

**Developmental Validation Studies of RSID™-Saliva
Lateral Flow Immunochromatographic Strip test for the
forensic detection of Saliva**

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Table of Contents

Introduction	2
Configuration of the Salivary Amylase lateral flow test	5
Quantification of salivary amylase strip tests	7
Sensitivity Testing: Saliva Extract and Human Oral Swab Extract	9
RSID™-Saliva Limit of Detection	11
Body Fluid Specificity	14
Species Specificity	17
Testing for High Dose Hook Effect	18
Stability Testing of RSID™-Saliva	21
Detection of saliva and STR analysis from Forensic -like Samples	23
Aluminum coke can, plastic coffee lid	
Plastic and glass water bottle	
Cigarette butts, envelopes	
References	33
Acknowledgements	33

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

Introduction

The identification of human saliva can be important for both legal and investigative purposes. There is often a need to determine if saliva was left or deposited on evidence collected at crime scenes, on discarded samples, or on other evidence items such as envelopes, aluminum cans, glass or plastic bottles, coffee mugs or fabric to (a) reconstruct what may have occurred during the crime and/or (b) to determine which items of evidence should be processed for DNA-STR testing. Current methods to determine the presence of saliva have significant drawbacks including lack of specificity, lack of sensitivity, and lack of integration into current DNA-based protocols. In addition, current saliva detection methods require significant time and effort by crime laboratory personnel. Here we present RSID™-Saliva, a new, lateral flow immunochromatographic strip test for human saliva detection and we illustrate experimental results demonstrating that this test is accurate, reproducible, easy to use, highly specific for human saliva and can identify saliva from a variety of materials and surfaces.

Current crime laboratory methods used to identify saliva generally assay for the enzymic activity of α -amylase. This enzyme is widely distributed in animals, plants, bacteria, and fungi, (Svensson, 1988). In humans, two main isozymes of α -amylase exist, salivary and pancreatic, and current methods used to detect α -amylase enzyme activity cannot distinguish between these different α -amylase isozymes. Thus, the

current enzyme based methods (*i.e.*, those methods using Phadebus or similar substrates) used to detect saliva will not distinguish between the many sources of this enzyme as bacterial, fungal and pancreatic α -amylase all score positive with this assay.

The Rapid Stain IDentification Test (RSID™) for saliva from Independent Forensics is a lateral flow immunochromatographic strip test designed to detect the presence of human salivary α -amylase, an enzyme found in high quantity in human saliva. The enzyme's physiological role is to aid in the digestion of dietary starches. The RSID™ -Saliva uses two anti-salivary amylase monoclonal antibodies in a lateral flow format which detects the *presence* of salivary amylase, rather than the *activity* of the enzyme. Here we detail studies on the sensitivity, body fluid specificity, species specificity, and stability of the RSID™ -Saliva as well as numerous experiments demonstrating the ability of the test to detect human saliva from a variety of objects that are typically encountered in forensic laboratory case work.

The new Rapid Stain IDentification (RSID™) Saliva is designed for fast, easy, and reliable detection of human saliva; test development is complete within 10 minutes and the stated limit of detection of the assay is 1 μ l of human saliva (nominal or experimental limit of detection (LOD) is much lower). The detection protocol can be completely integrated into standard forensic laboratory procedures for DNA analysis. The test detects saliva from envelopes, glass bottles, cans, swabs, and plastic lids *BEFORE* they are processed for DNA-STR analysis. Test sensitivity has been adjusted such that if saliva is detected, using the provided protocol, there should be sufficient

biological material for generating an STR profile. Suggested protocols are included in the technical documentation included in each kit.

RSID™-Saliva Buffer components

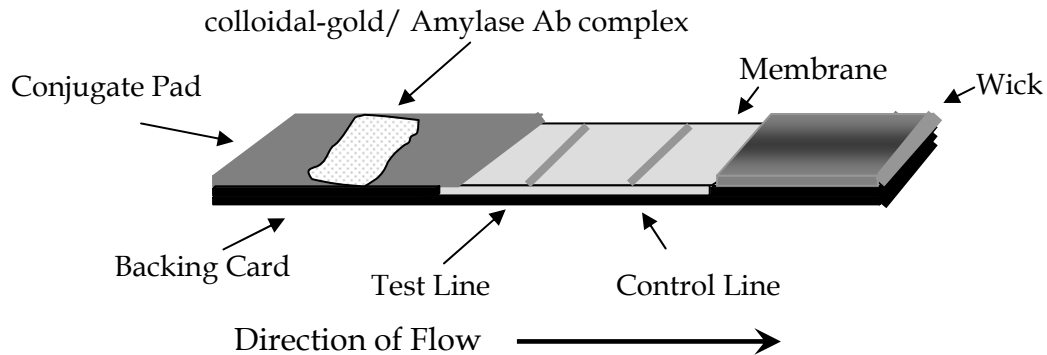
The RSID™-Saliva laboratory kit includes extraction buffer (25 ml) and running buffer (5 ml) that is required for use with the RSID™-Saliva kit. Each experiment included in this validation document uses RSID™-Saliva extraction buffer for extraction of the sample/s (extraction volumes are specified); the final volume of the sample to be tested is brought to 100 µl with RSID™-Saliva running buffer. RSID™-Saliva extraction buffer + RSID™-Saliva running buffer (100 µl) is loaded onto the cassette and results are recorded after 10 minutes.

RSID™-Saliva extraction buffer is designed to efficiently extract the protein α -amylase from questioned stains and swabs. RSID™-Saliva running buffer is 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 buffer 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).

Configuration of the salivary amylase lateral flow test

The RSID-Saliva™ test is an immunochromatographic assay that uses two monoclonal antibodies specific for human salivary α -amylase. The system consists of overlapping components (conjugate pad, membrane, and wick), assembled such that the tested fluid is transported from the conjugate pad to the membrane and is finally retained on the wick (see figure below). The conjugate pad and membrane are pre-treated before assembly such that the user need only add his/her extract in running buffer 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 anti-human salivary α -amylase monoclonal antibodies. The sample redissolves the colloidal gold-labeled anti α -amylase antibodies, which will bind salivary amylase if it is present in the sample. Salivary α -amylase-colloidal gold antibody complexes are transported by bulk fluid flow to the membrane phase of the test strip. These complexes, if present, migrate along the membrane and are bound at the 'test line' by the second anti-salivary α -amylase antibody, creating a red 'line' (see figure below; note that this figure depicts an RSID™-Saliva strip test that has already been developed with saliva present in the sample and therefore both the test and control lines are visible on the membrane; test and control lines are not visible on an unused strip test).

Fig: Schematic Components of Lateral Flow Immunochromatographic Strip Test



Uncomplexed colloidal gold-labeled mouse antibody will progress along the membrane and be bound by anti-mouse antibody at the 'control line,' again creating a red line. A red line at the 'test' position indicates the presence of human saliva, while a red line at the 'control' position indicates that the strip test is working correctly. When performed correctly and functioning properly, all RSID™-Saliva test strips should produce a line at the control position.

The control line is made by 'striping' goat anti-mouse antibody onto the membrane component of the lateral flow strip test; the deposited antibody will retain colloidal-gold anti- α -amylase mouse monoclonal antibody that migrates past the test line. The line closest to the sample well is the test line and indicates that human α -amylase is present in the sample. The test line is made by 'striping' a mouse monoclonal anti- α -amylase antibody onto the membrane component of the strip test; complexes of colloidal gold-labeled anti- α -amylase mouse monoclonal antibody that are formed in solution upon addition of the sample to the sample well and have progressed through the conjugate pad and membrane (or allowed to wick up the conjugate pad

when the strip is tested outside of a plastic housing, e.g., in a 12 x 75 test tube) will be retained at the test line. A red control line must be visible at 10 minutes after sample addition in order to interpret results.

