Developmental Validation Studies of RSID™-Semen A Lateral Flow Immunochromatographic Strip test for the Forensic Detection of Seminal Fluid

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Developmental Validation Study of Rapid Stain Identification Test for Semen (RSID™-Semen) by Independent Forensics

Introduction

Semen is the most common form of body fluid evidence encountered or sought in sexual assault cases. In screening or examining sexual assault evidence, semen or other body fluids, may be present on a variety of surfaces including sample collection swabs (sexual assault evidence collection kits or crime scene collection kits), articles of clothing, bed sheets, towels, flooring, condoms, and feminine hygiene products. These evidence samples are often stored for many years: testing for body fluid identification and DNA profiling must be able to reliably, specifically and with high sensitivity detect semen from a variety of sources. Current forensic laboratory identification methods for semen are presumptive, *i.e.*, they use methods that are not specific for seminal fluid, but provide a criminalistic basis for continued processing of the tested exhibit. These methods include dye-enhanced acid phosphatase testing and antigen P30 testing or use generalized staining (*e.g.*, KPIC stain) with microscopic examination in an attempt to identify sperm. These detection protocols are not inherently specific for semen, or sperm, and are therefore open to legal and scientific challenge. In addition they are prone to false positives, false negatives and thus inefficient, time consuming and expensive.

Current methods in general practice to determine the presence of human semen in sexual assault evidence involve testing for acid phosphatase activity as well as testing for the presence of prostate specific antigen, sometimes called antigen p30. Acid phosphatase testing is a well documented presumptive test for the presence of semen (Brauner 1992, Brauner and Gallili 1993, McCloskey *et al.* 1975, Schiff 1978, and Forensic Science Symposium on the Analysis of Sexual Assault Evidence, FBI academy, 1983). However, acid phosphatase activity is not confined to semen or prostatic tissue, and in fact, the acid phosphatases found in vaginal secretions and lysozomes are all genetically

identical (Sensebaugh 1978). Immunoassays detecting the presence of p30 are available, but recent studies have found p30 to be present in substantial levels in amniotic fluid and breast milk (Lovgren *et al*. 1999; Yu & Diamandis 1995), as well as serum from females and female urine (Breul *et al*. 1994 and 1997). In addition, low concentration of p30 can be detected in vaginal fluid (Macaluso *et al*. 1999).

The following is a validation summary of RSID™-Semen (**R**apid **S**tain **Id**entification), a lateral flow immunochromatographic strip test that uses two monoclonal antibodies in a lateral flow format which detects the presence of semenogelin, a unique protein specific to seminal fluid. Semenogelin is the major protein component of human semen, and together with fibronectin, gives rise to the gel-like coagulum of newly ejaculated semen (Lundwall *et al*. 2002). Interestingly, semenogelin is the substrate for the P30/PSA protease and as all biochemistry students know, there is considerably more substrate than enzyme in biological reactions.

Here we present experimental evidence demonstrating that our test for semenogelin test is accurate, reproducible, easy to use, and highly specific for human semen and can identify semen from a variety of materials and surfaces. In addition, we describe studies showing lack of cross-reactivity with vaginal fluid, as well as studies on the sensitivity, body fluid specificity, species specificity, and stability of the RSID™-Semen.

Configuration of the Semenogelin lateral flow test

RSID™-Semen is an immunochromatographic assay that uses two monoclonal antibodies specific for semenogelin. This 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, running 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 semenogelin monoclonal antibodies. The diluent redissolves the colloidal gold labeled anti α-semenogelin antibodies which will bind semenogelin if it is present in the sample. Semenogelin-colloidal gold antibody complexes are transported by bulk fluid flow to the membrane phase of the test strip.

Quantification of semenogelin strip tests

To quantify the results of the strip tests used in the validation studies, the intensity of the test strip line is scored by visual comparison against a standard. This visual chart consists of a series of graded reddish lines from faint to strong against which the observed control and test lines are given an intensity score. The operator compares the test line of the strip test against the score sheet, and records the observed intensity; this method minimizes operator variance and provided quantitative data for Quality Control/Quality Assurance and was used for these validation studies. RSID™-Semen is a qualitative test for the detection of semenogelin.

Sensitivity – Testing Semen Extract and the high dose hook effect

For sensitivity studies, we tested semen extract, in which 50 µL semen was deposited on a cotton swab and allowed to air-dry. The cotton batting of the cotton swab was cut off, placed in a 1.5 ml microfuge tube, and extracted in 1 mL RSID™-Semen extraction buffer for 1 hour at room temperature. Assuming 100% extraction efficiency, each µL of extract will contain approximately 50 nL of semen (concentration of the extract, 50 nL semen/µL extract). To assess the threshold of the high dose hook effect, we tested increasing amounts of the semen extract. A *high dose hook effect* refers to the decrease in test line intensity seen with increasing amount of antigen in the extract. This can lead to a false negative on immunochromatographic strip tests when very high levels of target are present in the tested sample. Under these conditions, unbound semenogelin antigen reaches the test line *before* the colloidal gold-labeled antibody thereby occupying the test line with non-labeled anti-semenogelin resulting in a

decrease signal can produce a false negative result. Please see Table 1 for the dilutions of extract tested. The ten dilutions of semen extract (as well as a sample lacking semen extract for a negative control) were tested with RSID™-Semen by adding the indicated semen extract to a final volume of 100 µL with RSID™-Semen running buffer and placing the 100 µL in the sample window of the cassette. The control and test lines in the strip test window were evaluated after 10 minutes.

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 1: Semen Extract Dilutions Tested.

1 2 3 4 5 6 7 8 9

Fig. 1: Sensitivity of RSID™-Semen with Semen Extract

Results- Sensitivity of Semen Extract

The strongest band intensity was seen with 0.5, 1, and 5 µL of semen extract after 10 minutes (see fig. 1 lanes 5, 6, and 7, respectively). Although a weaker band was

observed with the 1 µL @ 1:20 dilution (2.5 nL semen), this sample was clearly positive after 10 minutes (see fig. 1, lane 2). This indicates that RSID™-Semen can detect as little as 2.5 nL human semen. The signal slightly decreased with 10 and 20 µL semen extract (see photo above, lane 9), indicative of a high dose hook effect.

Testing the threshold for high dose Hook effect

To determine the threshold of the high dose Hook effect with increasing concentrations of semen, we tested semen extract in which 50 µL semen was extracted into smaller volumes than our standard 1 mL RSID™-Semen extraction buffer, *i.e.*, 200 µL and 400 µL, to increase the semen concentration of the extract. Assuming 100% extraction efficiency, each µL of extract from the 400 µL extraction will contain approximately 125 nL of semen (concentration of 125 nL semen/µL extract). The entire volume from the 200 µL extract was tested, which was approximately 100 µL liquid (after absorption of the extract liquid into the cotton swab). The volumes of semen extract were tested with the semenogelin strip tests and the equivalent amounts of semen are listed in the Table 2.

Lane	Vol of semen ext	Equ vol of semen
1	0	0
2	5 µL (1 mL ext)	250 nL
3	1 µL (400 µL ext)	125 nL
4	5 µL (400 µL ext)	625 nL
5	25 µL (400 µL ext)	3.125 µL
6	50 µL (400 µL ext)	6.25 µL
7	100 µL (400 µL ext)	12.5 µL
8	~50 µL (200 µL ext)	50 µL

Table 2: Volume of Semen Extract used for Testing High Dose Hook Effect.

The signal from 1 µL of the 400 µL extraction was positive after 10 minutes (fig. 2. lane 3), and was comparable with the signal from 5 µL extract of the 1 mL extraction volume (lane 2 in photo below). As we increased the extract volume to 5 µL extract (from the 400 µL extract), the signal decreased (see lane 4 in photo below). The signal from extract volumes of 25, 50, and 100 µL (3.125, 6.25, and 12.5 µL semen; lanes 5, 6, and 7, respectively) were roughly equivalent to the "buffer only" signal (lane 1 in photo below), indicating false negative results. Furthermore, the signal from the complete extract volume from the 200 µl extract (~50 µl) was ~0 after 10 minutes, also indicating a false negative result (lane 8 in photo below).

Fig. 2: Dose Dependent High Dose Hook Effect

Conclusion: A high dose hook effect is present on RSID™-Semen. The effect can produce a false negative result at high semen concentrations – specifically when analyzing samples that load greater than 3 µL of semen into the sample well.
This issue can be readily addressed by using a dilution of the sample to insure that the amount of tested semen is below the high dose hook effect threshold. In general, a simple dilution of 1:20 is sufficient to allow proper function of the lateral flow strip test. We will therefore analyze samples exhibiting a high dose hook effect by diluting the sample 1:20 and retesting with RSID™-Semen.

