Developmental Validation of
RSID™ Universal Buffer
with
RSID™-Semen, RSID™-Saliva, and RSID™-Blood

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Introduction

The new RSID™– Universal Buffer is designed for use with Independent Forensics' RSID™-Saliva, RSID™-Semen and RSID™-Blood tests. When using RSID™– Universal Buffer, forensic labs can now extract one sample using a single buffer, and test for three different body fluids: ***one sample, one buffer, three body fluid tests***. The use of a single buffer will enable laboratories to minimize sample consumption without compromising the specificity or sensitivity for the forensic detection of saliva, semen, and blood.

The original formulation of the RSID™ body fluid detection laboratory kits contained a dual buffer system: an extraction buffer and a separate running buffer specific for each kit; six buffers in all. RSID™-Saliva, RSID™-Semen and RSID™-Blood extraction buffers were designed to efficiently extract the specific antigen (α-amylase, semenogelin, and glycophorin A) from questioned stains and swabs. RSID™-Saliva, RSID™-Semen, and RSID™-Blood running buffers were designed to dissolve the antibody-colloidal gold conjugate from the conjugate pad, maintain an extract at the appropriate pH, and facilitate correct running of the test. Components of the extraction and running buffers include buffer and salts (Tris, NaCl, KCl) for physiological stability, a chelating agent (EDTA) for stability, detergents and surfactants (Triton X-100 and Tween 20) for extraction efficiency and solubility maintenance, protein (BSA) for reducing non-specific adsorption and loss, and a preservative (sodium azide).

The components of the RSID™- Universal Buffer are similar to the components of the original extraction and running buffers of the RSID™-Saliva, RSID™-Semen, and RSID™-Blood laboratory kits. However, the components have been optimized to allow ***one sample, one buffer, three body fluid tests*** without compromising sensitivity or specificity of the analysis.

RSID™**–** Saliva **w**as the first test developed by Independent Forensics and the initial RSID™– Saliva laboratory kits did not include provided buffers for extraction of samples or running of the RSID™ **–** Saliva cassettes. For optimal sensitivity and specificity and to encompass the wide variety of samples encountered by forensic labs,

Independent Forensics developed a dual buffer system consisting of an extraction buffer for extraction/soak of samples and a running buffer for optimal running of the strip test.

The next commercially released product was RSID™-Semen and this development paralleled that of RSID™-Saliva with the inclusion of optimized extraction and running buffers for the detection of semen, to again facilitate the sampling of a wide range of sample types encountered in forensic laboratories. Finally, the same basic approach was used for the development of RSID™-Blood and the RSID™-Blood extraction and running buffers. All three tests were robust, sensitive, and specific and are simple and easy to use. However, the use of individual extraction and running buffers for each RSID™ test requires that three separate portions from one evidence sample would be required to test for all three body fluids using RSID™-Saliva, RSID™-Semen, and RSID™-Blood. Although clearly feasible, this is not an ideal work flow for evidence samples of limited size.

Based on the stated need of our customers to conserve the amount of sample required for body fluid testing, we have developed a single buffer that can be used for both extraction/soak of the sample and running of the three RSID™ tests: RSID™-Universal Buffer. Using the RSID™-Universal buffer, the specificity and sensitivity of RSID™-Saliva, RSID™-Semen, and RSID™-Blood remain identical with a stated detected limit of <1 µL of the cognate body fluid. However, the experimental limit of detection of RSID™-Blood is slightly less sensitive in aged samples due to slightly less efficient extraction from the substrate. The following validation document summarizes our findings testing RSID™-Universal Buffer with RSID™-Saliva, RSID™-Semen, and RSID™-Blood and comparing side-by-side results against the original dual buffer system in the laboratory kits.

RSID™-Saliva – Sensitivity Using RSID™-Universal Buffer

This experiment demonstrates the sensitivity of RSID™-Saliva using the dual buffer system and the RSID™-Universal Buffer with a positive control swab where 50

μL of saliva was deposited on a sterile cotton swab and allowed to air-dry (our standard and recommended positive control sample). The cotton batting was removed from the swab using laboratory clean technique and placed in a 1.5 mL microcentrifuge tube and extracted in either 1 mL of RSID™-Saliva extraction buffer (see fig. 1, top panel) or 1 mL RSID™-Universal Buffer (see fig. 1, lower panel) for 1 hour at room temperature. Assuming 100% extraction efficiency each microliter of extract/soak will contain 50 nL (0.05 μL) of saliva. For the dual buffer system, the specified amounts of RSID™-Saliva extract were added to RSID™-Saliva running buffer to a final volume of 100 μL (volumes are noted on the cassettes, see fig. 1). For RSID™-Universal Buffer, the specified volumes of RSID™-Universal Buffer extract were added to RSID™-Universal Buffer for a final volume of 100 μL (volumes are noted on the cassettes, see fig. 1). All tested volumes of extract/soak from the RSID™-Saliva extraction buffer were scored positive (see fig. 1, lanes 1-5, top panel); identically, extraction in RSID™-Universal Buffer (fig. 1, lanes 1-5, bottom panel) were also scored positive. This experiment was repeated with 3 separate lots of RSID™-Saliva with identical results.