Quantification of salivary amylase strip tests results

In order to maintain test-to-test consistency throughout the validation studies of RSID™-Saliva, strip test results were quantified by comparing the intensity of the observed results (i.e., how dark the test and control lines were) with a published reference set of test and control lines. This score sheet, which consists of a series of graded reddish lines is visually compared to all results. In addition, a digital picture of the results was also recorded: both quantitative and pictorial results are presented. RSID™-Saliva is not a quantitative test for the amount of saliva present in a given sample. This procedure was used for the Development Validation and the design of internal QA/QC production standards and is not used for the forensic application of the test. RSID™-Saliva is a qualitative test and therefore test results are to be interpreted as data for the presence or absence of α -amylase and by extension, saliva.

Specimens

Human saliva, blood, and urine samples were obtained voluntarily from laboratory staff and deposited on sterile cotton swabs in aliquots of 50 μ l. Unwashed semen was obtained from a local sperm bank and deposited on sterile cotton swabs in aliquots of 50 μ l. Human breast milk samples were obtained from SRI (Richmond, CA). Briefly, human breast milk was collected from lactating mothers in a manner that would

preclude contamination with other body fluids and deposited on sterile cotton swabs and air dried. Human fecal samples were obtained from a library of body fluid samples obtained under IRB supervision. Post-coital vaginal swabs were obtained from volunteers (laboratory staff). Animal saliva samples were kindly provided by the Brookfield Zoo, Brookfield, Illinois.

Preparation of body fluid extracts

For body fluid extracts (saliva, semen, blood, urine, and breast milk), 50 µl of fluid was 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 1 ml of RSID™-Saliva extraction buffer for 1 hour at room temperature. Assuming 100% extraction efficiency each microliter of extract will contain 50 nl (0.05 µl) of whole fluid. Oral swab extracts were made by swabbing the inside of an individual's cheek for 10 seconds with a cotton swab, and extracting the swab in 1 ml RSID™-Saliva extraction buffer for 1 hour at room temperature. Negative control extracts were made in an identical manner, but omitting the addition of body fluid to the swab before extraction.

Experimental samples were prepared by combining the noted volume of extraction solution with sufficient running buffer to produce a final volume of 100 µl (RSID™- Saliva extract sample volume + RSID™- Saliva running buffer = 100 µl). Most samples were tested on strips placed in cassettes, but for photographic clarity, some

experiments were performed in 12 x 75 mm test tubes; in all cases results were recorded 10 minutes after sample addition.

DNA extraction and STR DNA Analysis

DNA was extracted from swabs used to sample a plastic coffee lids, and an aluminum soda can, and from cigarette butt paper, using a Chelex extraction protocol. The extracted DNA was amplified using Identifiler (ABI) following a low copy number protocol. The amplification reactions were run on an ABI Prism 310 Genetic Analyzer and analyzed with Genescan (v. 3.7) and Genotyper (v 3.7) using an allele threshold of 75 RFU.

Sensitivity Testing: Saliva Extract and Human Oral Swab Extract

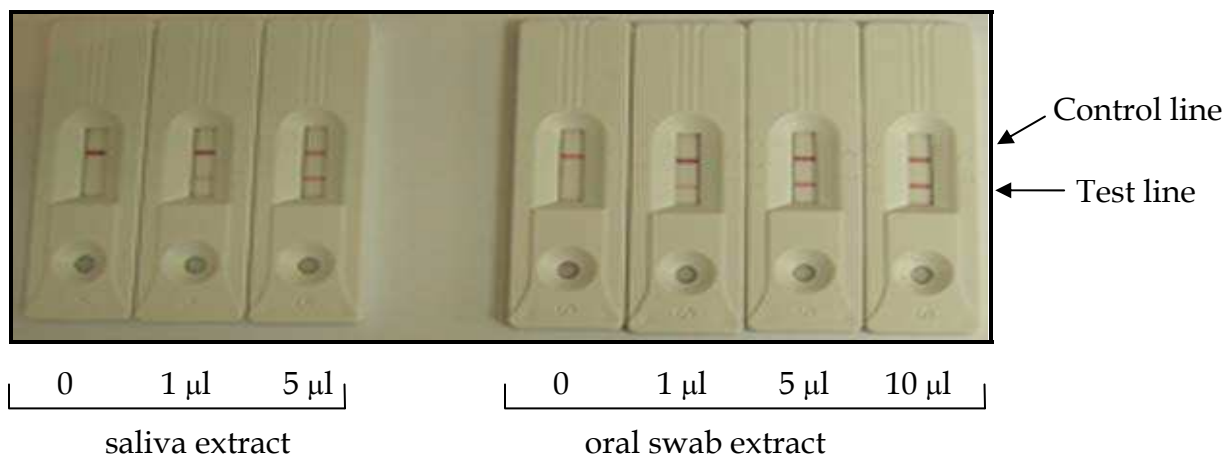
Methods: For sensitivity studies, we tested saliva extracts and human oral swab extracts. For saliva extracts, 50 µl of human saliva was deposited on a sterile cotton swab and allowed to air-dry. The end of the swab with the cotton batting was cut off using laboratory clean technique and placed in a 1.5 mL microcentrifuge tube. The swab head was extracted in 1 mL of RSID™-Saliva extraction buffer for 1 hour at room temperature. We calculate that the extract will contain approximately 50 nL (0.05 µL) of saliva (assuming 100% extraction efficiency) per microliter of extract. An oral swab extract was made by swabbing the inside of an individual's cheek for 10 seconds with a cotton swab, and extracting the swab in 1 ml RSID™-Saliva extraction buffer for 1 hour at room temperature in a 1.5 ml microcentrifuge tube. Negative control extracts were

made in an identical manner, but omitting the addition of saliva or oral extract to the swab before extraction.

Two volumes of saliva extract were generally tested, 1 μL (equivalent to ~ 50 nL saliva) and 5 μL (equivalent to ~ 250 nL saliva) by adding the indicated volume of saliva extract to RSIDTM-Saliva running buffer and bringing the total volume to 100 μL . The full 100 μL , containing both extract and running buffer, was placed in the sample window of the cassette. Extracts from oral swabs were tested in an identical manner; the quantity of saliva in an oral swab could only be estimated; we assume that 10 μL of an oral swab extract is equivalent to ~ 0.5 μL of saliva. The control and test lines in the test strip window were scored after 10 minutes.

Results- Sensitivity of Saliva Extract and Oral Swab Extract

After 10 minutes, the test line of the 1 and 5 μL saliva extracts were scored as positive, respectively (see photo, left panel). These results indicate that the limit of detection for RSIDTM-Saliva is approximately 50 nL (0.05 μL) of saliva. This experiment was repeated with the buccal swab extract (see photo, right panel) and the results for 1, 5, and 10 μL of oral extract were scored as positive, whereas 0 μL extract was negative.