Diluting semen samples 1:20 when RSID™-Semen signal is low

If the signal from RSID™-Semen is low, a possible high dose hook effect may be occurring. To address this, a dilution of the sample by a factor of 1:20 and retesting

with RSID™-Semen will resolve the issue as one of two possible results will be observed: 1) an ***increase*** in signal is observed after the 1:20 dilution, an indication of a high dose hook effect in the first tested sample or 2) a decrease in signal after the 1:20 dilution indicating that there was a limiting amount of semen in the first sample tested.

To determine if diluting a sample by a factor of 20 is sufficient to address a high dose hook effect, we diluted a semen extract in which 50 µL semen was extracted in 400 µL or 200 µL RSID™-Semen extraction buffer by a factor of 20. We tested the same volume of extract that demonstrated a high dose hook effect, but with a 1:20 dilution prior to addition to RSID™-Semen; i.e., 5, 25, 50, and 100 µL of the 400 µL extract (fig. 3, lanes 3, 4, 5, and 6) and 50 µL of the 200 µL extract (fig3., lane 7).

The 5 µL extract from the 1 mL extraction volume sample was positive after 10 minutes (fig. 3, lane 2). The signal from the 400 µL extract at 1:20 dilution was scored at similar intensity for the 5, 25, 50, and 100 µL volumes tested, after 10 minutes (fig. 3, lanes 3, 4, 5, and 6); all results scored at 10 mins. The 1:20 dilution of the 200 µL extract was positive after 10 minutes (fig. 3, lane 7).

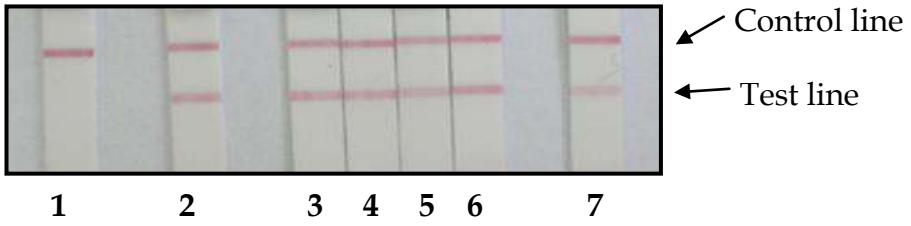


Fig. 3: Dilution Determination Test – High Dose Hook Effect

Conclusion: A simple 1:20 dilution of the tested sample will determine if a weak signal seen on RSID™-Semen is a result of high dose hook effect or the result of limiting semen in the tested sample. Importantly, the dilution calculation demonstrates consistency with the known limit of detection for RSID™-Semen. A dilution of a sample that originally contained approximately 3-5 µL, and thus would likely exhibit a high dose hook effect, by a factor of 20 would bring the sample in the

range of approximately 150-250 nL semen, an amount that produces a strong signal using RSID™-Semen.

Body Fluid Specificity – Testing extracts from swabs of human blood, saliva, semen, and urine alone or as a mixture of body fluids.

Here we test the ability of non-cognate body fluids to interfere or reduce the specificity of RSID™-Semen to detect seminal fluid; 50 µL of saliva, urine, semen, or blood were each deposited on separate cotton swabs and allowed to air-dry. The cotton batting of the swab was removed using laboratory clean technique and placed in 1.5 mL microcentrifuge tube and extracted into 1 mL RSID™-Semen extraction buffer for 2 hours at room temperature. Extracts from body fluids were combined with and without semen to evaluate potential cross-reactivity and possible inhibition due to the presence of other body fluids. Semen extract, 5 µL, was tested in the presence of 25 µL of extract from the other three body fluids (saliva, blood & urine, see fig. 4, lane 6), or all three body fluids (25 µL of each extract, blood, saliva & urine, see fig.4, lane 7) were combined and tested on RSID™-Semen. All tested volumes were brought to 100 µL with RSID™-Semen running buffer. Assuming 100% extraction efficiency, 25 µL of body fluid extract from these samples is approximately 1.25 µL whole body fluid.

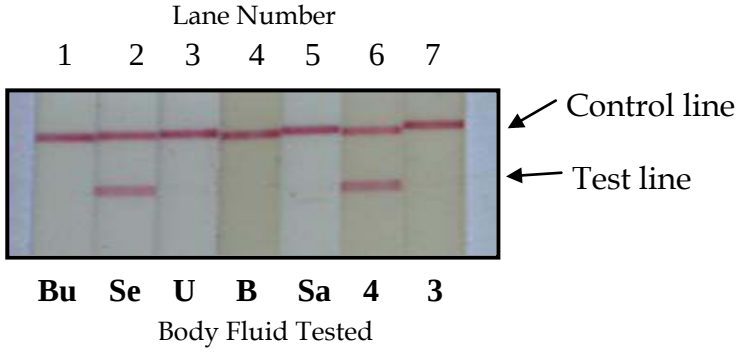


Fig. 4 – Mixed Body Fluid Test on RSID™-Semen

Testing extract from swabs of human blood, saliva, semen, and urine alone or as a mixture of body fluids (fig. 4) clearly demonstrates that the specificity of RSID™-Semen is unaffected by non-cognate body fluids (fig.4, compare lanes 3, 4, and 5 with lane 7) and that these added body fluid extracts do not interfere with the sensitivity of the test (fig. 4, compare lane 2 and lane 6).

<u>Conclusions:</u> There is no cross-reactivity of urine, blood, or saliva extract, either alone or as a mixture with RSID™-Semen. In addition, the sensitivity of semen extract is not affected by the presence of these other body fluids.

Stability of RSID™-Semen

We have previously demonstrated that RSID™-Semen is both specific and sensitive for human semen. Another critical component for the use of these tests is their stability; here we demonstrate the robustness of this product by storage at elevated temperature. In general, storage of the strip tests at 37° C for one month mimics storage at room temperature for one year.

We have tested 0, 1 µL (~50 nL semen), and 5 µL (~250 nL semen) semen extract with RSID™ -Semen strip tests after storage of the strips at 37° C for 1.5 months.

The stability of RSID™ -Semen strip tests was not affected by storage at 37° C for 1.5 months, as compared to strip tests stored at room temperature. The sensitivity of 1 and 5 µL semen extract was the same under both conditions (data not shown).

Conclusions – No significant change in sensitivity of RSID™ -Semen after storage at 37° C for 1.5 months was observed. We have therefore set the expiration date of RSID™ –Semen at 18 months. Extensive work since the release of this product has demonstrated the accuracy of this shelf life.

Detection of Semen from Forensic Exhibit-like Samples

We have clearly established that RSID™ -Semen can detect semen from laboratory prepared control samples; here we demonstrate that RSID™ -Semen can detect semen from samples likely to be encountered in forensic laboratory case work. We present experiments testing for the presence of semen from multiple types of fabric, as well as numerous experiments demonstrating that RSID™ -Semen does not cross-react with vaginal fluid, an important aspect of the RSID™ -Semen over semen detection using acid phosphatase and PSA-based tests.

Test sample 1: Testing woman's undergarment for semen detection using RSID™ -Semen

Independent Forensics was presented with a pair of woman's black undergarments with a request for semen detection. The area of visible stain on the undergarment was swabbed with a moistened sterile cotton swab, and the swab was allowed to air-dry. For a negative control, an area of the undergarment without a visible stain was swabbed with a moistened cotton swab and the swab was allowed to air-dry. Both the positive and negative swabs were extracted in 300 µL RSID™ –Semen extraction buffer for 2 hours and 10 µL of the extracts were tested with RSID™ –Semen strip tests. Positive and negative controls were included as a matter of course - 1 µL of authentic semen extract and a "buffer only" sample.

The signal from 1 µL semen extract and 10 µL of "positive" stain were scored identically after 10 minutes and were strong positives (see fig. 5, lanes 2 and 4). Both the "buffer only" and extract from the "negative" stain were negative after 10 minutes (see fig. 5, lanes 1 and 3). In conjunction with our analysis of the extracts on RSID™ -Semen, we also tested the "positive" and "negative" extracts for semen using the Abacard PSA. Identical volumes of the extract were tested on both the PSA and RSID™ -Semen strips. The results from the Abacard PSA card revealed a negative result from 10 µL of the "positive" stain after 10 minutes (see fig. 6). This same extract was also tested using dye-enhanced acid phosphatase detection; the stain was only "weakly"

positive. Certainly from this evidence sample, the PSA and acid phosphatase tests were less sensitive than RSID™-Semen.