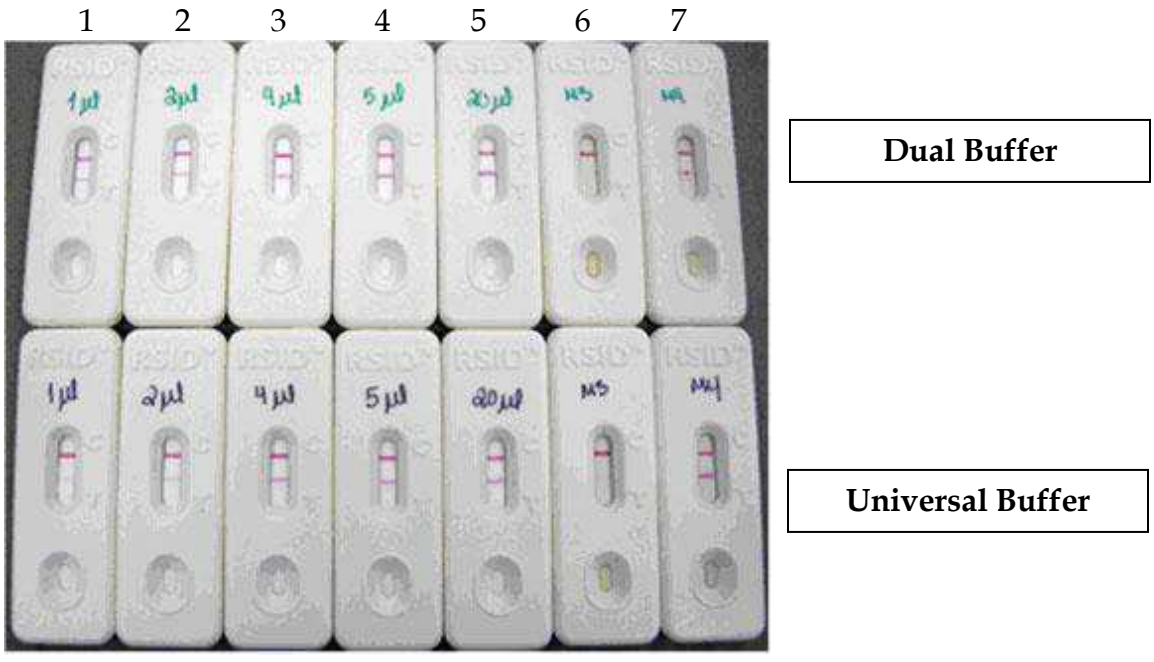


Fig. 1: Extraction Comparison and Body Fluid Mixtures

Body Fluid Specificity Testing: Mixed Extracts (blood, semen, urine) with and without added saliva extract.

In order to evaluate potential cross-reaction or inhibition of RSID™-Saliva when using RSID™-Universal Buffer, an experiment using mixed body fluids was performed. Here, 50 µL of semen, blood, and urine, were deposited on individual swabs and allowed to air-dry. Extracts from these swabs were prepared either in their original buffer (semen in RSID™-Semen extraction buffer, saliva in RSID™-Saliva extraction buffer, blood in RSID™-Blood extraction buffer, and urine in RSID™-Urine buffer) or each extract was prepared in RSID™-Universal Buffer. Combinations of body fluid extracts in their original buffer were tested with or without saliva (20 µL of each body fluid extract and added to final volume of 100 µL with RSID™-Saliva running buffer); these results were compared with body fluid extracts prepared in RSID™-Universal Buffer and added to a final volume of 100 µL with RSID™-Universal Buffer (20 µL of each body fluid extract). Positive signals were observed only in mixtures containing all four body fluid extracts, regardless of whether each body fluid was extracted in its original buffer (see fig. 1, lane 7, top panel) or RSID™-Universal Buffer (see fig.1 , lane 7, bottom panel). Results of three body fluid mixtures without saliva were negative (see fig. 1, lane 6, both panels). With both dual buffer systems and RSID™-Universal Buffer, the mixture of blood, semen and urine produced only a band at the control line with no visible signal at the test line (see figure below, lane 6, top and bottom panel). Sufficient volumes of extract, 20 µL of each extract equivalent to 1 µL of each body fluid, were tested to insure that even low levels of cross-reactivity, if present, would be observed.

Experimental Limit of Detection, RSID™-Saliva using RSID™ Universal Buffer.