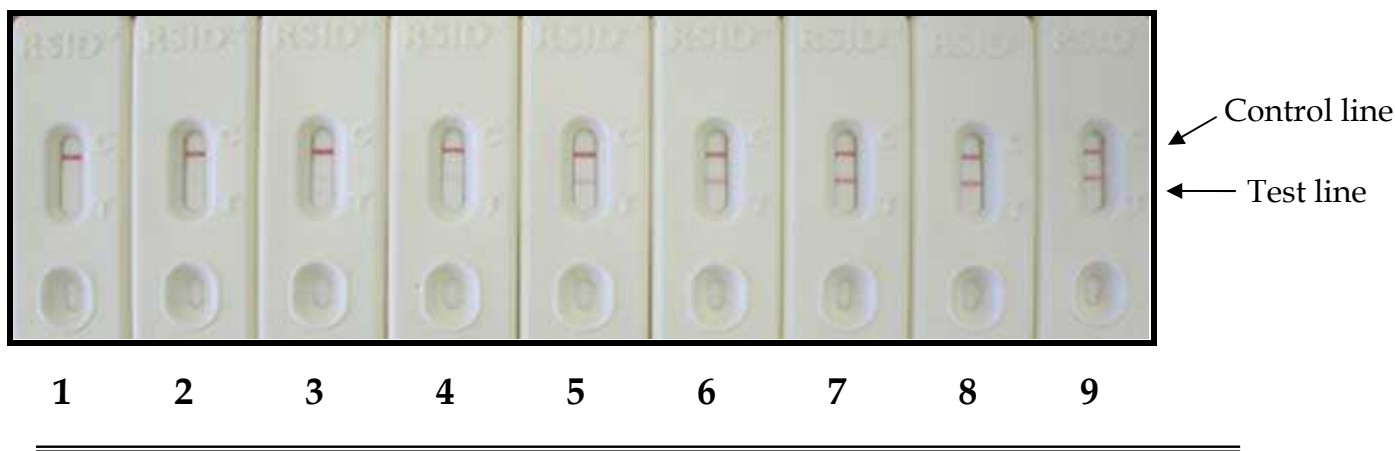


RSID™-Saliva Limit of detection Experiment.

In this experiment a broad range of saliva extract volumes were analyzed with RSID™-Saliva in order to better determine the lower limit of detection. Positive control extract, 50 µl of saliva deposited on a sterile cotton swab and extracted in 1.0 ml of RSID™-Saliva extraction buffer, was used throughout. Equivalent saliva volumes are calculated assuming 100% extraction efficiency.

Strip	Extract Amount (µl)	RSID™-Saliva RB	Equivalent Saliva (µl)	Score
1	0	100	0	-
2	1 µl from 1:10 dilution	99	0.005	-
3	1 µl from 1:5 dilution	99	0.01	-
4	1 µl from 1:2 dilution	99	0.025	-
5	1 µL	99	0.05	+
6	2 µL	98	0.1	+
7	5 µL	95	0.25	+
8	10 µL	90	0.5	+
9	20 µL	80	1	+

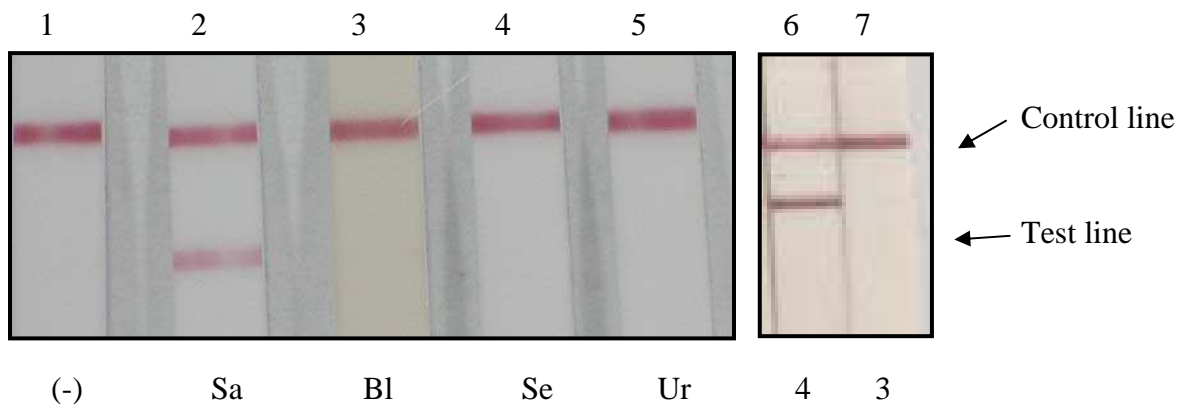
Results: A clear signal at the test line can be observed for strips 5-9. Detection limit is therefore ~50 nL (0.05 μ L) of saliva.



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

In order to evaluate potential cross-reaction or inhibition of RSID™-Saliva, extracts from human body fluids (saliva -Sa, blood -Bl, semen -Se, and urine -Ur, prepared as described above) were tested on RSID™-Saliva (see figure below). Individual extracts of saliva, blood, semen and urine reacted as expected with only saliva extracts providing a positive result (see figure below, strips 2, 3, 4 and 5). Combinations of extracts with or without saliva were also tested; only the mixture containing all four body fluid extracts gave a positive signal (blood, semen, urine and saliva, see figure below strip 6), while 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, strip 7). Again, strips were analyzed in 12 x 75 test tubes for photographic clarity; identical results were obtained with strips held in plastic cassettes (data not shown). Sufficient volumes of

extract, 25 µL of each extract equivalent to 1.25 µL of each body fluid, were tested to insure that even low levels of cross-reactivity would be observed, if present. For comparison, a negative control was included in the experiment (see figure below, strip 1). As an additional test of specificity, extracts of saliva, blood, semen and urine were combined at different ratios (1:1, 1:3, and 3:1) and tested with RSID™-Saliva. Again, RSID™-Saliva did not cross-react with mixed extracts from urine, blood, or semen at any ratio tested (data not shown). Taken together, these experiments demonstrate that RSID™-Saliva does not cross react with the body fluids tested. The presence of semen, blood, and urine does not interfere with the detection of saliva, an important issue since multiple body fluids are often present on evidence collected at crime scenes.

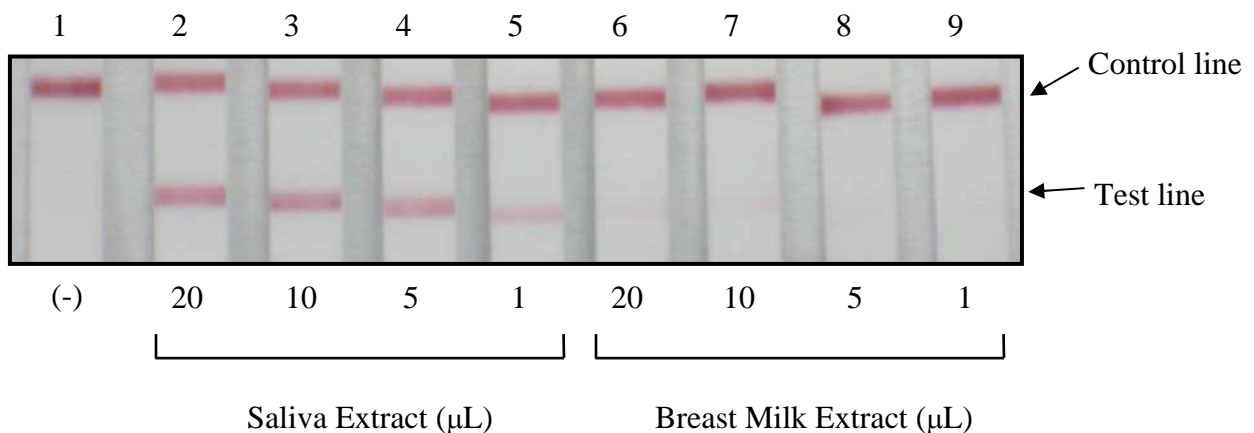


Specificity of RSID™-Saliva: Detection of Salivary α -Amylase in Human Breast Milk and Fecal samples

It is well-documented that human breast milk contains low-levels of salivary α -amylase that is probably present as an aid to carbohydrate digestion in infants (8, 9). Therefore, we tested if human breast milk would give a positive signal with RSID™-Saliva.