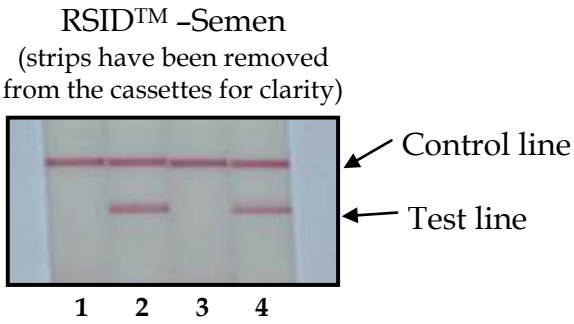


Fig. 5: Detection of Semen from SAE – stain on women's underwear

Fig. 6: Analysis of questioned stain using Abacard PSA (Antigen p30) Test, used as directed for sample.

Test sample 2: Testing cuttings from post-coital vaginal swabs; comparing results with and without condom use.

We received clippings from post-coital vaginal swabs collected after intercourse with and without the use of a condom. We tested two post-coital vaginal samples with the use of a condom, and two post-coital samples at Day 0 (same day) and Day 14 (14 days post intercourse) without the use of a condom. Clippings from these swabs were extracted in 100 µL RSID™ –Semen extraction buffer for 1 hour at room temperature and 10 µL of the extract was tested with RSID™ -Semen.

The intensities of 10 µL extract of samples #1 and #2, Day 0 and 14 post-coital with the use of a condom were zero, negative result, after 10 minutes (lane 3 and 4 in digital photo below), similar to the "buffer only" signal (lane 1 in photo below).
10 µL extract from the Day 0 and 14 samples post-coital without the use of a condom (see figure below, lanes 5 and 6, respectively) were also tested: day 0 samples scored strongly, while the day 14 sample was negative. This result was repeated when a second set of independent swabs were analyzed, (see figure below, strips 7 and 8 which represent RSID™ -Semen results from Day 0 and Day 14 post-coital without a condom).

These results indicate that RSID™ -Semen detects semen from post-coital vaginal swabs at Day 0 without the use of a condom. These data indicate that RSID™ -Semen does not cross react with extracts from vaginal swabs and appears specific for semen.

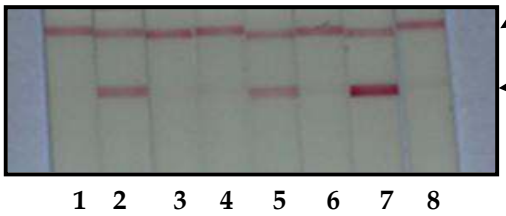


Fig. 7: RSID™-Semen Analysis of Post-coital vaginal swabs

Strip 1 – Buffer only
Strip 2 – 1 µL semen extract
Strip 3 – #1 Day 0, post-coital with condom
Strip 4 – #2 Day 14, post-coital with condom
Strip 5 – #3 Day 0, post-coital, no condom
Strip 6 – #4 Day 14, post-coital, no condom
Strip 7 – #5 Day 0, post-coital, no condom
Strip 8 – #6 Day 14, post-coital, no condom

Fig. 7 Legend –Sample Map

The observed results coincide identically with the expected presence of semen in that the samples obtained from intercourse with a condom are negative, but samples tested post intercourse without a condom test positive for semen. The lack of semen detection fourteen days post-coital is expected, and is interpreted as demonstrating the specificity of the test for semen.

Test sample 3: Testing post-coital vaginal swabs without condom use.

We obtained samples from a volunteer consisting of post-coital vaginal swabs without the use of a condom on days 0, 1, 2, 3, 4, 5, 6, 7 and 9 post sexual intercourse. Each swab was extracted in 200 µL RSID™ -Semen extraction buffer for 1 hour at room temperature and 20 µL extract was tested with the RSID™ –Semen test. "Buffer only" and 1 µL semen extract was included for a negative and positive control, respectively.

The intensity of 20 µL extract from Day 0 and 1, were strong positives after 10 minutes (see fig. 8, lanes 3 and 4) and 20 µL extract from Day 2 was weak positive after 10 minutes (see fig. 8, lane 5). The extracts from Day 3-9 swabs were negative after 10 minutes (fig. 8, lanes 6-11). This demonstrates that RSID™ -Semen can detect semen from this set of post-coital vaginal swabs collected on Day 0, 1, and 2.

To determine if male DNA could be analyzed by DNA-based STR testing after testing with RSID™ -Semen, DNA was extracted from the samples tested in the post-coital experiment and analyzed for male DNA using Y-filer. A partial DNA profile was obtained from swabs PC 0, 1, and 2, whereas no profiles were obtained from swabs PC 3, 4, 5, 6, 7, and 9 (see electropherograms of Y-Filer analysis, below).

RSID™ -Semen can detect semen from post-coital vaginal swabs from Day 0, 1, and 2, but not days 3 through 9 from this set of vaginal swabs. Using Y-filer DNA-STR kits, a DNA profile can be obtained from the samples that were considered "positive" using RSID™ -Semen, demonstrating a correlation between a positive RSID™ -Semen signal and the ability to acquire a DNA profile from the donor.

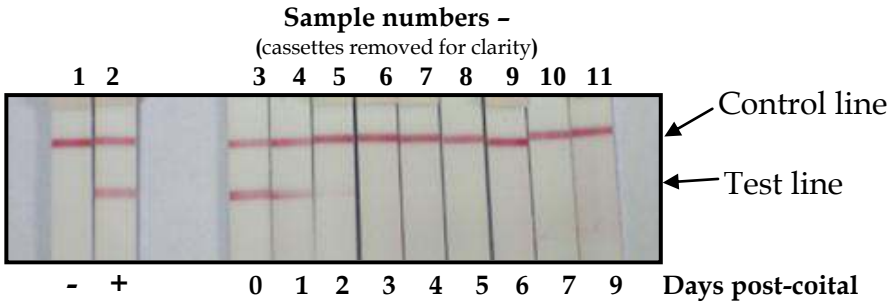
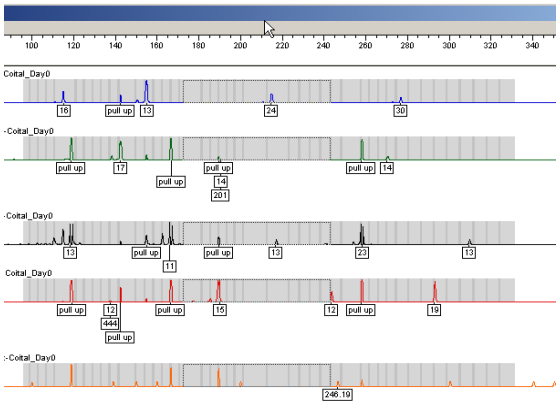
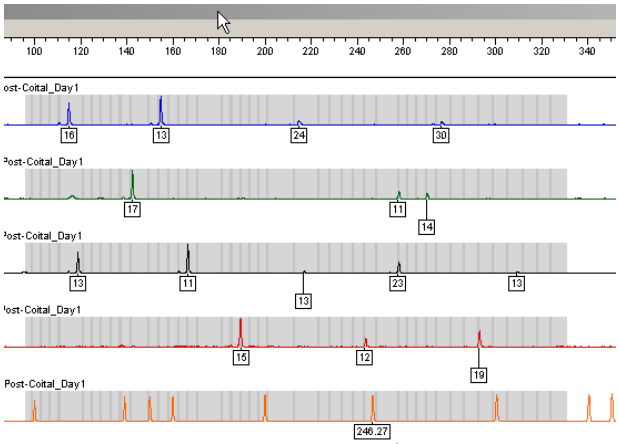


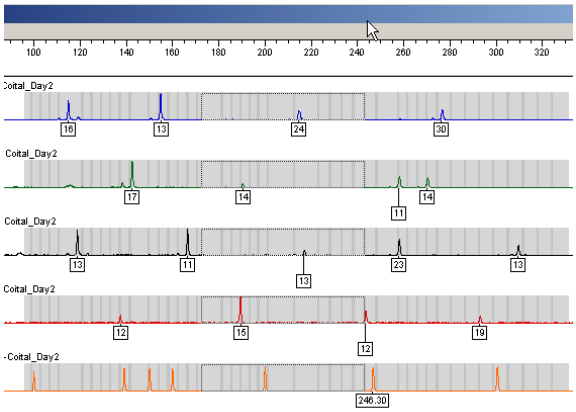
Fig. 8: Post-coital samples tested on RSID™-Semen – Time course



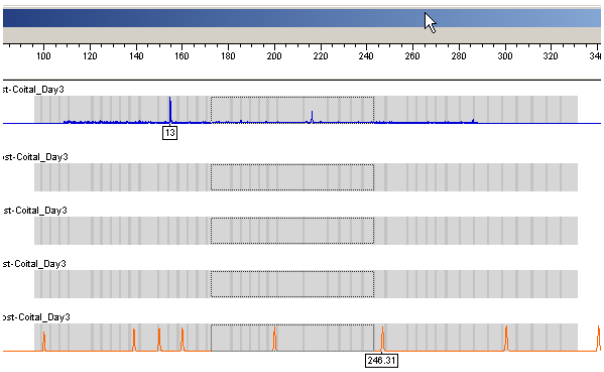
Day 0 , Y-STR analysis



Day 1, Y-STR analysis



Day 2, Y-STR analysis



Day 3-9, Y-STR analysis

Fig. 9: Y-STR analysis of Post-Coital samples

As can be seen in Fig. 9, RSID™ -Semen detection correlates well with the Y-STR DNA profiles from vaginal swabs. [Note: This will not be the case for vasectomized males or for males with low sperm count].

