Developmental Validation of RSID™-Urine

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Introduction

The body fluid urine is comprised primarily of salts and water. The cell content of urine is very low and is derived mainly from epithelial cells shed from the urinary tract as well as some erythrocytes and leukocytes that escape from the glomeruli. The importance of urine detection in forensic analysis is often minimized because of the insensitivity of current urine tests and the low success rate for obtaining DNA profiles with evidence samples stained with this body fluid. A common detection method for urine relies on visual examination for stains with characteristic appearances. Concentrated urine stains will generally fluoresce under alternate light sources, but diluted stains are harder to detect and odor is a common detection method.

Historically, methods for urine detection have relied on identifying small organic compounds: urea and creatinine. Both of these compounds are found in numerous other body fluids such as sweat, blood, saliva, and semen. Urea is present at high levels in urine, approximately 1400 to 3500 milligrams per 100 milliliters. Creatinine is present at one-tenth these values, with average concentrations of 105 to 210 milligrams per 100 milliliters. While urea and creatinine are found at relatively high levels in liquid urine, they may be difficult to detect in stains, since the absorption of liquid urine into fabrics (the most commonly found type of urine sample) dilutes and disperses the urine components. Testing for urea relies on urease, an enzyme that breaks down urea and releases ammonia and carbon dioxide. Ammonia is detected using an indicator chemical, such as Nessler's reagent (mercuric iodide in potassium iodide) or DMAC (p-dimethylamino-cinnamaldehyde) (Stuart H. James & John J. Nordby, Forensic Science: an introduction to scientific and investigative techniques). Creatinine is detected by applying a saturated solution of picric acid in toluene or benzene to a stain extract which will chemically combine with creatinine to form creatinine picrate, an easily detectable colored product.

Tamm-Horsfall protein (THP) is the most abundant protein present in human urine and was originally described by Tamm and Horsfall in 1950 (Tamm I, Horsfall FL 1950). It is secreted by the thick ascending limb of the loop of Henle and then excreted

into urine at a rate of 50-100 mg/day (Hoyer & Seiler 1979; Dulawa et al. 1985). THP is a monomeric protein of ~85 kD that is heavily glycosylated by polyantennary sialate (up to 30% of the molecular weight can be carbohydrate). Although the physiological role of THP is unknown, its synthesis and membrane expression by cells of the thick ascending limb of the loop of Henle have led to the suggestion that it might be involved in ion transfer (Hoyer & Seiler 1979; Richet G, 1983). THP is specific to urine, however, and is found in many animal species.

Configuration of the RSID™–Urine lateral flow test

RSID™–Urine is an immunochromatographic assay that uses polyclonal rabbit antibodies specific for Tamm-Horsfall protein (THP). This lateral flow test system consists of overlapping components treated 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 pretreated before assembly such that the user need only add his/her extract in diluent buffer (provided in the kit) 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 blue, pre-dispersed latex-beads conjugated to anti-human THP polyclonal antibodies. The diluent redissolves the latex bead labeled anti THP antibodies which will bind Tamm Horsfall if it is present in the sample. THP-latex bead-antibody complexes are transported by bulk fluid flow to the membrane phase of the test strip. The membrane has been prepared such that antibodies laid down on the membrane are invisible until the test is performed. The immobilized anti-THP antibodies on the test line (*i.e.*, on the membrane) capture the THP-antibody-latex bead complexes, producing a blue line at the test position. If no THP is present in the sample, latex bead-conjugated antibody-antigen complexes are not formed, and latex bead anti-THP complexes will not accumulate at the test line. Each strip test is designed with a control, consisting of anti-rabbit IgG deposited on an invisible line on the membrane; these anti-rabbit antibodies will capture rabbit

antibodies flowing past the test line producing a visible blue line at the control position (see Fig. 1). The control line should always give a positive blue line thereby demonstrating that the sample fluid was transported through the length of the test, and that the components of the strip test were assembled correctly and the test is working appropriately.