Samples of human breast milk (50 μ L) (kindly provided by SRI, Richmond CA and described in *Specimens*) were extracted and various volumes of breast milk extract- 1, 5, 10 and 20 μ L, equivalent to 0.05, 0.25, 0.5 and 1.0 μ L of human breast milk- were analyzed with RSID™-Saliva and compared side by side with equivalent volumes of authentic human saliva (see figure below, strips 1-10).

As expected, RSID™-Saliva demonstrates a weak positive result with extracts prepared from human breast milk (see figure below, strips 6, 7, and 8). By comparing equivalent volumes of saliva and human breast milk (see figure below, strips 2, 3, 4, and 5) we estimate that breast milk is at least twenty fold less reactive on RSID™-Saliva than authentic human saliva (see figure below, strips 2 and 7, strips 3 and 8).



Since the majority of saliva is swallowed, we expected RSID™-Saliva to detect salivary α -amylase in fecal samples. Six fecal samples from a human stain library were extracted in 1 mL RSID™-Saliva extraction buffer for 1 hour at room temperature and 5, 20, and 100 μ L of extract were analyzed with RSID™-Saliva. 100 μ L of extract from each of the six samples showed a weak positive while the other extract volumes were negative (data not shown). In the same experiment, 1 μ L of saliva extract (50 nL of equivalent saliva) produced a strong positive, indicating that saliva is many times more reactive on RSID™-Saliva test strips than fecal samples. Due to the unknown amount of fecal matter present on the swabs, direct quantitative comparison with α -amylase levels in saliva is not possible. This finding must be considered when anal swabs from sexual assault evidence kits are tested with RSID™-Saliva.

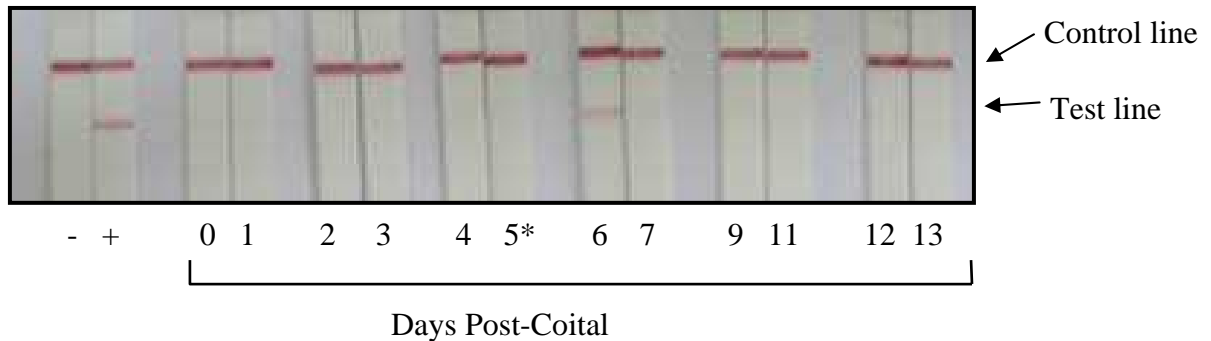
A significant disadvantage of using α -amylase as a forensic indicator for saliva is the distribution of this enzyme in human breast milk and feces, thereby making any test using α -amylase a presumptive test. When using RSID™-Saliva, some conclusions based on the signal intensity must be carefully considered. Fecal swabs tested on RSID™-Saliva only generate a weak RSID™-Saliva positive, as do human breast milk samples. A weak RSID™-Saliva positive signal can indicate either minimal amounts of saliva, a fecal sample or breast milk sample, or inefficient sample extraction. RSID™-Saliva cannot overcome the biological distribution of α -amylase, but as the relative concentration of α -amylase varies considerably between these three body fluids, a strong positive RSID™-Saliva result indicates, but does not prove, the presence of saliva.

Specificity of RSID™-Saliva: Testing Extracts from Vaginal Swabs

The ability to detect human saliva from sexual assault evidence is an important issue for forensic scientists. Therefore, we tested the ability of RSID™-Saliva to reliably identify saliva from a series of vaginal swabs obtained from a subject with a well defined sexual contact history. Post-coital swabs collected at 0-7, 9, and 11-13 days following intercourse without a condom, were extracted with RSID™-Saliva extraction buffer and analyzed with RSID™-Saliva test strips. Contact history included both semen deposition (day 0) and oral contact (day 5). To increase the stringency of the test, swabs were extracted in 300 µL of extraction buffer and 20 µL of this extract was combined with 80 µL of RSID™-Saliva running buffer and then tested on RSID™-Saliva test strips.

The results clearly demonstrate that in this sample set, RSID™-Saliva does not cross-react with post-coital vaginal swab extracts as no signal was observed from samples taken 0, 1, 2, 3, 4, or 5 days post intercourse (see figure below, strips 0, 1, 2, 3, 4, and 5, respectively). However, oral contact on day 5 was confirmed using RSID™-Saliva when day 6 vaginal swabs were tested (see figure below, strip 6 designated by arrow). No other RSID™-Saliva positive samples were observed from this experimental series, demonstrating the specificity of RSID™-Saliva; RSID™-Saliva results correlated precisely with the known sexual history of the samples. The lack of cross-reactivity of the vaginal fluid extracts observed in this experiment is representative of results seen with over 20 additional subjects, in which no signal was detected in extracts from vaginal swabs with no reported presence of semen (data not

shown). This supports the conclusion that RSID™-Saliva does not cross-react with vaginal fluid.



These data indicate that using mock sexual assault samples, RSID™-Saliva does not cross react with semen or vaginal fluid and can easily and specifically detect saliva from collected vaginal swabs. It should be noted that we have demonstrated body fluid specificity using RSID™-Saliva for only the tested human body fluids of semen, saliva, urine, blood, and vaginal fluid as well as detection of α -amylase in breast milk and fecal samples. We have not tested RSID™-Saliva on samples obtained from cadavers or other decomposing specimens; forensic lore states that cadaver samples present particularly difficult body fluid identification issues.

Species Specificity of RSID™-Saliva: Testing of Animal Samples

Saliva swabs from various animal species, both exotic and companion animals, were kindly provided by the Brookfield Zoo, Brookfield, Illinois. Extracts were prepared as previously described and 25 μ L of each extract was tested with RSID™-Saliva. No cross reactivity was observed with saliva from the following animals: dog,

opossum, guinea pig, woodchuck, cow, domestic cat, domestic rabbit, tokay gecko, cuckoo, mongoose, chameleon, domestic pig, llama, sheep, horse, goat, grey gull, ferret, hedgehog, skunk, lion, tiger, rhinoceros, marsh snake, Sykes monkey, Capuchin monkey, tamarin, and marmoset. A positive signal was obtained from the saliva of gorilla (data not shown).