Test sample 4: Testing post-coital vaginal swabs, no condom use.

We obtained samples from two additional volunteers (V2 and V3) who provided post-coital (PC) vaginal swabs without the use of a condom on Days 0, 1*, 2*, 3*, 17, and 19 (#V2) and Days 0, 1, 2, 6, 8, and 10 (#V3) post coital. Interestingly, these samples contained menstrual blood (indicated by the (*)). Swabs were extracted in 200 µL RSID™ -Semen for 1 hour at room temperature and 20 µL of this extract was tested with RSID™ -Semen. Controls included "Buffer only" and 1 µL of authentic semen extract.

Results: - *V#2*- The signal from 20 µL of extract from samples PC 0, 1*, and 2* was positive after 10 minutes, although the signal from PC0 was weakly positive. The extract from samples PC 3*, 17, and 19 was negative after 10 minutes. Our expectation was that the highest semen detection would come from the PC0 extract; the sample immediately following intercourse. We therefore interpreted the low signal as a possible high dose hook effect. This was tested by diluting the extracts from PC 0, 1, 2, and 3 by 1:20 fold and re-running the diluted samples on new RSID™ -Semen strips (fig. 11).

Results: - *V #3*- The signal from 20 µL extract from samples PC 0, 1, and 2 was positive after 10 minutes, whereas extract from PC 6, 8, and 10 was negative after 10 minutes. We would expect the highest signal indicating detection of semen at PC0 (immediately following intercourse), therefore, similarly to sample #2, the weak signal from samples PC 0 to PC2 indicate a possible high dose hook effect. This was tested by diluting the extracts from PC 0, 1, 2, and 6 by a factor of 1:20 and re-running the diluted samples on new RSID™-Semen strip tests (fig. 11).

To determine if male DNA could be analyzed from these post-coital samples, DNA was extracted and analyzed for male DNA using Y-filer DNA-STR kits. DNA extraction and DNA-STR analysis was performed on samples PC0, PC1*, PC2* and PC3* (V #2) and PC0, PC1, PC2 and PC6 (V #3). Partial profiles, i.e., 16 out of 17 loci, were obtained from PC0 and PC1*, 9 out of 17 loci for PC2*, no DNA profile was obtained from PC3*. These results correlate exactly and completely with the semen detection results obtained with RSID™ -Semen for all samples tested (DNA-STR data not shown). Partial profiles 16 out of 17 loci, were obtained from PC0, 10 loci from PC1 and 2 loci from PC2, no DNA results were obtained from PC6 (V #3), again demonstrating excellent correlation between RSID™-Semen results and DNA-STR analysis.

RSID™ -Semen detects semen from post-coital vaginal swabs at Day 0, 1, and 2, and the presence of menstrual blood does not interfere with detection, specificity or sensitivity of semen detection using RSID™ -Semen. RSID™ -Semen detection correlates with the DNA-STR data obtained from Samples #2 and Samples#3.

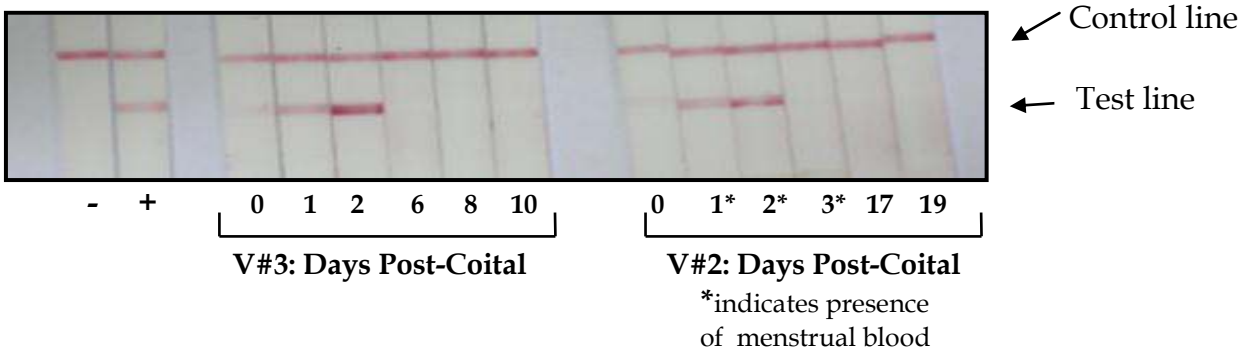


Fig. 11: Post-coital Detection of Semen with RSID™-Semen

High dose hook effect in post-coital vaginal samples. Test of proposed dilution Factor of 1:20.

The post-coital vaginal samples taken on PC 0, 1, 2, and 6 (V #3) and samples taken on, PC0, 1*, 2* and 3*, (V#2) were tested for high dose hook effect by testing a dilution of the extract into RSID™ –Semen running buffer and adding these diluted samples on fresh RSID™-Semen strips. The diluted samples were re-tested on RSID™ -Semen using 20 µL of the diluted extract brought to 100 µL with running buffer.

Results: *V #2.* The diluted extract, 20 µL from the PC0 and PC1* samples demonstrated stronger test line signal than the undiluted samples after 10 minutes (see fig. 12, lanes 7 and 8); diluted extracts from PC2* and PC3* were still negative after 10 minutes (see fig. 12, lanes 9 and 10). The samples from PC0 and PC1*gave a stronger test line intensity after dilution demonstrating a high dose hook effect. The weak signal from the diluted PC3* indicates a limiting semen in the extract. We observed a similar high dose hook effect for sample set #3 where an increase in test line intensity after dilution was noted for PC0 and PC1 (see figure below, lanes 3 and 4, respectively). The decrease in signal for PC 2 after dilution demonstrates that this sample did not demonstrate a high dose hook effect (see figure below, lane 5).

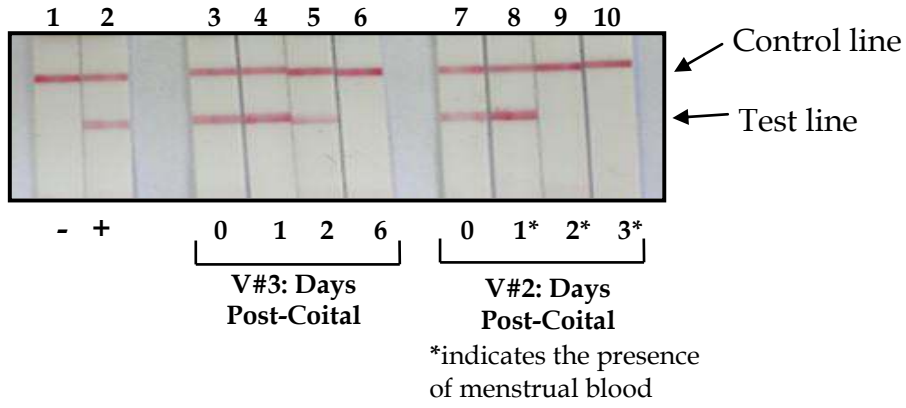


Fig. 12: Assessment of High Dose Hook effect from Post-Coital

Conclusion: A dilution factor of 1:20 is sufficient to restore a positive semen signal from a high dose hook effect using RSID™ -Semen.

Test sample 5: Dual analysis of vaginal swabs with RSID™ -Semen and RSID™ – Saliva

A set of vaginal swabs with a well defined contact history were analyzed with both RSID™ -Semen and RSID™ -Saliva in order to verify specificity and lack of interference. Swabs were extracted in 300 µL RSID™ –Semen extraction buffer and 20 µL of extract was used with RSID™ -Semen and RSID™ -Saliva. Contact history included both oral and semen contact.