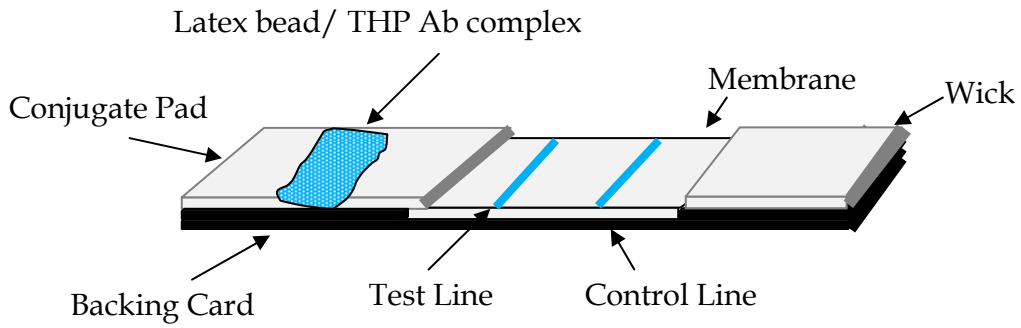


Figure 1: Diagram of **RSID™–Urine** Lateral Flow Strip Test

Quantification of RSID™–Urine strip test results

Strip test results were evaluated using a color intensity score sheet. The intensity of the test strip line was noted by visual comparison against a standard, which consisted of a series of graded blue lines, from faint to strong, where each displayed line was given an intensity score value. The operator compared the test line of the strip test against the score sheet, and recorded the observed intensity; this method minimizes operator variance and provided quantitative data for production QA/QC and validation.

Sensitivity of RSID™–Urine

The levels of THP in human urine vary widely throughout the day due to differences in hydration state, diet, and genetic variation. To assess the varying levels of

THP, urine samples were collected from individual volunteers throughout the course of one day. To determine if the color of the urine sample correlated with the intensity of the test line on **RSID™–Urine** cassettes, each urine sample was recorded as light (L), medium (M), or dark (D) yellow. From each urine sample, 50 µL of liquid urine was deposited on several cotton swabs and allowed to air-dry. Each swab was extracted in 1 mL RSID™–Urine buffer for 1 hour at room temperature and 100 µL was loaded onto the cassette. Assuming 100% extraction efficiency, this was approximately 5 µL of urine. Results were recorded after 15 minutes. Results of analyzing sequential time collection of urine during a full day from three volunteers (#1, #2, #3) are shown in Fig. 2. Samples from an additional 9 volunteers were also analyzed (data not shown). The color intensity of the urine sample is noted below each cassette (L, M, or D).