High Dose Hook Effect

The high dose hook effect can induce a false negative result on some lateral flow immunochromatographic strip tests when high levels of target antigen are present in the tested sample. The false negative result due to a high dose hook effect occurs when the amount of target antigen in the sample is sufficiently high that a significant amount of target antigen remains unbound by the colloidal gold-labeled antibody in the conjugate pad. Free antigen then migrates to the membrane ahead of the labeled antibody-antigen complexes, thereby occupying the bound antibody on the test line with unlabeled antigen and leaving no sites for the gold labeled antibody-antigen complexes. By blocking the test line with unlabeled antigen, the test result appears negative. Most forensic laboratory personnel are familiar with high dose hook effects and test a dilution of the questioned stain extract to insure that the observed result is a true negative, and not due to a high dose hook effect. We evaluated RSID™-Saliva with increasing amounts of saliva extract to evaluate RSID™-Saliva's response to high levels of antigen. Positive control extracts of 0, 5, 25, 50, 75, and 100 µL were prepared and run on RSID™-Saliva (see figure below, strips 1-6, respectively). Note that at all extract

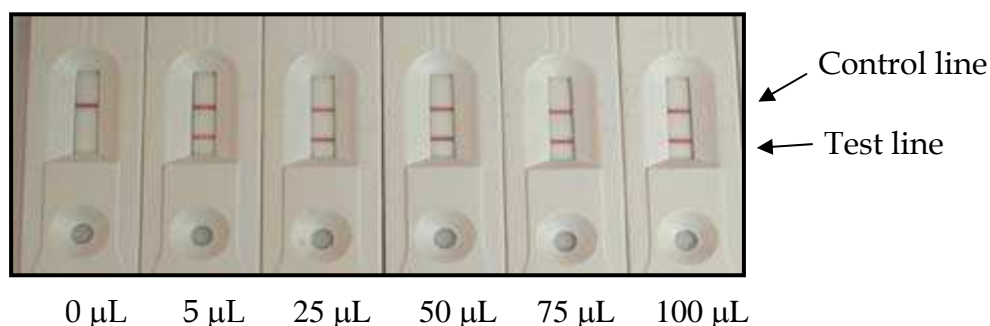
volumes tested (even the equivalent of 5 μ L saliva) no decrease in the intensity of the test line demonstrating a complete lack of high dose hook effect for RSID™-Saliva at all tested concentrations.

This observation led us to more stringent tests of high dose hook effect in which the concentration of the positive control extract was increased by decreasing the extract volume from 1 mL to 400 μ L (2.5X more concentrated), and finally to extracting the positive control swab in 200 μ L in order to produce an even more concentrated extract (5X more concentrated than standard positive control). In addition, larger volumes of these more concentrated extracts were used on RSID™-Saliva test strips. These experimental approaches for preparing highly concentrated saliva extracts were designed to demonstrate the functional upper limit of saliva detection by RSID™-Saliva. For these experiments, strip tests were run in 12 x 75 mm test tubes (see figure below). A standard positive control swab with 50 μ L of saliva was extracted in 400 μ L of extraction buffer and 50 μ L and 100 μ L of this extract was run on RSID™-Saliva strips (see figure below, strips 2 and 3). For comparison, 20 μ L of a sham extract was included as a negative control (see figure below, strip 1). A fresh positive control swab was extracted in 200 μ L and the entire extract was tested on an RSID™-Saliva (due to absorption of liquid by the cotton swab, ~100 μ L of fluid was remaining and loaded onto the cassette). Again no evidence of a high dose hook effect was observed (see figure below, strip 4). These increased volumes of highly concentrated saliva extract were tested numerous times with the same result: no evidence of a high dose hook effect was observed including any reduction of the test line intensity (data not shown).

Users of RSID™-Saliva can expect no false negative results due to high dose hook effects.

The lack of high dose hook effect will facilitate the integration of RSID™-Saliva into DNA forensic laboratory protocols, as a wide range of saliva concentrations, and stain sizes, can be tested without performing dilutions of questioned stain extracts.

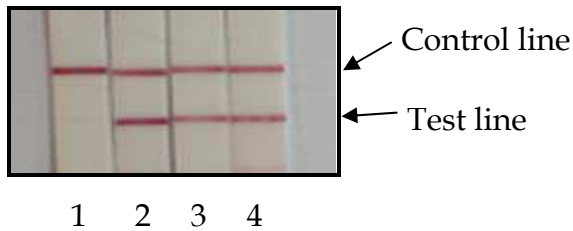
Test for High Dose Hook Effect (I)



As an additional test for High dose hook effect, the concentration of the saliva extract was increased such that 50 μL of saliva on a sterile swab was extracted into 400 μL or into 200 μl RSID™-Saliva extraction buffer. From these concentrated saliva extracts, 50 μL and 100 μL of the 400 μL extraction and the entire volume from the 200 μL extract were tested on RSID™-Saliva (due to absorption of liquid by the cotton swab, ~100 μL of fluid was remaining and loaded onto the cassette).

Results : No diminution of signal was observed for the concentrated saliva extracts even at the highest concentration of saliva tested. No high dose hook effect was observed.

Test for High Dose Hook Effect (II)



Test strips have been removed
from cassettes for clarity

Strip 1- Buffer only

Strip 2- 50 μ L saliva extract (50 μ L saliva extracted in 400 μ L)

Strip 3- 100 μ L saliva extract (50 μ L saliva extracted in 400 μ L)

Strip 4 - 60 μ L saliva extract (50 μ L saliva extracted in 200 μ L)

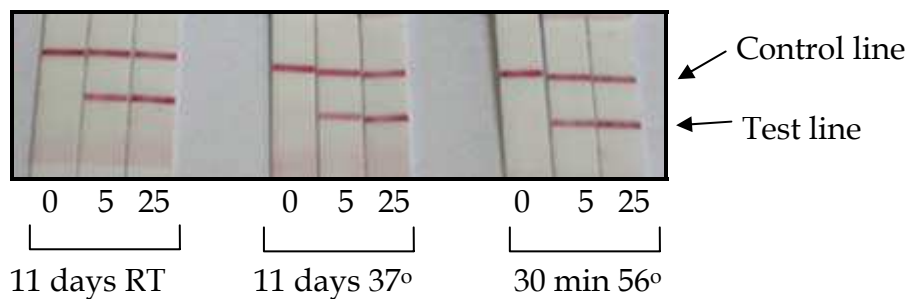
Conclusion: At all saliva extract volumes tested, no high dose hook effect was observed with RSID™-Saliva and users can expect to observe no false negative results due to high dose hook reactions.

Stability Testing of RSID™-Saliva

We have previously demonstrated that RSID™-Saliva is both specific and sensitive for human saliva. Here we test the stability of the assembled cassettes by storage at elevated temperatures. Assembled strip tests were stored at 37°C to increase aging and potential degradation of the strips and subjected to a heat shock of 56°C, again to test stability of the assembled test cassettes.

Extracts from positive control swabs were prepared and 0, 5 and 25 μ L of extract (equivalent to 0, 0.25 and 1.25 μ L of saliva) were tested with RSID™-Saliva which has been stored at 37°C for 11 days (condition designed to mimic storage for ~134 days at room temperature) and with RSID™-Saliva that had been exposed to 56°C for 30 minutes.

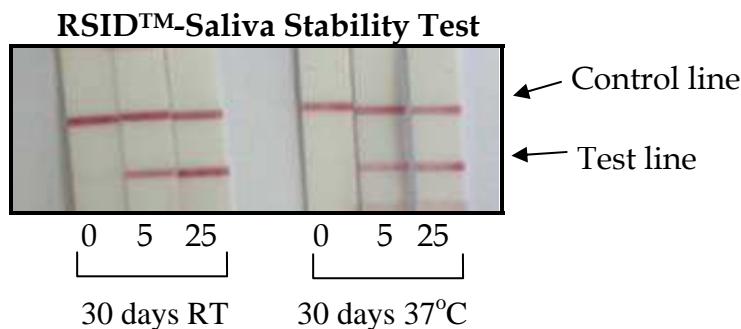
Stability Test, RSID™-Saliva



Test strips have been removed from cassettes for clarity

We performed an additional stability experiment meant to simulate 1 year storage of RSID™-Saliva. Here we tested 0, 5 μ L, and 25 μ L of positive control extract (equivalent to 0, 0.25 μ L and 1.25 μ L saliva) with RSID™-Saliva after storage of the strips at 37°C for 30 days and compared the results with RSID™-Saliva that has been stored at room temperature for the same amount of time.

