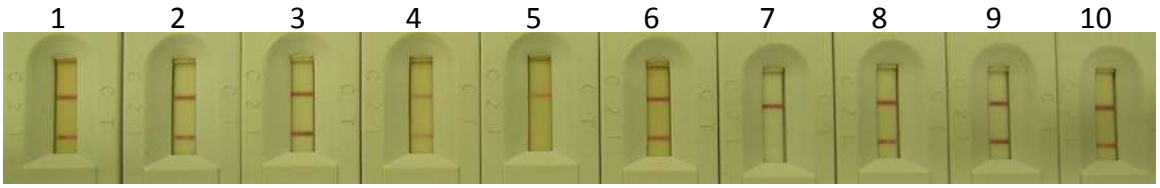
Conclusions: **RSID™-Blood** is able to detect blood from a variety of samples and from a diverse collection of individuals.

Experiment: Blood detection from simulated sample evidence stains

Rationale: To be an effective forensic detection method for human blood, **RSID™-Blood** must be able to detect blood from a wide variety of stains and surfaces. Here we test **RSID™-Blood** for its ability to detect blood from a variety of simulated evidence samples.

Methods: Human blood was deposited and spread onto a cotton shirt and dropped onto a laminate countertop. Various cleaning solutions were added to the stains on cotton. Stains were sampled with a 5 mm punch (cotton) and by sponging with a water moistened swab (cotton and laminate countertop). Punches and swabs were extracted in **RSID™-Blood** Extraction Buffer for 2 hours at room temperature. Extracts (100 µl) were analyzed with **RSID™-Blood** cassettes. After 10 minutes, the test line intensity were evaluated.

Figure 6.



Strip#	Sample
1	Unwashed (drop)
2	Pine Cleanser (drop)
3	Antibacterial Aerosol (drop)
4	Liquid Soap (drop)
5	Laundry Detergent (drop)
6	Alcohol (drop)
7	Bleach (drop)
8	Unwashed (smear)
9	Swabbed dried drop (laminate countertop)
10	Swabbed dried drop (cotton)

Results: **RSID™-Blood** gave a positive signal from all stains, drops and smears with the exception of the blood sample pre-treated with laundry detergent (see Figure 6). The laundry detergent extract certainly contained large concentrations of phosphate-based ionic detergents that are known to inhibit antibody-antigen interactions. Other cleaning solutions certainly affected test line intensity, but did not produce a false negative result.

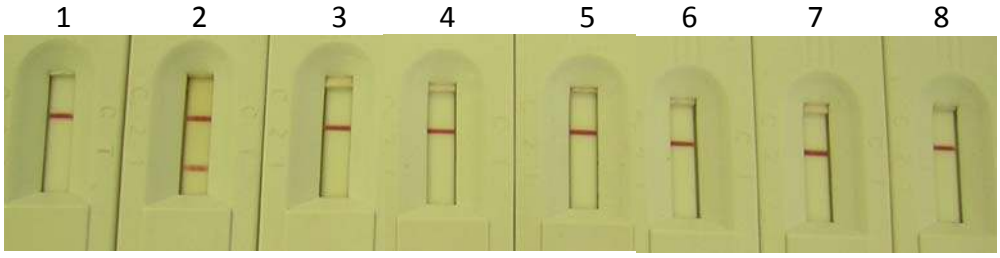
Conclusions: **RSID™-Blood** is able to detect blood from a variety of treated blood stains. The presence of commercial detergents in extracts may interfere with **RSID™-Blood** detection.

Experiment: Red/brown stain analysis.

Rationale: A body fluid test for human blood must be able to distinguish human blood from solutions and substances that can have the appearance of human blood, so-called red/brown stains. We therefore tested **RSID™-Blood** with a variety of red/brown stains produced from food items.

Methods: Food products that could cause stains that visually resemble blood were dropped onto cotton fabric and air dried overnight. Punches (5 mm) were removed from the stains and extracted in 200 µl **RSID™-Blood** Extraction Buffer at room temperature for 2 hours. Extracts (100 µl) were analyzed with **RSID™-Blood** cassettes. Sham extract (negative control from clean swab extract) and 20 µl blood extract (positive control, equivalent to 1 µl blood) were analyzed for comparison. After 10 minutes, the test line intensity was evaluated (see Figure 7).

Figure 7.



Strip#	Extract
1	100 µl Sham
2	20 µl Blood
3	100 µl Ketchup
4	100 µl Sweet and Sour Sauce
5	100 µl Diet Pepsi
6	100 µl Coffee
7	100 µl Hot Chocolate
8	100 µl Tea

Results: No tested extract gave a positive result with **RSID™-Blood**. No false positives were observed.

Conclusions: **RSID™-Blood** can readily distinguish between authentic blood and red/brown stains produced from food stuffs.