To determine if the experimental limit of detection is affected when using RSID™-Universal Buffer for extraction and running with RSID™-Saliva strip tests, saliva extracts were prepared in either RSID™-Saliva extraction buffer or RSID™-

Universal Buffer (from a standard positive control swab, 50 µL saliva deposited on a cotton swab and allowed to air dry) and various volumes of extracts were tested in duplicate corresponding to the following amounts of saliva (see table 1, below).

Lane	Vol of saliva ext	Equiv vol of saliva
1,2	1 µL @ 1:2	25 nL
3,4	1 µL	50 nL
5,6	5 µL	250 nL
7,8	10 µL	500 nL
9,10	20 µL	1.0 µL

Table 1: Volume of Saliva Extract used for Experimental Limit of Detection.

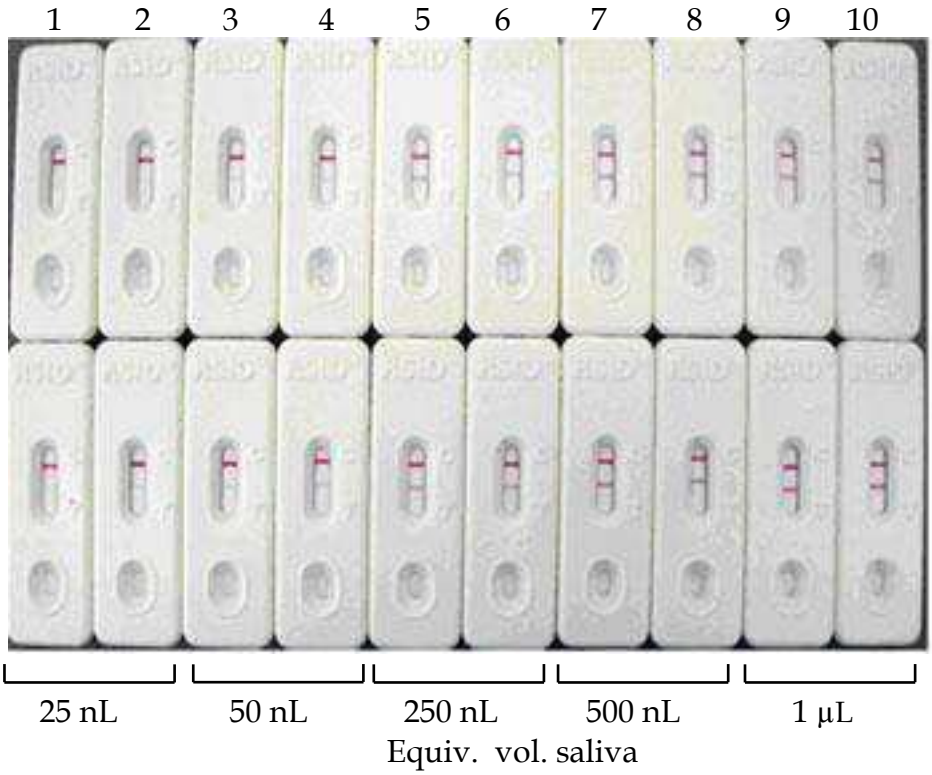


Fig. 2 Experimental Limit of Detection, RSID™-Saliva with Universal Buffer.

Due to the difficulty in photographing faint positive results, fig.2 does not provide the clearest result of the experimental limit of detection, however scoring of the cassettes clearly demonstrated that less than 25 nL of saliva could be reliably and reproducibly detected using either the dual or Universal buffer systems. Additional experiments, data not shown, demonstrated that as little as 5 nL of saliva could be scored as positive, again, with no difference observed between the tested buffer systems.

Detection of Saliva from Forensic-like sample: Drinking glass

To test the effect of using RSID™-Universal Buffer for the detection of saliva on a real world sample such as a drinking glass, two drinking cups (substrate, glass) were filled with water and the water consumed by a volunteer. The glasses were individually sampled with a damp swab. The head of each cotton swab was removed with scissors and one swab was extracted in 300 µL RSID™-Saliva extraction buffer whereas the second cotton swab was extracted in 300 µL RSID™-Universal Buffer; both extracts soaked for 1 hour at room temperature. Results from testing 20 µL of RSID™-Saliva extract in extraction buffer added to 80 µL RSID™-Saliva running buffer are shown in fig. 3, lanes 5 and 6. These strips clearly score positive for saliva. As per protocol, a negative control was included in which a blank cotton swab was extracted in 300 µL RSID™-Saliva extraction buffer and 20 µL of the RSID™-Saliva extract was added to 80 µL RSID™-Saliva running buffer and added to the RSID™-Saliva cassette (see fig. 3, lanes 7 and 8, right panel); as noted, samples were run in duplicate. Results from testing 20 µL of the RSID™-Universal Buffer extract added to 80 µL of RSID™-Universal Buffer and added to the RSID™-Saliva cassette are shown in fig. 3, , lanes 1 and 2, right panel. Again, a negative control was included in which a blank cotton swab was extracted in 300 µL RSID™-Universal Buffer and 20 µL of the RSID™-Universal Buffer extract was added to 80 µL of RSID™-Universal Buffer and added to the RSID™-Saliva cassette, and again, samples were run in duplicate (see fig. 3, lanes 3 and 4, right panel).