Results: Test strips stored under conditions to mimic storage at room temperature for one year showed a small but measurable decrease in signal intensity. Positive control saliva extracts, 5 and 25 μ L scored positive (respectively) for test strips stored at 37°C for one month. Similarly, positives were observed for test strips stored at room temperature for one month. Overall sensitivity of RSID™-Saliva was not significantly affected.



Test strips have been removed from cassettes for clarity

Conclusions: RSID™-Saliva cassettes are stable to storage without significant loss of sensitivity.

Detection of Saliva from Forensic Exhibit-like Samples

We have clearly established that RSID™-Saliva can detect saliva from a laboratory prepared control sample; here we demonstrate the ability of RSID™-Saliva to detect saliva from samples likely to be encountered in forensic laboratory case work. In addition we show that RSID™-Saliva can be incorporated into DNA-STR analysis and suggest protocols such that saliva detection can be performed prior to DNA-STR analysis.

Test Sample 1: Aluminum Coke can

Test Sample 2: Plastic coffee cup lid

Test Sample 3: Plastic Water Bottle

Test Sample 4: Glass Water Bottle

Test Sample 5: Cigarette Butts

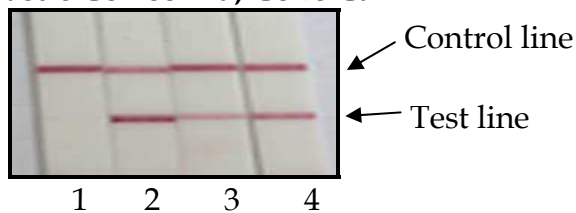
Test Sample 6: Clippings from swabs used to sample plastic coffee lids (2) and aluminum cans (2).

Procedure: Sterile cotton swabs were moistened with ddH₂O and used to 'sponge' the can lip and 'pop-top' opening of the can, and coffee cup lip. The swabs were extracted in 300 µL RSID™-Saliva extraction buffer for 2 hours at room temperature. 25 µL of the extract was removed for RSID™-Saliva testing and the remaining contents of the tube (including the swab batting) were processed for DNA extraction and STR analysis as

per laboratory protocol. A buccal swab/oral swab used as a positive control was extracted and processed in an identical manner.

Results: Samples from the plastic coffee lid and 'Coke can' were scored positive, respectively (see digital photo below). DNA extraction, multiplex PCR and STR analysis on an ABI310 capillary electrophoresis instrument gave complete DNA-STR profile (15 loci + amelogenin) from the Coke can and a partial DNA-STR profile from the coffee lid (10 loci + amelogenin).

**RSID™-Saliva,
Plastic Coffee Lid, Coke Can**

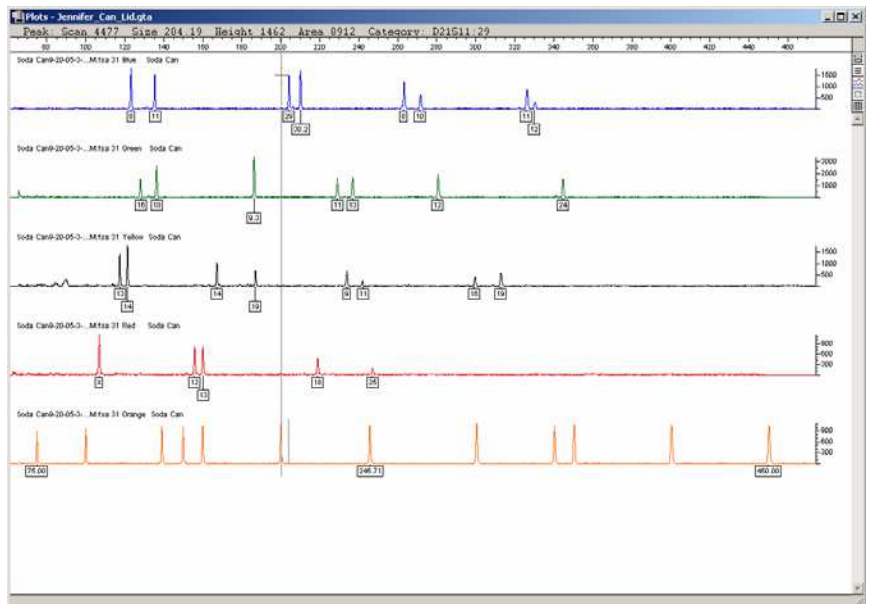


- Strip 1 - Buffer only
- Strip 2 - 25 µL oral swab extract
- Strip 3 - 25 µL plastic lid extract
- Strip 4 - 25 µL Coke can extract

Test strips have been removed from cassettes for clarity

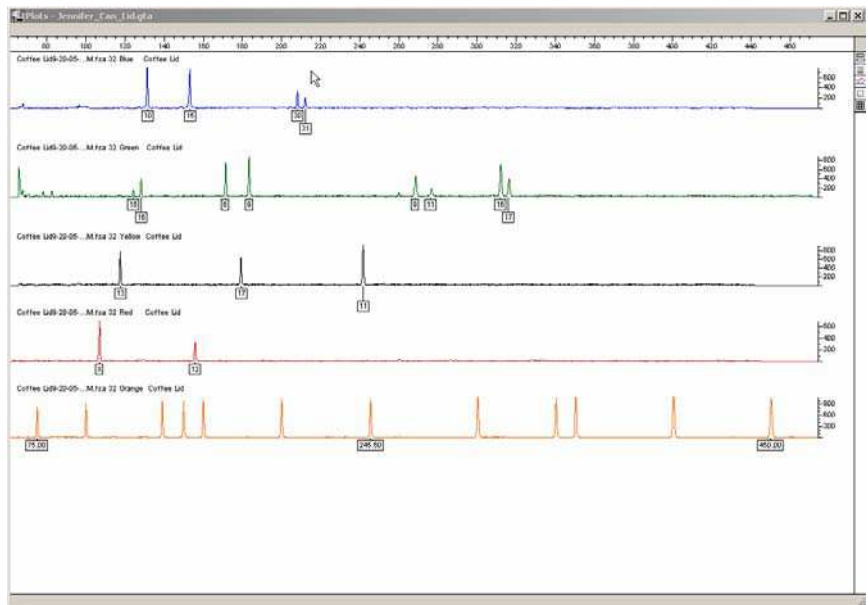
Identifiler STR profile of 'Coke can' sample, processed for stain ID with RSID™-Saliva and for DNA-STR analysis.

Single Tube extraction protocol used.



Identifiler STR profile of 'coffee lid' sample processed for stain ID with RSID™-Saliva and for DNA-STR analysis

Single Tube extraction protocol used.