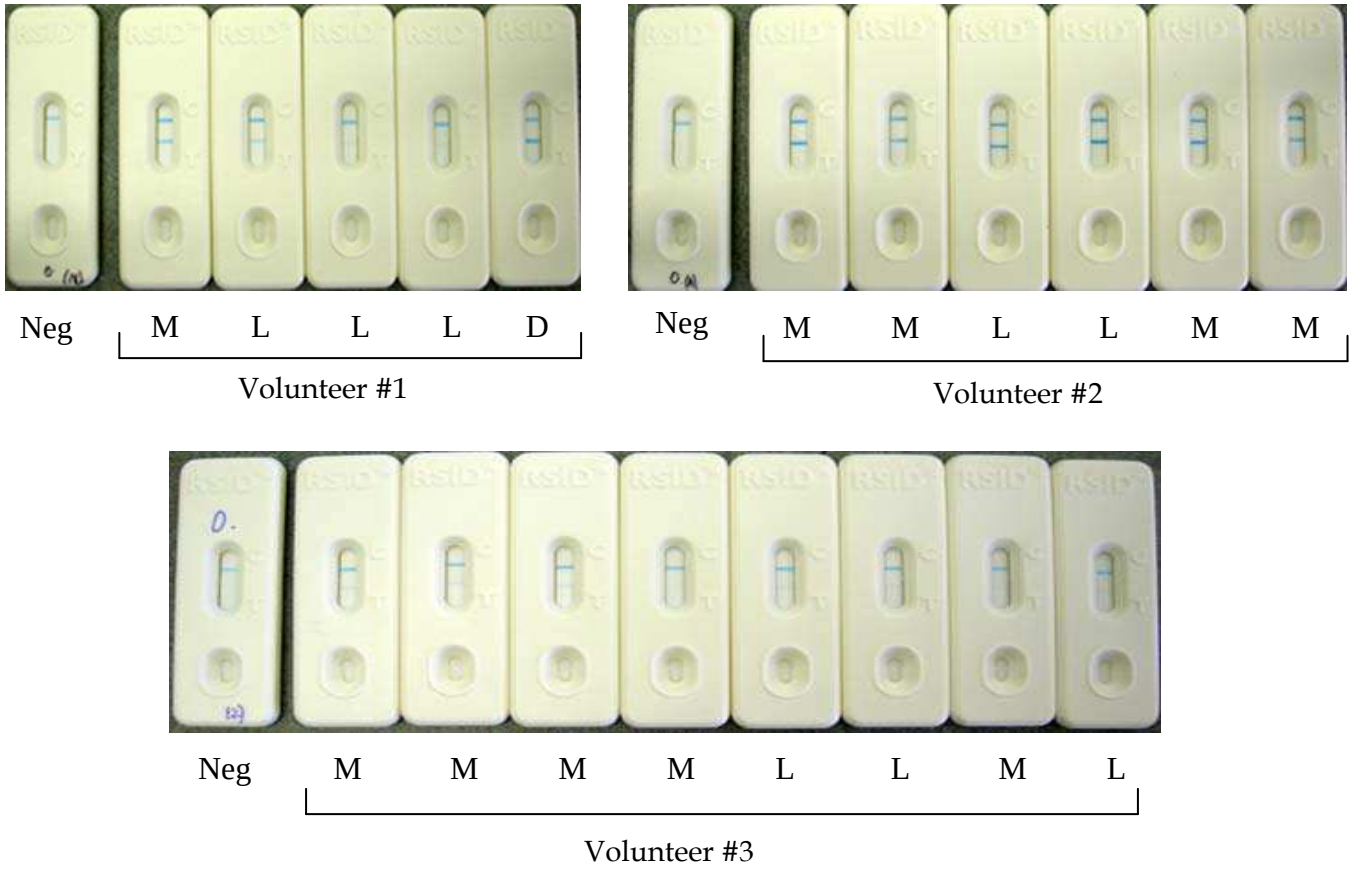


Fig. 2. One Day Time Course of THP in Three Volunteers

A negative control (neg) of 100 µL **RSID™–Urine** buffer only, was included with each set of samples.

The results of the analysis for THP using **RSID™-Urine** from each individual were completely self-consistent such that darker urine samples produced stronger test lines and lighter urine samples produced weaker test lines (Fig. 2, volunteers #1 and #3). Some individuals, example volunteer #2, produced a strong reaction on the test line regardless of the color intensity of the sample (Fig. 2, volunteer #2 top right panel). We noted two types of individuals, those that varied their THP output during the day and thus demonstrated an excellent correlation of urine color with THP, and other individuals for whom THP is strongly expressed irrespective of urine color.

Determining the sensitivity of detection of urine using **RSID™–Urine** is difficult due to the clear variability in THP levels from individual to individual as well as the variation in THP levels due to diet and hydration. In order to control for hydration level, urine samples were classified by color (L, M, D) from each individual before testing. In addition, urine samples from multiple individuals were also compared in order to try and control for personal variation in THP secretion. Extractions were prepared from swabs on which 50 µL of urine had been deposited and air-dried, and subsequently extracted in 1 mL **RSID™–Urine** buffer for 1 hour at room temperature. A standard volume of this extract (100 µL) of was tested on **RSID™–Urine** cassettes (see Fig. 3). Results were recorded after 15 minutes. Assuming 100% extraction efficiency, the equivalent volume of urine tested was approximately 5 µL.

The results from the light, medium, and dark samples were variable, as expected, but the majority of the samples were scored positive (Fig. 3). These results emphasize that the levels of THP are quite variable and thus preclude the possibility of establishing a definitive limit of detection for urine using **RSID™–Urine**. However, we have found after extensive testing, that using the equivalent of 5 µL of urine routinely gives a positive result (Fig. 3 and data not shown) and have therefore designated 10 µL as the stated limit of detection for **RSID™–Urine** as an added margin for urine identification.

The normal, physiological range of THP is such that we recommend that questioned samples and evidence cuttings be extracted in a minimum volume of (200-300 µL) of **RSID™–Urine** buffer. We have also determined that directly testing from a fabric cutting, as opposed to using a swab adsorption technique increases the sensitivity of **RSID™–Urine**.