The results, fig. 3, clearly show positives where expected and show negatives where expected, demonstrating that RSID™ Universal buffer is just as effective as the dual buffer system for identifying saliva from this substrate.

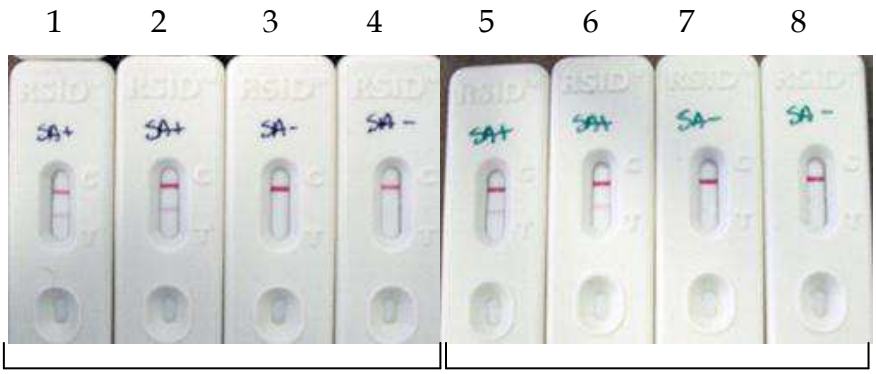


Fig. 3: Detection of Saliva From Drinking Container: Glass

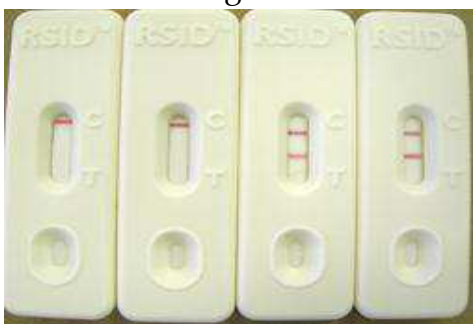
In order to insure that RSID™-Universal Buffer can be used as a general approach to detecting saliva, additional tests with different substrates were performed. Saliva detection from the lid of a Starbucks coffee cup, a ceramic coffee mug, and a plastic Nalgene drinking container were all attempted using RSID™-Saliva and RSID™-Universal Buffer (fig. 4).

The results from attempting to detect saliva from these different substrates using RSID™-Saliva with RSID™-Universal buffer with an identical protocol as above (fig.4) clearly demonstrate the flexibility and sensitivity of this approach and illustrates the effectiveness of RSID™-Universal buffer when used on forensic-type samples.

Ceramic coffee mug

Clear plastic water bottle

Red Nalgene bottle



Starbucks coffee cup lid

Fig. 4 – Saliva Detection from Different Substrates

RSID™-Semen – Sensitivity Using RSID™-Universal Buffer

The sensitivity of RSID™-Semen with RSID™-Universal Buffer was examined using a positive control swab; 50 µL of semen deposited on a sterile cotton swab and allowed to air-dry. The cotton batting was removed using laboratory clean technique and placed in a 1.5 mL microcentrifuge tube and extracted in either 1 mL of RSID™-Semen extraction buffer (fig.5, top panel) or 1 mL RSID™-Universal Buffer (fig. 5, bottom panel) for 1 hour at room temperature. Assuming 100% extraction efficiency each microliter of extract will contain 50 nl (0.05 µL) of semen. Testing of 1, 2, 4, 5, and 20 µL of extract from the RSID™-Semen extraction buffer showed clear positives (lanes 1-5, top panel), identical to extraction in the RSID™-Universal Buffer (lanes 1-5, bottom panel). Reproducible results were obtained in these series of experiments using 3 separate lots of RSID™-Semen (data not shown).

Fig. 5: Comparison RSID™-Semen: Dual Buffer and RSID™-Universal Buffer

RSID™ -Semen Experimental Limit of Detection (LOD).

The experimental limit of detection for RSID™-Semen was determined using both the cognate dual extraction and running buffer system and RSID™-Universal buffer. Various volumes of positive control extract were tested as shown (Table 2) as increasing volumes of semen extract were tested either with the dual buffer system or with RSID™-Universal Buffer.