Test Sample 3: Plastic Water Bottle

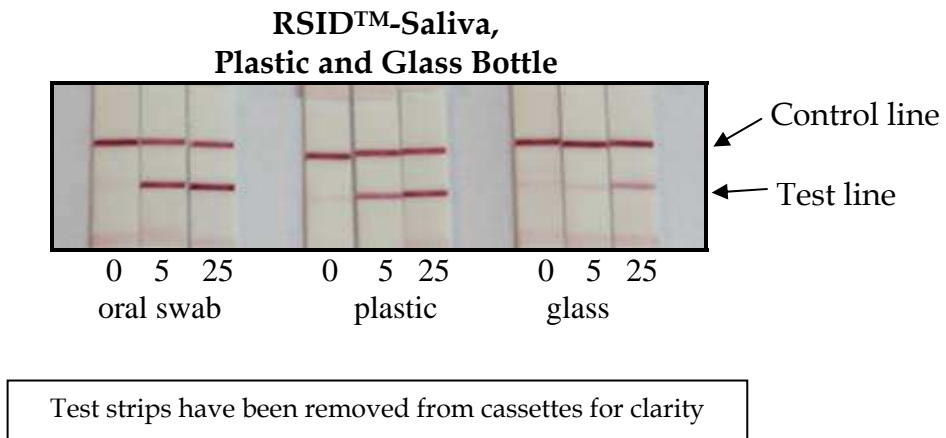
Test Sample 4: Glass water bottle (Perrier)

Procedure: Moistened sterile cotton swabs were used to 'sponge' the openings of both bottles and subsequently extracted in 200 μ L RSID™-Saliva extraction buffer for 2 hours at room temperature. A total of 30 μ L of the extract was removed for RSID™-Saliva testing, i.e., 5 and 25 μ L aliquots, were used for analysis with RSID™-Saliva. The remaining extract (including the swab) was used for DNA extraction and STR analysis. Oral swab extract (in 1 mL) was used as a positive control.

Results: Saliva from Sample 3, the plastic water bottle was readily detected and RSID™-Saliva was scored positive for both 5 and 25 μ L extract, respectively (see figure below). Saliva extract from the glass bottle also scored positive with 5 and 25 μ L of extract, respectively (see figure below).

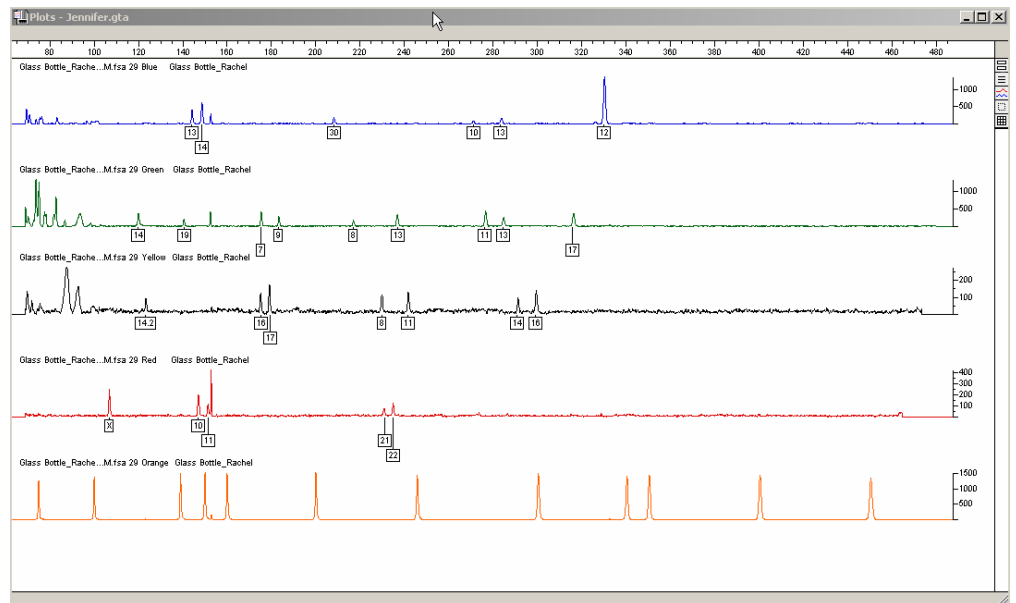
STR analysis did however provide a full profile (15 loci + amelogenin) from the glass bottle, whereas only two loci were obtained from the plastic bottle. Correlating the

intensity of the RSID™-Saliva test results with the observed DNA-STR results may not be straightforward: a number of variables including person to person variation, extraction methods, and amplification kit used, may all affect the ability of the analyst to obtain a full DNA profile from the tested sample.



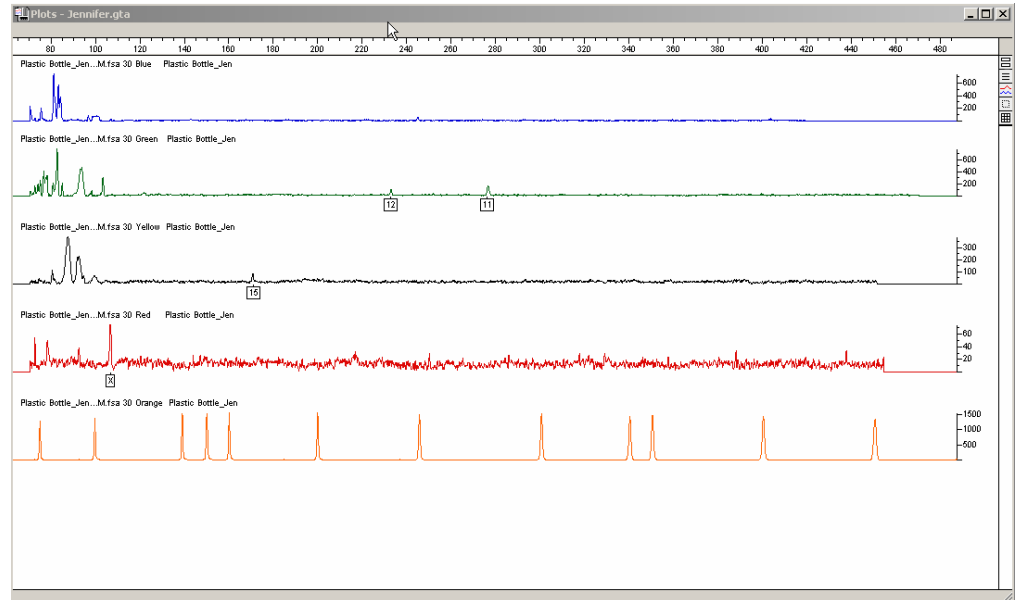
Identifiler STR profile of 'Glass bottle' sample, processed for stain ID with RSID™-Saliva and for DNA-STR analysis.

Single Tube extraction protocol used.



Identifiler STR profile of 'Plastic bottle' sample, processed for stain ID with RSID™-Saliva and for DNA-STR analysis.

Single Tube extraction protocol used.

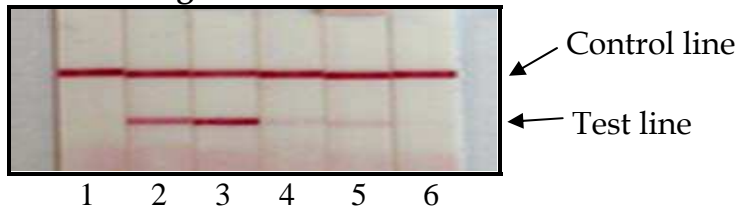


Test Sample 5: Saliva detection from Cigarette butts.

Procedure: Two received cigarette butts (samples 5a and 5b) were sampled using moistened sterile cotton swabs which were subsequently extracted in 200 μ L of RSID™-Saliva extraction buffer; an aliquot of the extraction was used for RSID™-Saliva (25 μ L) while the majority of the extract was processed for DNA-STR analysis.

Results: Positive control saliva extracts gave positive results for 5 and 25 μ L of saliva extract (respectively), 25 μ L of samples 5a and 5b gave test lines of low intensity but were clearly above background levels. Sample 5a was analyzed for Y-STRs and provided clear data for 14 loci (see below).