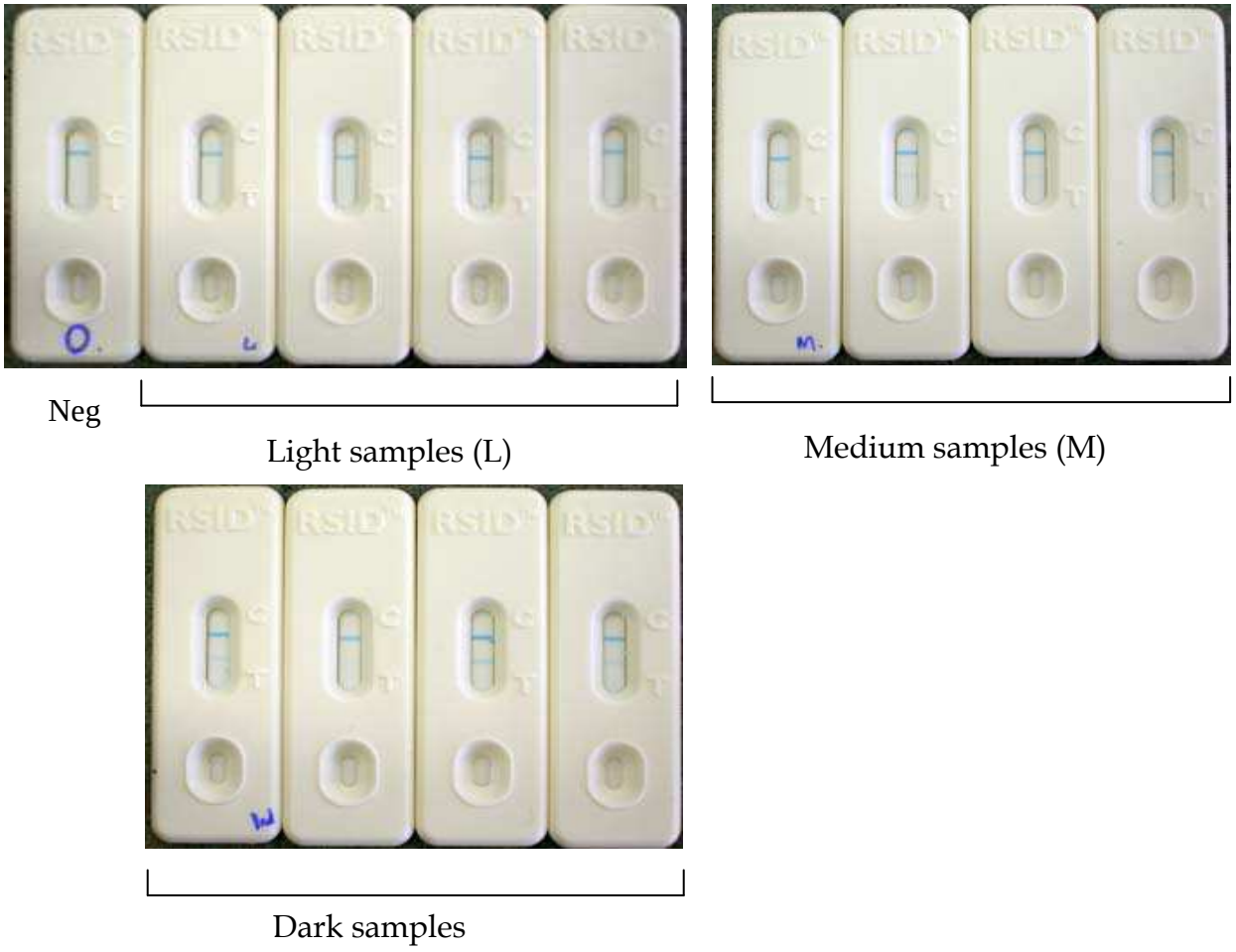


Fig. 3. **RSID™-Urine** analysis of identified light, medium or dark urine samples.

RSID™–Urine: Testing Fabric vs. Cotton Swab

The most common type of sample in forensic laboratories from which urine detection is requested is from fabric. We therefore tested urine stains from several types of fabric including polyester, cotton and denim. The fabric samples (Table 1) were prepared by depositing 50 µL of urine from light and dark urine samples on several pieces of polyester, cotton, and denim fabric. As a control, the same urine samples were deposited on cotton swabs and allowed to air dry.