Lane	Vol of semen ext	Equ vol of semen
1	0	0
2	1 µL @ 1:20	2.5 nl
3	1 µL @ 1:10	5 nl
4	1 µL @ 1:5	10 nl
5	1 µL @ 1:2	25 nl
6	1 µL	50 nl
7	5 µL	250 nl
8	10 µL	500 nl
9	20 µL	1.0 µL

Table 2: Volume of Extract used for LOD

1 2 3 4 5 6 7 8 9

1 2 3 4 5 6 7 8 9

Fig. 6: RSID™ Semen LOD Determination

A positive signal was detected with as little as 2.5 nL of seminal fluid equivalent (see figure below, lane 2). This limit of detection is similar to the limit of detection of RSID™-Semen when using the dual buffer system (data not shown). A positive signal was seen at all volumes of semen extract tested (see below).

RSID™-Semen - Body Fluid Specificity Testing: Mixed Extracts (blood, saliva, urine) with and without added semen extract.

In order to evaluate potential cross-reaction or inhibition of RSID™-Semen when using RSID™-Universal Buffer, an experiment using mixed body fluids was performed. Here 50 µL of semen, saliva, blood, or urine was deposited on a swab and allowed to air-dry. Extracts from semen, saliva, blood, and urine were prepared either in their original buffer (semen in RSID™-Semen extraction buffer, saliva in RSID™-Saliva extraction buffer, blood in RSID™-Blood extraction buffer, and urine in RSID™-Urine buffer) or each extract was prepared in RSID™-Universal Buffer. Combinations of extracts with or without semen (20 µL of each body fluid extract) were tested; only the mixture containing all four body fluid extracts gave a positive signal (see fig. 7 lanes 2 and M4), regardless of whether each body fluid was extracted in its original buffer (see fig. 7, lane 2) or RSID™-Universal Buffer (see fig. 7, lane M4), whereas the mixture of blood, saliva and urine extract produced only a band at the control line with no visible signal at the test line (see fig.7 lanes 1 and M3). Sufficient volumes of extract, 20 µL of each extract, equivalent to 1 µL of each body fluid, were tested to insure that even low levels of cross-reactivity would be observed, if present.

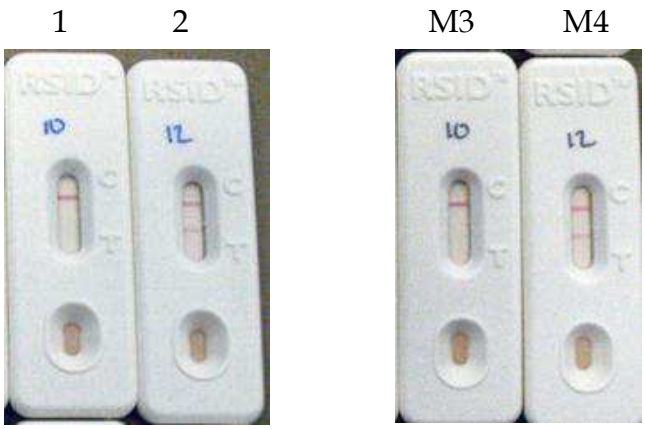


Fig. 7: RSID™-Semen and Mixed Body Fluids

RSID™-Semen – Testing post-coital samples

To evaluate the use of the RSID™-Universal Buffer with real world samples such as swabs from sexual assault evidence kits, post-coital swabs with known semen content were tested. Both aged (swabs collected in 2006) and recently collected swabs (2010) were evaluated (swabs were collected 10 hours post coitus). Swabs were extracted in 300 µL RSID™-Universal Buffer for 1 hour at room temperature and 20 µL of each extract was tested using RSID™-Semen (see fig. 8). In order to evaluate the possible high dose hook effect of using RSID™ Universal buffer, 100 µL of each extract was also tested on RSID™-Semen strips. As per protocol, a negative and positive control was included with each set of strips.

20 µL and 100 µL of extract from both the 2006 and 2010 swabs were clearly positive (see fig. 8) indicating that the RSID™ Universal buffer works well for extraction of PC samples and testing with RSID™-Semen.