Results, RSID™ -Semen: A positive signal was observed from 20 µL of extracts from PC 0, 1, and 2 (see fig.13A, lanes 3-5). Extracts from Days 3-18 had no signal using RSID™ –Semen (see fig13A, lanes 6- 13, 16). These results correlate *exactly* with the provided contact history.

Results, RSID™ -Saliva: A positive saliva signal was observed from 20 µL extract of PC 6 (see fig.13B, lane 9), whereas no signal from RSID™ -Saliva was detected in any other samples (see fig. 13B). These results correlate *exactly* with the provided contact history.

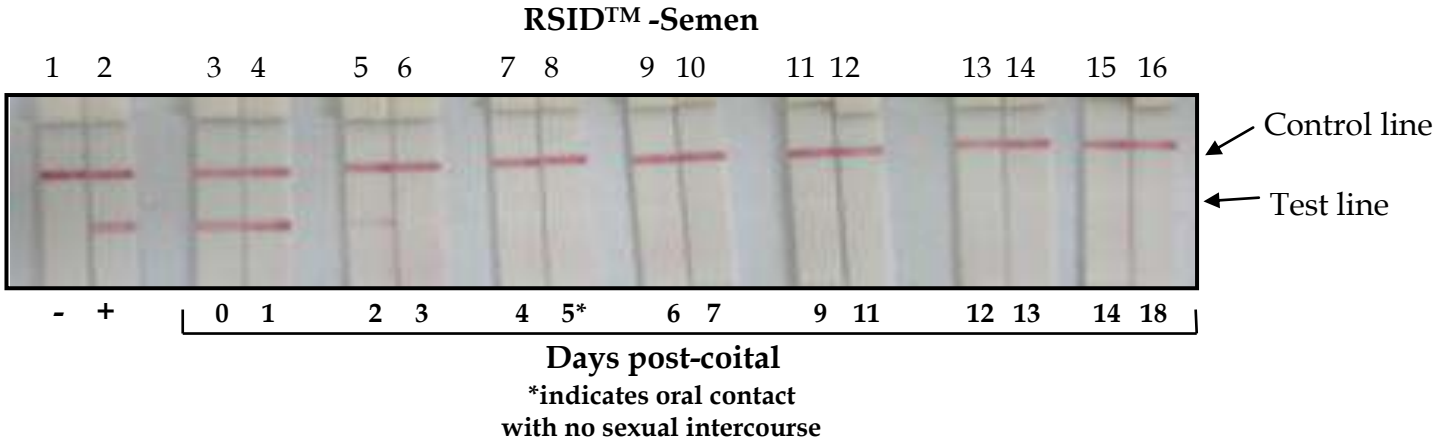


Fig. 13A: RSID™-Semen analysis of post-coital samples

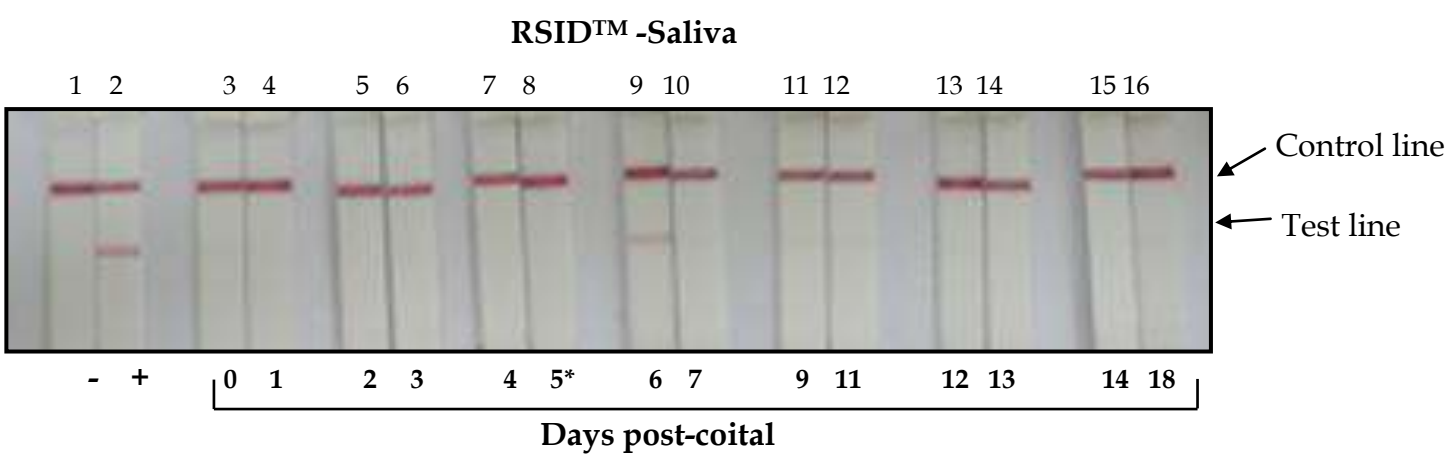


Fig. 13B: RSID™-Saliva analysis of post-coital samples

Conclusions: No cross-reaction between the two body fluid tests was observed in any sample. In addition, the analysis using RSID™ demonstrated the specificity of the two tests in that the results precisely correlate with the known sexual history of the samples. No non-specific cross-reaction is seen with RSID™ -Saliva or RSID™ -Semen. RSID™ -Semen and RSID™ -Saliva can easily and specifically differentiate semen from saliva contact on vaginal swabs.

Test of Exhibit Extraction Procedure. Fabric Types and Cutting *vs.* Swabbing

An important issue prior in the analysis of forensic evidence is the method used to obtain the test sample, extract or soak. Two general approaches are in forensic laboratory practice: cuttings and/or transfer to moistened swab. Here we directly compare these two methods on a variety of fabric substrates: swabbing the stain with a moistened cotton swab or extracting a cutting of defined size (here a 5 mm diameter circle made with a stainless steel Harris punch). Tester samples were prepared by depositing 50 µL of semen onto the following types of fabrics: 1) cotton chambray, 2) flannel cotton sheet, 3) cotton twill, 4) cotton denim), 5) nylon lace, 6) nylon knit jersey, and 7) cotton sheet. Each fabric type was sampled using a ddH_20 - moistened cotton swab or by excising a 5 mm diameter circle using a SS Harris punch. Each swab was

extracted in 200 µL RSID™-Semen extraction buffer for 1 hour at room temperature and each 5 mm punch was extracted in 100 µL RSID™-Semen extraction buffer for 1 hour at room temperature. A test of these two different sampling approaches by using 20 µL of the swab extract and 10 µL of the 5 mm punch extract, each brought to a final volume of 100 µL by the addition of RSID™ -Semen running buffer was performed. Controls included "Buffer only" and 1 µL semen extract.

The results clearly show that the 10 µL extract from the 5 mm punch/cutting worked well with all fabric types tested, such that a strong positive signal was observed after 10 minutes (see fig.14, lanes 4, 6, 8, 10, 12, 14, and 16). Extracts from the swab sampling method were not nearly as efficient and was clearly fabric type dependent. The signal from 20 µL of swab extract from the nylon lace and nylon knit jersey was strong for both fabrics after 10 minutes (see figure below, lanes 11 and 13, respectively). However, the signal from swab extracts from all cotton fabrics was low (see figure below, lanes 3, 5, 7, 9, and 15).

The extracts used for RSID™-Semen were subsequently tested for DNA-STR using Y-Filer in order to determine if a correlation between RSID™-Semen signal intensity and male DNA content could be observed. The extracts and swabs were extracted for DNA analysis as per protocol. The results (data not shown) were clear: partial DNA profiles ranging from 10 to 14 loci were observed from the 5 mm punch extracts while only 4-6 loci were observed from the swab extracts.