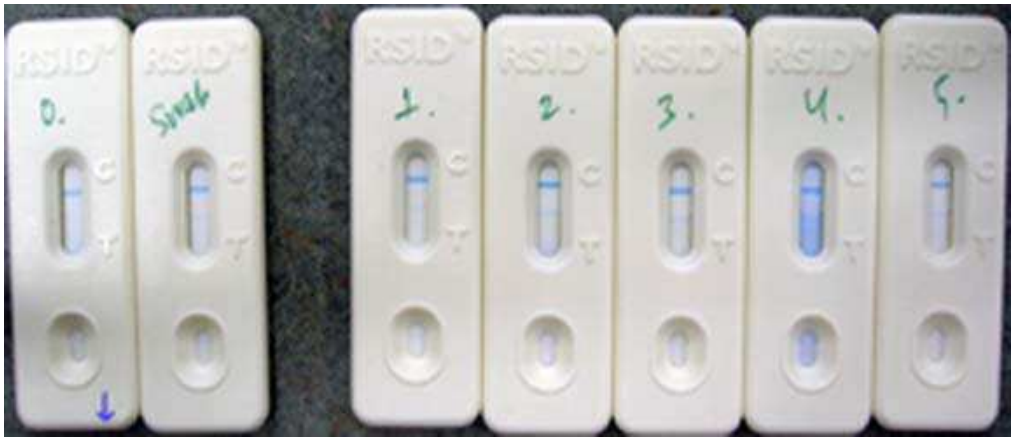
Table 1: Fabric and Swabs Tested with **RSID™–Urine**

Sample	Sample Type	% Stain Sampled
Swab	Cotton Swab	100
1	Cotton Swatch	8
2	Denim	25
3	Black Cotton Twill	64
4	Blue T-shirt	44
5	Polyester	5.5

Samples were extracted from a uniform cutting obtained by using an 8 mm Harris punch to excise a defined piece of each fabric. As each type of fabric produced a different sized stain, due to the "spreading" of the liquid, the percentage of the stain sampled in the 8 mm diameter cutting was noted (Table 1). This was calculated by dividing the area of the Harris punch by the area of the stain and multiplying by 100 (Table 1). The polyester fabric exhibited the most spreading, and therefore the 8 mm punch sampled the least of the 50 µL of urine added to the fabric (~5.5% of the stain), whereas the cotton twill exhibited the least spreading and thus almost 64% of the stain could be tested from this substrate (Table 1).

The results from testing different fabric types with **RSID™–Urine** revealed that sampling a smaller percentage of a urine stain from different fabrics resulted in a similar, or even stronger signal as compared to sampling the whole stain from a cotton swab (fig. 4A and 4B, both light and dark urine samples). This observation was likely

due to greater extraction efficiency of the urine stain from the fabrics than from a swab. The signal from extraction of the polyester fabric was similar to the signal obtained from the cotton swab extraction (fig. 4) indicating that extraction from fabric enhances the sensitivity of **RSID™–Urine**. Since the majority of urine samples received by forensic laboratories are on fabric, the sensitivity of **RSID™–Urine** may be higher than indicated from extraction from a swab.