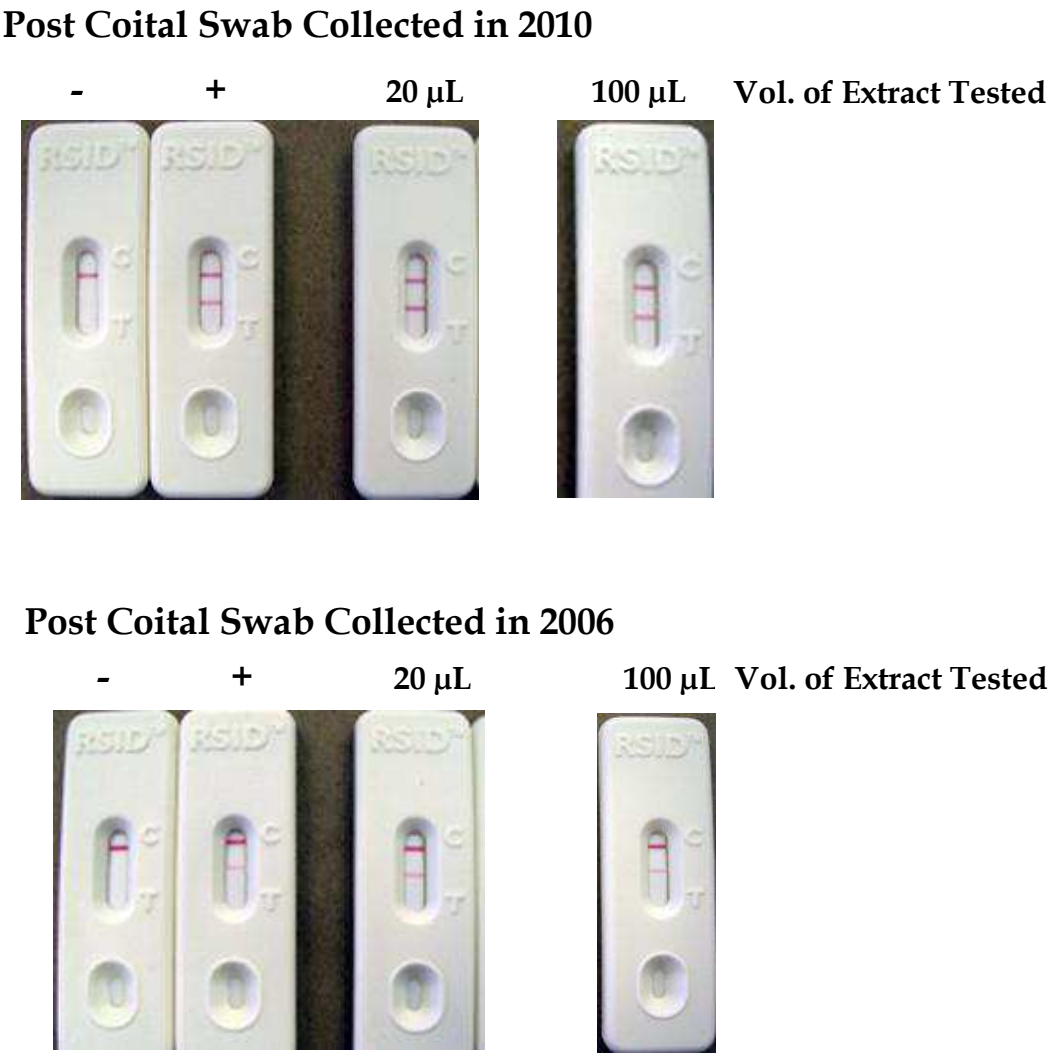


Fig. 8: RSID™-Semen, Post-Coital samples and Universal Buffer.

RSID™-Blood – Sensitivity Using RSID™-Universal Buffer

The sensitivity of RSID™-Blood using RSID™-Universal Buffer was examined using a positive control; 50 µL of blood deposited on a sterile cotton swab and allowed to air-dry. The cotton batting was removed using laboratory clean technique and placed in a 1.5 mL microcentrifuge tube and extracted in either 1 mL of RSID™-Blood extraction buffer (see fig. 9, top panel) or 1 mL RSID™-Universal Buffer (see fig. 9, bottom panel) for 1 hour at room temperature. Assuming 100% extraction efficiency

each microliter of extract will contain 50 nl (0.05 µL) of body fluid. Testing of 1, 2, 4, 5, and 20 µL of this extract from the RSID™-Blood extraction buffer showed clear positives (lanes 1-5, top panel), similarly to extraction in the RSID™-Universal Buffer (lanes 1-5, bottom panel). Identical results were seen with all repetitions of this experiment using three separate lots of RSID™-Blood. This experiment was performed with a blood sample less than one year old.

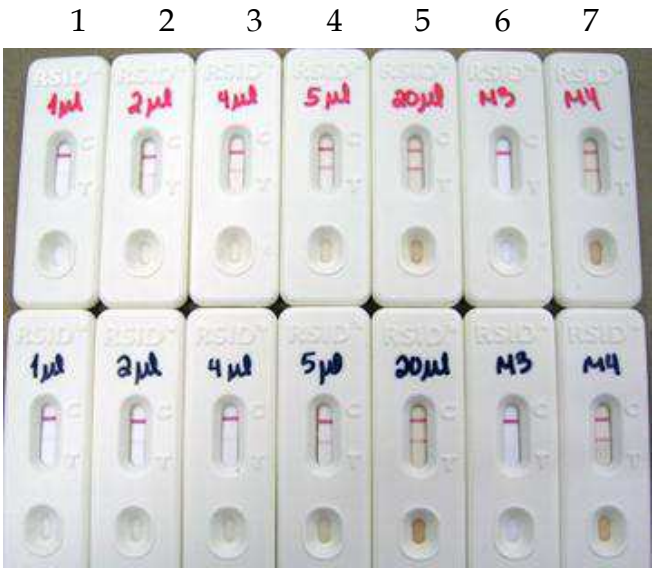


Fig. 9: Extraction Comparison, RSID™-Blood. Dual Buffer system *vs.* Universal Buffer.