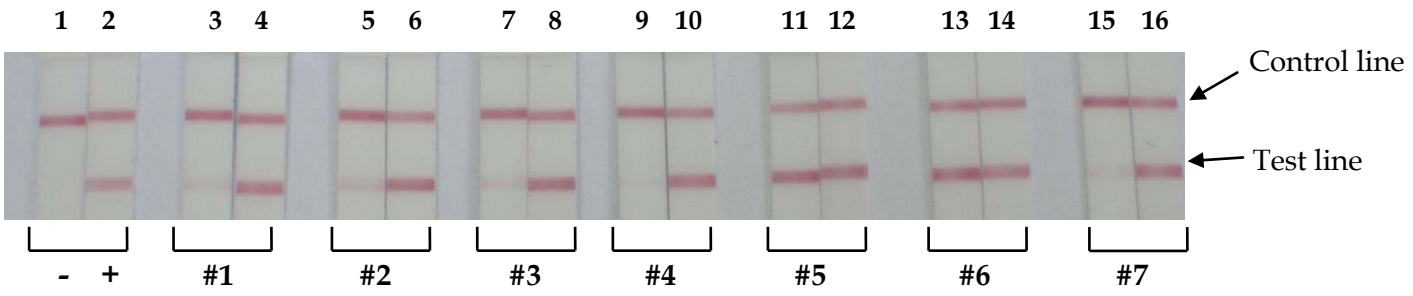


Fig. 14: RSID™-Semen – Swab absorption vs. Cutting

Fig. 14 Legend: Fabric types tested include: cotton chambray (set #1, lanes 3 & 4), flannel cotton sheet (set #2, lanes 5 & 6), cotton twill (set #3, lanes 7 & 8), denim (Levi's) (set #4, lanes 9 & 10), nylon lace (set#5, lanes 11 & 12), nylon knit jersey (set #6, lanes 13 & 14) and cotton sheet (set #7, lanes 15 & 16).
Each experimental pair demonstrates RSID™-Semen strip intensity after testing either a cutting from the stained fabric (lanes 4, 6, 8, 10, 12, 14 and 16) or a swab used to absorb the stain (lanes 3, 5, 7, 9, 11, 13 and 15).

Conclusions: The results clearly indicate that the efficiency of semen detection is much greater using 10 μL of a 100 μL total volume extract derived from a small cutting, that from attempting to swab-absorb the stain on a swab that is then extracted. We interpret this finding as a confirmation of the general observation that primary evidence is superior to secondary items. We can further strongly suggest that stains on non-porous surfaces can be sensitively and specifically sampled using a swab-absorb technique, but that stains on porous or absorptive material should be tested using cuttings or punches. Here we have standardized the cutting surface area using a 5 mm punch. The results obtained with RSID™-Semen were consistent with DNA extraction and Y-STR analysis, further pointing out the usefulness of the technique.

Note: We have calculated the approximate sizes of 50 μL *drops of semen on the tested fabrics and then determined the percentage of the stain sampled using a 5 mm punch to obtain a cutting. The size of the stain varied, as expected and the standard cutting was approximately 9.5% of the total stain area (range 3.2% to 20%, median 7.7%).*

Test of RSID™-Semen Cross Reaction with human breast milk

Objective: To determine whether RSID™-Semen cross reacts with extracts from human breast milk.

Methods: Breast milk, collected in a manner that was careful to avoid saliva contamination (breast was washed prior to collection with a freshly cleaned breast pump), was received from the Serological Research Institute. The liquid sample, 50 µL of breast milk, was pipetted onto a cotton swab that was subsequently air-dried and extracted in 1 mL of RSID™-Semen extraction buffer. This extract, 20 µL of breast milk extract, was analyzed with RSID™-Semen. Also, mixtures of extracts from body fluids (saliva, SA; blood, BL; urine, UR; and breast milk, BM; with and without semen, SE) were analyzed to test for cross-reactivity and interference. As a control, 5 µL from an authentic semen extract (50 µL of semen pipetted on to a cotton swab which was air-dried and extracted in 1 mL of RSID™-Semen extraction buffer) and negative control (clean unused cotton swab extracted in 1 mL of RSID™-Semen extraction buffer) extracts were analyzed for comparison.

The 20 µL of breast milk extract was negative after 10 minutes (see figure below, lane 3). Furthermore, the signal from the mixture of 20 µL each saliva, blood, urine, breast milk extract and 5 µL semen extract was positive (see figure below, lane 5), showing no interference of semen detection in the presence of the other body fluids. The mixture of the body fluids (20 µL each extract) without semen extract was negative (see figure below, lane 4) and the positive and negative controls were positive and negative after 10 minutes, as expected (lanes 2 and 1, respectively).

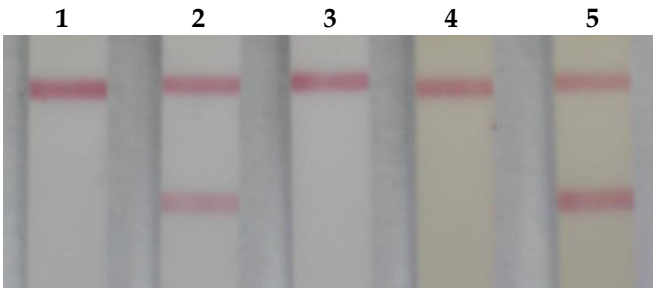


Fig. 15: RSID™- Semen and Human Breast Milk

Conclusion: RSID™ -Semen does not cross-react with human breast milk, and the presence of breast milk does not interfere with RSID™ -Semen detection of semen.

RSID™ -Semen: effects of consumer contraceptives and vaginal lubricants on semen detection I.

Objective: To determine whether commercially available consumer contraceptives (spermicidal gel and foam) and/or vaginal lubricant interfere with the ability of RSID™-Semen to detect the presence of semen from a fabric stain.

Methods: A prepared stain of semen was made with 100 µL of semen mixed with 100 µL of a vaginal lubricant (KY jelly) and two different vaginal contraceptives (VCF foam: 12.5% nonoxynol-9, and Ortho Options Conceptrol gel: 4.0% nonoxynol-9) individually. Then, 100 µL of the mixture was pipetted onto a cotton sheet, allowed to air dry, and stored for future use. As a control, 50 µL of semen was pipetted onto a different section of the same cotton sheet, this stain was analyzed side-by-side as an extraction control. Cuttings, 5 mm in diameter made with a stainless steel punch, were extracted in 100 µL of RSID™-Semen extraction buffer. An additional positive control made from 50 µL of semen pipetted on to a cotton swab, air-dried and extracted in 1 mL of RSID™-Semen extraction buffer was also used with an extraction control made from an unused swab extracted in 1 mL of RSID™-Semen extraction buffer. A standard volume, 10 µL of each extract, was added to RSID™ -Semen running buffer to a final volume of 100 µL analyzed with RSID™ -Semen.

Semen was detected in all samples tested, even in the presence of KY jelly, VCF foam, and Ortho Options Conceptrol (see fig. 16, lanes 3 , 4, and 5). These data clearly show that commercial personal lubricants and contraceptive spermicides do not interfere with RSID™ -Semen detection as no reduction in signal intensity was observed.

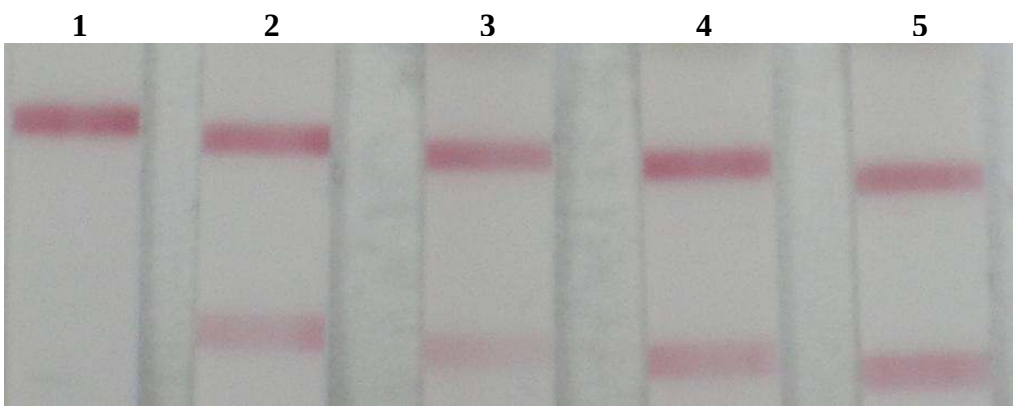


Fig. 16: RSID™-Semen and Spermicides and Lubricants

RSID™ -Semen: Test of Contraceptives and lubricants II.

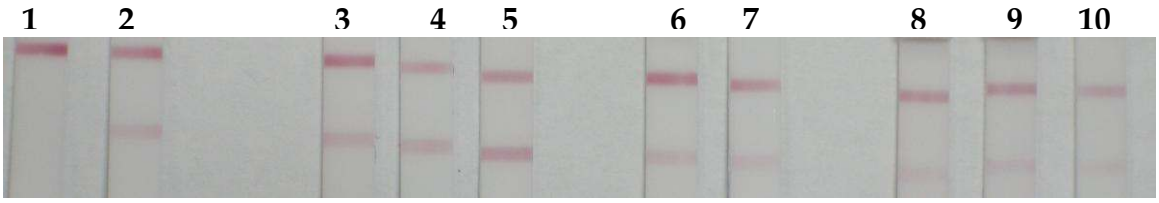
Objective: To determine whether contraceptives (condoms, spermicides) and/or vaginal lubricant interfere with the ability of RSID™ -Semen to detect the presence of semen.