(A). Light Urine Sample

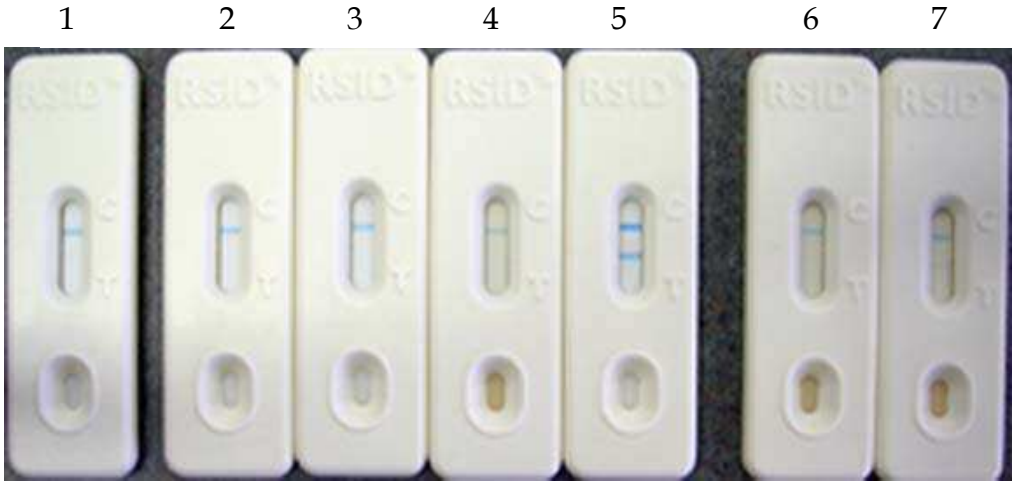
(B). Dark Urine Sample

Fig 4. Extraction of urine from different types of fabric

Body Fluid Specificity of RSID™–Urine

To determine if **RSID™–Urine** cross-reacts with other body fluids, we tested extracts from semen, saliva, blood, menstrual blood, and vaginal fluid on **RSID™–Urine** cassettes. Extracts of saliva, semen and blood were made from swabs on which 50 µL of the cognate fluid had been deposited and air-dried, and subsequently extracted in, respectively, 1 mL RSID™–Saliva, RSID™–Semen, and RSID™–Blood extraction buffer for 1 hour at room temperature. Urine extract was made from a cotton swab on which 50 µL urine had been deposited and air-dried, and extracted in 200 µL **RSID™–Urine** buffer for 1 hour at room temperature. Since the detection limit of RSID™–Saliva, RSID™–Semen, and RSID™–Blood is <1 µL body fluid, we tested 20 µL of each extract (equivalent to 1 µL body fluid, assuming 100% extraction efficiency) of saliva, semen, and blood extract alone on **RSID™–Urine** (Fig. 5, lanes 2, 3, and 4, respectively). Urine extract, 40 µL, was tested as a positive control (Fig. 5, lane 5). Extracts of semen, saliva, and blood (20 µL) were also tested in combination with and without urine extract (Fig. 5, lanes 6 and 7). In addition to individual body fluids, we also tested combinations of body fluids using 40 µL of urine extract added to 20 µL each of semen, saliva, and blood extract (Fig.5, lane 7, M4) for a total tested volume of 100 µL. The sensitivity of the M4 sample was clearly reduced when compared to urine extract alone (Fig. 5 top panel, compare lanes 5 and 7). This observation was examined further by testing dual fluid mixtures in combination with urine extract; the addition of blood extract inhibited the signal seen on **RSID™-Urine** (Fig. 5, lower panel, compare lanes 4 and 7) whereas the addition of semen or saliva extract had no effect on the signal observed from the urine extract (Fig. 5 lower panel, compare lanes 2, 3, and 7).

This result indicates that the presence of blood must be considered carefully when testing for urine with **RSID™-Urine** as the test sensitivity will be reduced. This effect can be overcome by testing a higher volume of the sample to overcome possible inhibition of the urine signal.



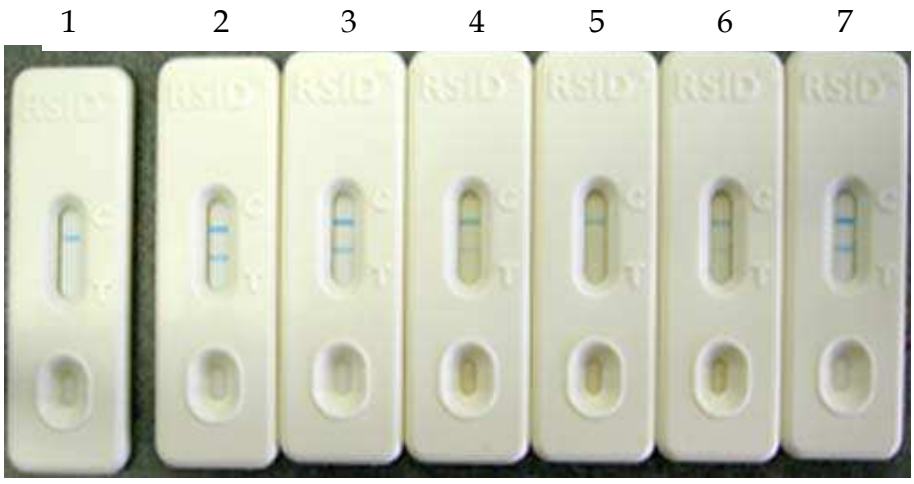


Fig. 5 Testing Different Body Fluid Extracts with **RSID™–Urine**

As the presence of blood inhibits the signal from urine extract when using **RSID™-Urine**, we sought to determine the amount of blood necessary to inhibit the signal from urine extract. We tested the effect of increasing amounts of extract from a blood swab on the effect of urine extract with **RSID™- Urine** starting with an equivalent of 10 µL of urine (i.e., 40 µL extract made from an extract containing 0.25 µL urine/ µL extract)

with 50 nl, 250 nl, and 1 µL equivalent volume blood (Fig. 6). Controls included a negative and a positive made from 10 µL urine alone. The results demonstrate a clear positive seen with 10 µL of urine alone, and decreasing signal with increasing amounts of blood extract in a dose dependent manner (Fig. 6). These results indicate that the presence of blood inhibits the detection of urine when using **RSID™–Urine**.