To test the detection of blood from an older sample, two cotton swabs on which 50 µL of blood had been deposited and air-dried in Oct 2006, were extracted in 1 mL of either RSID™ -Blood extraction buffer (see fig.10, top panel) or RSID™ -Universal buffer (see fig.10, bottom panel) and various volumes of extract were tested: 0, 2, 5, 10, 20, 50, and 100 µL. For the top panel, 2, 5, 10, 20, and 50 µL of extract were added to RSID™ –Blood running buffer for a final volume of 100 µL. An equivalent experiment using RSID™-Universal Buffer, is shown where the final volume of the extraction in RSID™-Universal buffer was made up to 100 µL with Universal buffer. The results

clearly demonstrate that with the dual buffer system, a positive signal could be readily observed with as little as 5 µL of extract whereas a positive signal could only be seen with 10 µL of extract when using the RSID™ -Universal buffer (see fig. 10, top panel lane 3 vs. fig. 10, lower panel lane 4). These positive results were difficult to photograph in fig. 10 but were clearly visible to the naked eye. The sensitivity of the blood extract from the older sample (made in 2006) was slightly lower when using RSID™ -Universal buffer as compared to the dual buffer system. This decreased sensitivity in experimental LOD was repeated with numerous older blood samples when using RSID™ -Universal buffer for extraction and running tests with RSID™ – Blood.