Methods: 1 mL of semen was pipetted into three different Trojan condoms (no lubricant, non-spermicidal lubricant, and spermicidal lubricant: 7% nonoxynol-9) and allowed to air dry overnight. On the next day the semen was sponged with a dry cotton swab which was subsequently air dried and extracted in 300 µL RSID™ –Semen extraction buffer.

An additional experiment using 100 µL of semen individually mixed with 100 µL of a vaginal lubricant (KY jelly) and two different vaginal contraceptives (VCF foam: 12.5% nonoxynol-9, and Ortho Options Conceptrol gel: 4.0% nonoxynol-9). The mixtures, 100 µL, was pipetted onto a cotton swab that allowed to air dry overnight and extracted subsequently extracted in 300 µL RSID™ –Semen extraction buffer. The

remaining 100 µL was pipetted onto a cotton sheet, allowed to air dry, and stored for future use. Positive controls were made with 50 µL of semen pipetted onto a cotton swab, extracted in 300 µL RSID™ –Semen extraction buffer to exactly match the experimental samples. Positive (50 µL of semen extracted in 1 mL RSID™ –Semen extraction buffer) and negative (sham, clean cotton swab extracted in 1 mL RSID™ –Semen extraction buffer) controls were included for comparison. A standard volume, 20 µL, of each extract was added to RSID™ -Semen running buffer to a final volume of 100 µL and analyzed with RSID™ -Semen.

Semen was detected in all samples except negative controls, and no reduction in sensitivity or interference was observed. The data clearly demonstrate that vaginal lubricant and spermicides do not interfere with semen detection using RSID™-Semen. The low signal intensity seen in some samples (fig. 17, strips and lanes 6-10) are the result of high dose hook effect (see fig. 18 and experiment below).

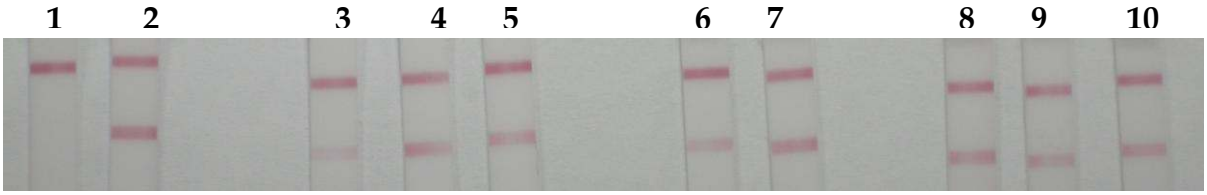


Strip	Figure Legend
1	sham
2	positive
3	KY + semen
4	VCF + semen
5	Conceptrol + semen
6	Semen alone
7	Semen alone
8	Semen from no lubricant condom
9	Semen from non-spermicidal lubricant condom
10	Semen from spermicidal lubricant condom

Fig. 17: RSID™-Semen and Spermicides and Personal Lubricants II.

RSID™ -Semen: Test of Contraceptives and lubricants. High Dose Hook Effect demonstration.

Results from the analysis of semen mixed with spermicides and lubricants demonstrated that RSID™-Semen is not inhibited by these compounds. The data clearly showed positive identification of semen from these mixtures. The test lines were weaker than expected and a high dose hook effect was suspected. Extracts were therefore re-tested on new RSID™-Semen strips after a 1: 20 dilution. In practice, 1 µL of extract was tested instead of the 20 µL initially used above; strips 6 through 10 were demonstrate the effect of dilution as a stronger signal was observed using 5% of the original aliquot (1 µL vs. 20 µL).



Strip	Figure Legend
1	sham
2	positive
3	KY + semen
4	VCF + semen
5	Conceptrol + semen
6	Semen alone
7	Semen alone
8	Semen from no lubricant condom
9	Semen from non-spermicidal lubricant condom
10	Semen from spermicidal lubricant condom

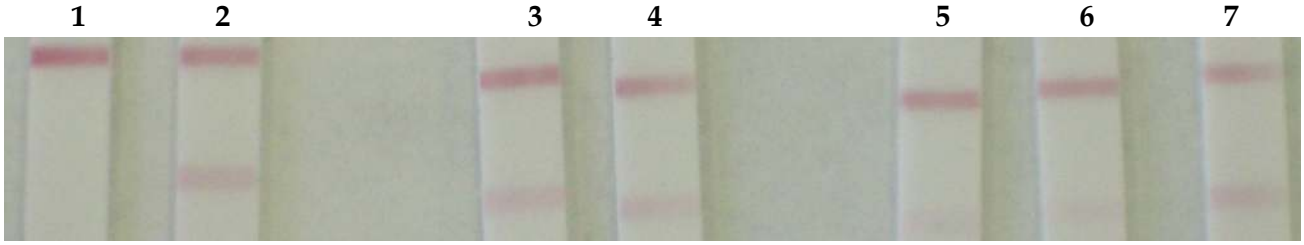
Fig. 18: Re-Test of RSID™-Semen and Spermicides and Lubricants: 1 µL vs 20 µL of Extract.

The data clearly show that semen was detected in all samples where semen was initially added and no loss of sensitivity or specificity was observed. Strips (and lanes) 8, 9 and 10 gave clear positive results suggesting that the weak signals seen in fig. 17 were due to high dose hook effect and not interference by the lubricants/spermicides in the condoms.

Semen Detection from Prophylactics using RSID™-Semen.

Objective: To determine whether prophylactics (condoms) interfere with the ability of RSID™ -Semen to detect semen.

Methods: Samples were prepared by adding 50 µL of semen into three different Trojan condoms (no lubricant, non-spermicidal lubricant, and spermicidal lubricant: 7% nonoxynol-9) and allowed to air dry overnight. On the next day the semen stains were sponged with a water moistened cotton swab which was subsequently air dried and extracted in 300 µL of RSID™ –Semen extraction buffer. Positive controls, 50 µL of semen pipetted onto a cotton swab and extracted in 300 µL of RSID™ –Semen extraction buffer were designed to match the experimental samples. Also, positive (50 µL of semen extracted in 1 mL of RSID™ –Semen extraction buffer) and negative (clean cotton swab extracted in 1 mL of RSID™ –Semen extraction buffer) controls were included for comparison. A standard volume of each extract, 20 µL was added to RSID™ -Semen running buffer to a final volume of 100 µL and analyzed with RSID™ -Semen.



Strip	Figure Legend
1	sham
2	positive
3	Semen alone
4	Semen alone
5	Semen from no lubricant condom
6	Semen from non-spermicidal lubricant condom
7	Semen from spermicidal lubricant condom

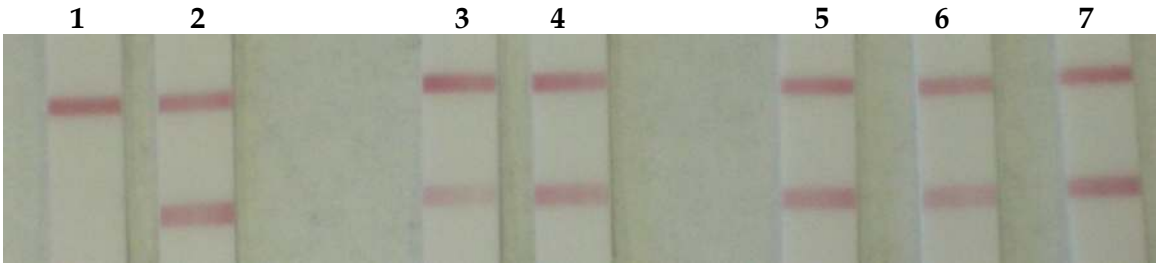
Fig. 19: RSID™-Semen and Condoms

Results: Semen was detected in all samples where appropriate semen and no change in specificity or sensitivity was observed. Sample 3, 4, 5, 6 and 7 were scored as faint positives; the putative high dose hook effect was tested, see fig. 20, below.

Semen Detection from Prophylactics using RSID™-Semen, continued.

Objective: To determine whether prophylactics interfere with the ability of RSID™ -Semen to detect the presence of semen. Test of observed faint positives.

Methods: Identical as above, however only 1 µL of extract was analyzed with RSID™ -Semen.