Fig 6. Testing Increasing Amounts of Blood with Urine Extract with **RSID™-Urine**

To determine if higher amounts of urine could overcome the inhibition caused by blood extract when using **RSID™-Urine**, we tested 5, 10, and 20 µL urine in combination with 1 µL equivalent volume blood (Fig. 7). Controls included a negative control and 10 µL urine alone as a positive control. A clear positive was seen with 10 µL urine alone and the signal from 5, 10, and 20 µL urine was similar in the presence of 1 µL blood and was reduced in comparison to the signal in the absence of blood (Fig. 7).

Figure 7. Testing Increasing Amounts of Urine Extract with Blood

To determine if the presence of blood could completely inhibit the signal from a genuine urine extract tested with **RSID™- Urine** and thereby possibly produce a false negative, liquid urine, 20, 50, and 100 µL, was mixed with 1, 5, or 20 µL of liquid blood in a microfuge tube and deposited on a cotton swab and allowed to air-dry. These swabs were extracted in 200 µL **RSID™-Urine** buffer for 1 hour at room temperature and the extract recovered using a Spin-Eze basket; this mixed body fluid extract, 100 µL, was added to an **RSID™-Urine** cassette. Results were recorded after 15 minutes. Note that half of the original extract volume (200 µL) was added to the **RSID™-Urine** cassette; the figures indicate the original volumes used to prepare the mixed body fluid swab.

The results for all three urine volumes were the same, therefore, only the results from 20 µL urine is shown in figure 8. Urine alone (20 µL) showed a clear positive and the signal was significantly reduced with 1 µL blood.

Fig. 8. Testing **RSID™-Urine** with Increasing Amounts of Blood

Species Specificity of RSID™–Urine

The protein sequences of THP from a variety of species are highly conserved with ~96-99% identity at the amino acid level from canine, bovine, equine, etc. To test the species specificity of **RSID™-Urine**, urine samples from different species were tested with **RSID™–Urine** (Table 2). The urine samples from various species were prepared by placing 100 µL of urine onto a cotton swab, and allowing the swabs to air-dry, and then extracting in 1 mL **RSID™–Urine** buffer. Extract was recovered and 100 µL placed in the sample well of **RSID™-Urine** cassettes; testing an equivalent of 10 µL of urine assuming 100% extraction efficiency. We found that a subset of non-human samples tested positive with RSID™–Urine; 1 of 2 gorilla samples, 2 of 3 equine samples, 1 of 2 rat samples , and half the canine samples (7 of 14) all tested positive with **RSID™–Urine**. Additional tests with these and more exotic species are underway. **RSID™–Urine** is specific for urine, but cannot be considered a human specific test.

Table 2. Summary of animal species tested with **RSID™–Urine**

Species	Number tested	Cross-reaction
Gorilla	2	Yes (1 of 2)
Cow	2	No
Cat	6	No
Dog	14	Yes (7 of 14)
Horse	3	Yes (2 of 3)
Alpaca	1	No
Sheep	2	No
Rabbit	2	No
Rat	2	Yes (1 of 2)
Opossum	1	No

RSID™–Urine: Testing for High Dose Hook Effect

A *high dose hook effect* refers to the false negative seen with immunochromatographic strip tests when very high levels of target are present in the tested sample. Under these conditions, unbound antigen can reach the test line *before* the labeled antibody thereby occupying the test line with non-labeled anti-antigen antibody, possibly resulting in a false negative result. Generically, lateral flow strip tests are susceptible to high dose hook effects – each specific test must be analyzed to determine if the particular configuration of strip, antibodies, and membrane could react together to produce a high dose effect false negative. Here we report on a series of experiments to test this question for **RSID™–Urine**; for these experiments 300 µL of urine were deposited onto a cotton swab, allowed to air dry, and extracted in 300 µL **RSID™–**

Urine buffer for 2 hours at room temperature. Extract, 0 – 100 µL, was added to **RSID™-Urine** cassettes and results recorded after 15 minutes (Fig. 9). The amount of extract tested was recorded on the cassette (Fig. 9). In order to verify our observation, this experiment was repeated on various production lots of **RSID™-Urine** with identical results (data not shown).

The strongest positive results on **RSID™-Urine** were seen when testing extract volumes of 10, 25, and 50 µL (fig. 9, lanes 5, 6, and 7, respectively). The signal observed on the cassettes decreased slightly at 75 and 100 µL extract volumes, indicating a weak to mild high dose hook effect (fig. 9, lanes 8 and 9). In functional terms, this is a large amount of tested urine (equivalent to 75-100 µL of urine) with no false negatives observed. In daily, practical terms, users of **RSID™-Urine** need not be concerned with false negatives due to high does hook effect, though a standard warning to exercise care and common sense when preparing extracts should be incorporated in laboratory SOPs.