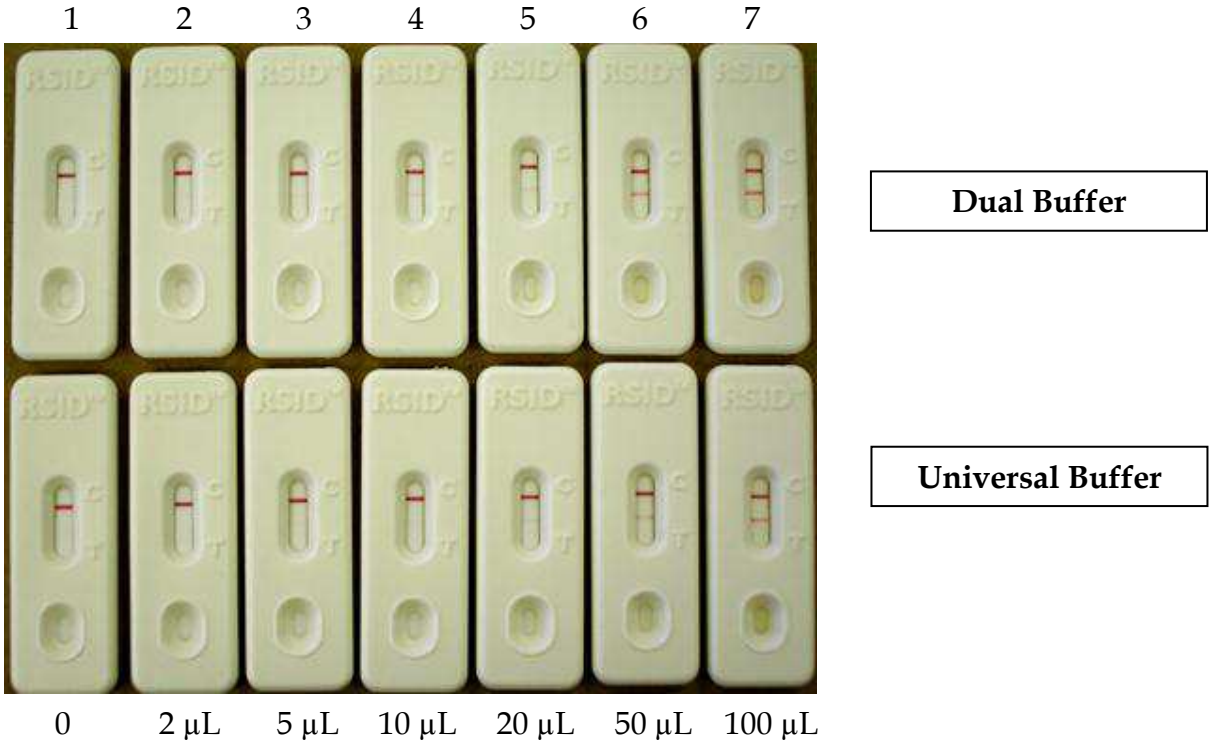


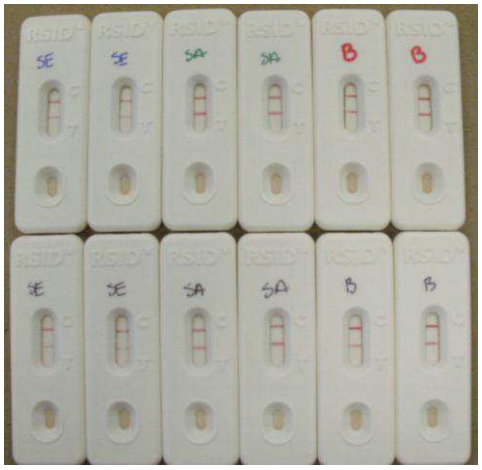
Fig. 10: Experimental LOD with RSID™-Blood, Dual Buffer System vs. Universal Buffer.

One Sample, One Buffer, Three tests

The RSID™ -Universal buffer will allow forensic analysts to use a single buffer to test one piece of evidence for the detection of three different body fluids, blood, saliva and semen. Here we examine the ability of RSID™-Universal buffer to extract body fluids from three different types of material, cotton fabric, denim, and a cotton swab and compare the dual buffer system with RSID™ Universal buffer for the detection of three human body fluids. In this series of experiments, 200 µL of each body fluid (semen, saliva, and blood) was mixed in a 1.5 mL eppendorf tube and 50 µL of this mixture was deposited on several examples of each of the three materials and allowed to air-dry. The cotton swab was tested with the dual system by extracting the entire swab head in a 1.5 mL tube with 1 mL RSID™ Extraction buffer [three swabs in total, one each for RSID™ -Saliva, RSID™ –Semen, and RSID™ –Blood extraction buffers] for 1 hour at room temperature. From each extract 20 µL was removed for analysis after bringing the volume to 100 µL with the cognate running buffer. For testing RSID™ -Universal buffer, a *single* cotton swab was extracted in 1 ml RSID™ -Universal buffer for 1 hour at room temperature. Aliquots of 20 µL were removed and added to final volume of 100 µL with RSID™ -Universal buffer for separate testing with RSID™ -Saliva, RSID™ –Semen, and RSID™ –Blood. To reiterate, a single cotton swab was extracted and used for testing all three body fluid tests using RSID™ -Universal buffer. All tests were performed in duplicate.

The results from the cotton swab were the same for RSID™ -Saliva, RSID™ –Semen, and RSID™ –Blood regardless of method of extraction (see fig. 11, top left panel). Extracting a single swab with RSID™ -Universal buffer and testing for three body fluids gave positive results for all three tests, similarly to extracting and testing three different swabs in their cognate dual buffer system.

Cotton Swab



Dual Buffer

Universal Buffer

Denim Fabric

Cotton Fabric

Dual Buffer

Universal Buffer

Fig. 11: One sample One buffer Three tests, Three substrates test.

Similar to the cotton swab, the same procedure for testing cotton and denim material was performed to compare extracting a single piece of fabric in RSID™ -Universal buffer with three separate extractions in RSID™-Saliva, RSID™-Semen or RSID™-Blood extraction buffer. The cotton and denim material was extracted from a 20 mm^2 cutting in 300 µL RSID™ -Universal buffer and in parallel, three separate 20 mm^2 cuttings of each fabric were extracted in 300 µL RSID™-Saliva, RSID™-Semen or RSID™-Blood extraction buffer for 1 hour at room temperature . For the RSID™ -Universal buffer,

aliquots of 20 µL were removed from the RSID™ -Universal buffer extraction for each of the three tests (tests were done in duplicate) and added to a final volume of 100 µL with RSID™ -Universal buffer. For testing with dual buffer system, 20 µL was removed from each of the three cognate buffer extracts from both types of fabric and added to final volume of 100 µL with cognate running buffer. The strip test used, RSID™-Saliva, RSID™-Semen or RSID™-Blood is marked on the cassette (SE, SA, B) in fig. 11.

Similar to the cotton swab, the results from the cotton fabric and denim were the same for RSID™ -Saliva, RSID™ –Semen, and RSID™ –Blood regardless of method of extraction (see fig. 11, bottom left panel and top right panel). Extracting a single cutting with RSID™ -Universal buffer and testing for three body fluids gave positive results for all three tests, similarly to extracting and testing three different cuttings in their cognate dual buffer system. These results are representative of several different experiments.

We have demonstrated that the RSID™ -Universal buffer can be used to extract a single sample followed by testing for three different body fluids using RSID™-Saliva, RSID™-Semen or RSID™-Blood (fig. 11). Some consideration should be taken if an aged blood sample is tested since use of RSID™ -Universal buffer results in slightly lower sensitivity with aged blood samples. To address this, more concentrated extract can be tested since RSID™-Blood does not exhibit the high dose hook effect.