Strip	Figure Legend
1	sham
2	positive
3	Semen alone
4	Semen alone
5	Semen from no lubricant condom
6	Semen from non-spermicidal lubricant condom
7	Semen from spermicidal lubricant condom

Fig. 20: RSID-Semen and Condoms, 1 µL vs. 20 µL

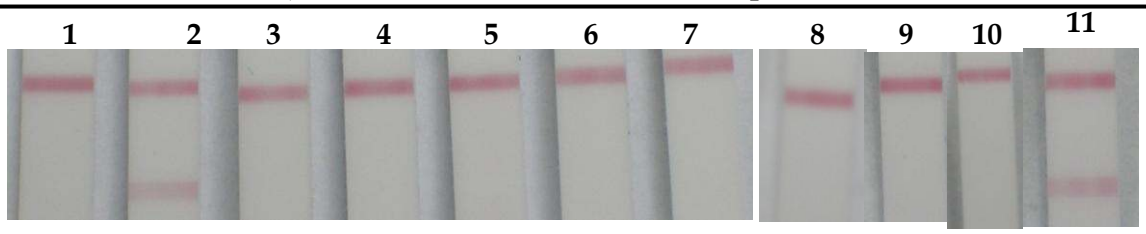
Results: Semen was detected in all appropriate samples with no reduction in sensitivity or change in specificity. The weak signal observed was due to high dose hook effect.

Conclusion: RSID™ -Semen is not affected by spermicides, prophylactics or lubricants. Users should be aware that high dose hook effect can give weak bands could produce false negative result. Weak or negative results should be retested at 1:20 dilution to avoid false negatives.

Animal Semen Cross Reactivity Test

Objective**:** To test the species specificity of RSID™ –Semen.

Methods: Air dried cotton swabs containing 50 µL of semen from the indicated species were extracted in 1.0 mL of RSID™ –Semen extraction buffer. Standard volumes, 20 µL and 1 µL (to address potential high dose hook effects) of the extract were used in a final volume of 100 µL of RSID™-Running buffer and applied to RSID™-Semen strip tests. Species tested: goat (fig. 21, strip 3), sheep (fig. 21, strip 4), pig (fig. 21, strip 5), bull (fig. 21, strips 6), dog (fig. 21, strip 7), horse (fig. 21, strip 8), mouse (fig. 21, strip9), and cat (fig. 21, strip 10). As an additional control human semen was mixed with a commercial 'extender' used to store purchased animal sperm to insure that no interference of this compound would confuse the observed results. Human semen was mixed with extender (50 µL human semen + 50 µL extender applied to a cotton swab which was air dried and extracted with 1 mL of RSID™ –Semen extraction buffer) and tested (fig. 21, strip 11). Positive (50 µL of semen extracted in 1 mL of RSID™ –Semen extraction buffer) and negative (clean cotton swab extracted in 1 mL of RSID™ –Semen extraction buffer) controls were included for comparison.



Strip	Figure Legend
1	5 µL sham
2	5 µL semen
3	20 µL goat
4	20 µL sheep
5	20 µL pig
6	20 µL bull
7	20 µL dog
8	20 µL horse
9	20 µL mouse
10	20 µL cat
11	5 µL Human semen + Extender

Fig. 21: Species Specificity of RSID™-Semen

Results: No cross reaction was present at the test line of **any** of the strips run with animal semen extracts. In addition, semen extender, often included in commercial sources of semen does not interfere (fig. 21, strip 11) with RSID™ -Semen detection.

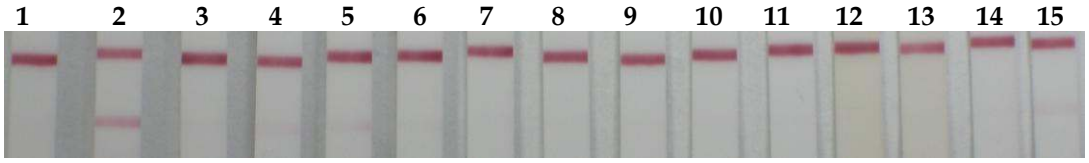
Conclusion: RSID™ -Semen is specific for human semen. Note: all tests were retested at 1:20 dilution to insure that no high dose hook effect false negative results were recorded. All retests also showed no cross-reaction with RSID™ -Semen (data not shown).

RSID™ –Semen & RSID™ -Saliva: Vaginal swab analysis.

Objective: To address the issue of possible cross-reaction of RSID™ -Saliva with vaginal fluid, to test additional samples with RSID™ -Semen, and to demonstrate a dual use of RSID™ for analysis of sexual assault evidence.

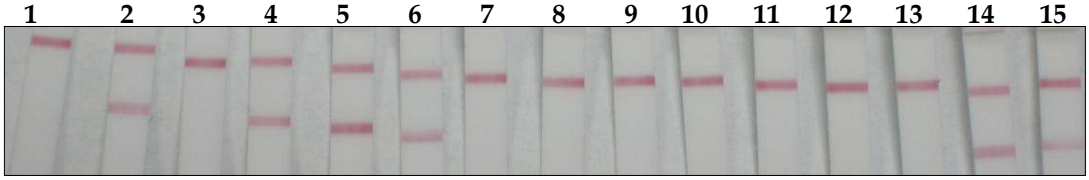
Methods: Extracts were made from vaginal swabs whose sample history was well documented. Samples from both penile and oral contact were analyzed. Swabs were extracted with 300 µL of RSID™-Saliva extraction buffer and 20 µL of this extract was analyzed with RSID™ -Saliva. Two volumes of extract, 20 µL and 1 µL were analyzed with RSID™ -Semen in order to ensure that no false negatives were observed due to high dose hook effect. All strips were brought to a final volume of 100 µL with running buffer.

Results: RSID™ -Saliva and RSID™ -Semen strips exhibited expected results that were completely congruent with the known contact history of the swabs. No cross decrease in specificity or sensitivity from these types of samples were noted. Saliva and semen detection were consistent with the details of the contact history.



Strip	Sample (µL)	# of days post-coitus
1	20 sham	n/a
2	5 saliva	n/a
3	20 vaginal swab 1	17
4	20 vaginal swab 2	0
5	20 vaginal swab 3	1
6	20 vaginal swab 4	2
7	20 vaginal swab 5	6
8	20 vaginal swab 6	8
9	20 vaginal swab 7	10
10	20 vaginal swab 8	12
11	20 vaginal swab 9	14
12	20 vaginal swab 10	15
13	20 vaginal swab 11	16
14	20 vaginal swab 12	0
15	20 vaginal swab 13	1

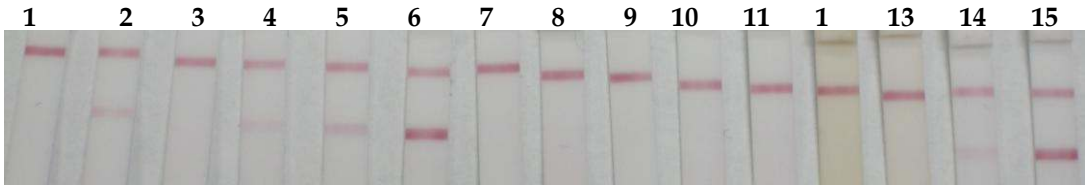
Fig. 21A RSID™ –Saliva Results – Testing of Vaginal Swabs



Strip	Sample (µL)	# of days post-coitus
1	20 sham	n/a
2	5 semen	n/a
3	20 vaginal swab 1	17
4	20 vaginal swab 2	0
5	20 vaginal swab 3	1
6	20 vaginal swab 4	2
7	20 vaginal swab 5	6
8	20 vaginal swab 6	8
9	20 vaginal swab 7	10
10	20 vaginal swab 8	12
11	20 vaginal swab 9	14
12	20 vaginal swab 10	15
13	20 vaginal swab 11	16
14	20 vaginal swab 12	0
15	20 vaginal swab 13	1

Fig. 21B: RSID™ –Semen Results – Testing of Vaginal Swabs

Possible High Dose Hook effect was examined by performing a 1:20 dilution and retesting on RSID™ -Semen



Strip	Sample (µL)	# of days post-coitus
1	20 sham	n/a
2	5 semen	n/a
3	20 vaginal swab 1	17
4	20 vaginal swab 2	0
5	20 vaginal swab 3	1
6	20 vaginal swab 4	2
7	20 vaginal swab 5	6
8	20 vaginal swab 6	8
9	20 vaginal swab 7	10
10	20 vaginal swab 8	12
11	20 vaginal swab 9	14
12	20 vaginal swab 10	15
13	20 vaginal swab 11	16
14	20 vaginal swab 12	0
15	20 vaginal swab 13	1

Fig. 21C: Retesting of Vaginal Swabs on RSID-Semen – Possible high dose hook effect.

Conclusion: RSID™ -Saliva shows no cross-reaction with vaginal extracts and is specific for saliva. RSID™ -Semen show no interference with vaginal extracts and is specific for seminal fluid.

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