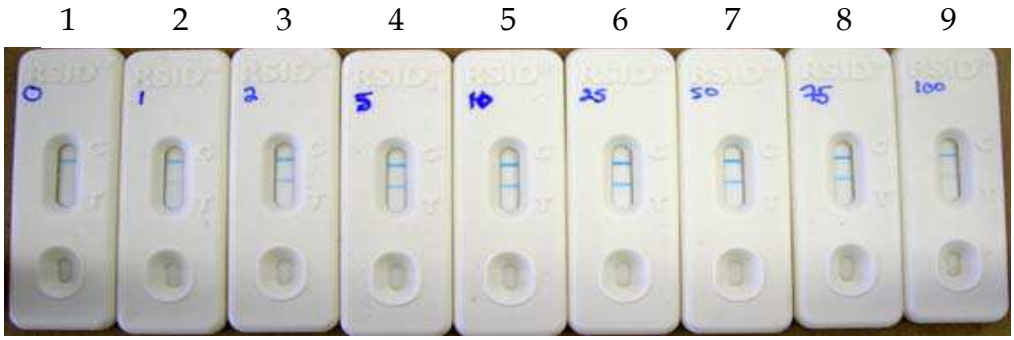


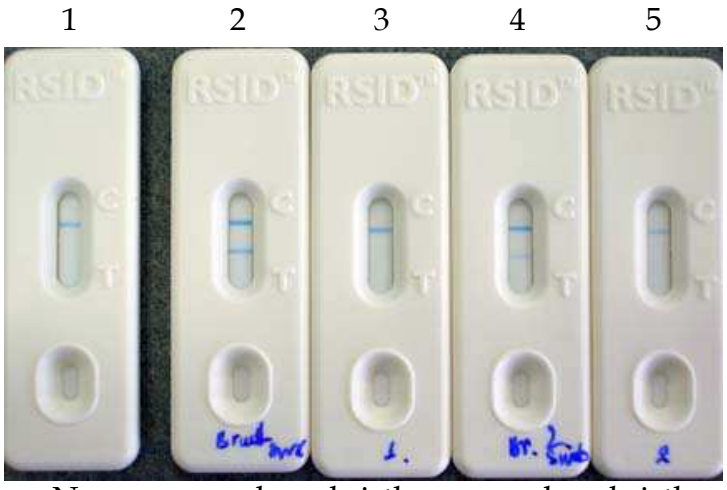
Fig. 9. High Dose Hook Effect of **RSID™–Urine**

Testing Casework Samples with RSID™–Urine

Independent Forensics has recently used **RSID™–Urine** for urine detection from two casework samples. One casework sample involved testing a toothbrush for the presence of urine. For this case, preliminary testing to determine the best method of evidence processing was performed. A positive control sample, toothbrush with added

urine, was processed by either cutting the bristles and extracting these in **RSID™–Urine** buffer for 1 hour at room temperature or by swabbing with a moistened swab and then extracting the swab in **RSID™-Urine** extraction buffer for 1 hour. Due to the individual variation in THP seen with urine samples, two different urine sources were used for this preliminary study (Fig. 10, top panel).

A. Toothbrush Control Study



B. Toothbrush exhibit

Fig. 10. Case Work Urine Detection using **RSID™–Urine**

The results of the preliminary study clearly showed that swabbing the toothbrush was a more effective and sensitive method of exhibit processing (Fig. 10, panel A, strips 2 & 4 vs. strips 3 and 5) for the detection of urine using **RSID™-Urine**. Importantly this observation was easily reproduced with both volunteer urine samples.

The case work sample was processed using both methods in the obvious sequence of first swabbing the evidence sample and then cutting the bristles and extracting those separately. The mandatory inclusion of positive and negative controls of buffer only and 10 µL urine (equiv volume) completed this test. The questioned swab and the bristles were extracted in 200 µL **RSID™–Urine** buffer for 1 hour at room temperature and 100 µL was placed on the cassette (fig. 10B, lower panel) and results were recorded after 15 minutes.

The extract from the swab of the toothbrush head was clearly positive after 15 minutes, whereas the extract from the cut bristles was negative (fig. 10B) indicating that the exhibit did indeed have urine. Note that for the testing of a toothbrush swabbing is clearly the superior method for identifying urine using **RSID™-Urine**.