**RSID™-Saliva,
Cigarette Butts**

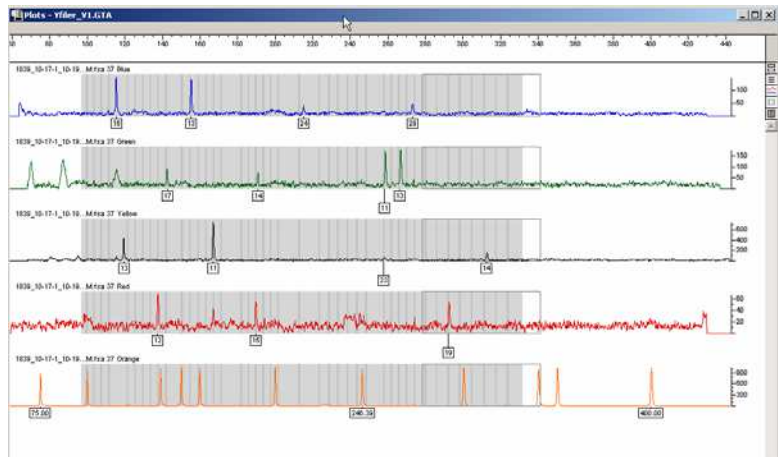


- Strip 1 - Buffer only
- Strip 2 - 5 µL saliva extract
- Strip 3 - 25 µL saliva extract
- Strip 4 - 25 µL extract Cig Butt #5a
- Strip 5 - 25 µL extract Cig Butt #5b
- Strip 6 - 25 µL extract Cig Butt neg

Test strips have been removed from cassettes for clarity

Cigarette Butt was analyzed for Y-STR using Y-Filer.

Single tube extraction protocol used for both stain ID and DNA-STR processing.



Conclusion: RSID™-Saliva detects saliva from cigarette butts.

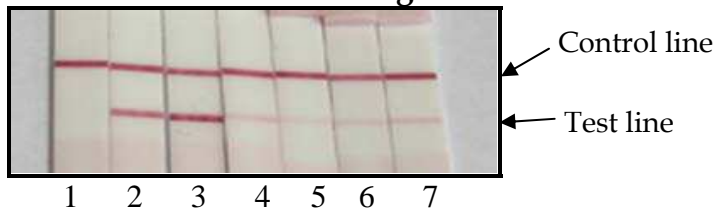
RSID™-Saliva Analysis of swab cuttings from swabs used to sample plastic lids and aluminum cans, alternative to single tube extraction protocol. Our laboratory protocol uses a single extraction step for both stain identification and DNA-STR analysis. The advantages of this approach are clear and include less sample loss (one tube for sample extraction, stain identification and DNA-extraction), less manipulation of the sample (cuttings and repeated testing of evidence swabs are eliminated), less chance of contamination (fewer procedural steps) and it eliminates variation due to inhomogeneous swabs. Many laboratories however, use an alternative approach, of testing small cuttings from swabs that were used to ‘sponge’ or sample questioned stains. Here we demonstrate that RSID™-Saliva can be used with cuttings obtained from swabs used to absorb questioned stains.

Test Sample 6: Extracts were prepared from cuttings from swabs used to sample two plastic coffee lids and two aluminum soda cans. Moistened swabs were used to ‘sponge’ the areas of 2 plastic coffee lids and 2 aluminum soda cans most likely to have been in contact with saliva. Swabs were allowed to dry in a protected environment and cuttings from the swabs were removed and placed individually in microcentrifuge tubes. These cuttings were extracted in 50 μL RSID™-Saliva extraction buffer for 1 hour at room temperature at which time $\sim 30 \mu\text{L}$ (all the volume available in the tube) was used for analysis with RSID™-Saliva.

Results: Clipping from the positive control scored clear positive for 5 and 25 μL saliva extract, respectively. Extracts from the swab cuttings, $\sim 30 \mu\text{L}$ each, scored weak positive but clearly above background signal (see figure below). The tested swabs were

processed for DNA-STR analysis with mixed results: one plastic lid provided a full profile (15 loci + amelogenin), the other provided a partial profile (13 loci + amelogenin) while analysis of the swabs from the cans gave partial profiles.

**RSID™-Saliva,
Test from Swab Cuttings**

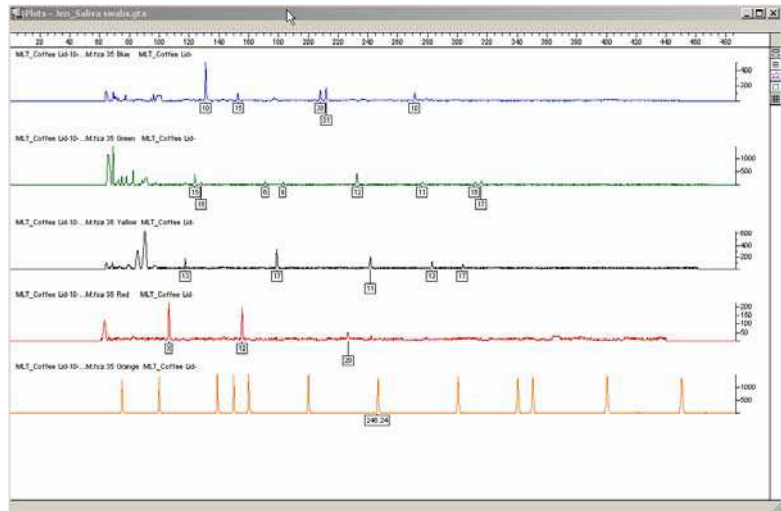


Test strips have been removed from cassettes for clarity

- Strip 1 - Buffer only
- Strip 2 - 5 µL saliva extract
- Strip 3 - 25 µL saliva extract
- Strip 4 - 30 µL 1st plastic lid
- Strip 5 - 30 µL 1st alum can clipping
- Strip 6 - 30 µL 2nd plastic lid clipping
- Strip 7 - 30 µL 2nd alum can(2) clipping

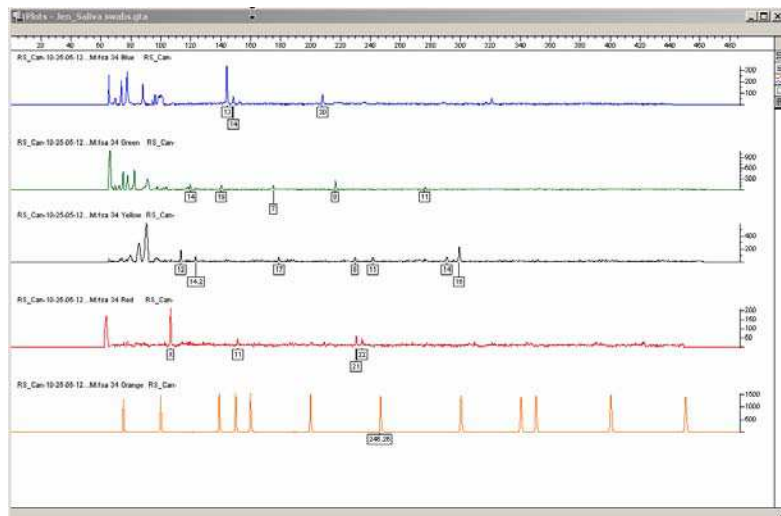
Identifiler STR profile of 'plastic coffee lid' sample processed for stain ID with RSID™-Saliva and for DNA-STR analysis.

Multiple cutting protocol used for stain ID and DNA-STR analysis.



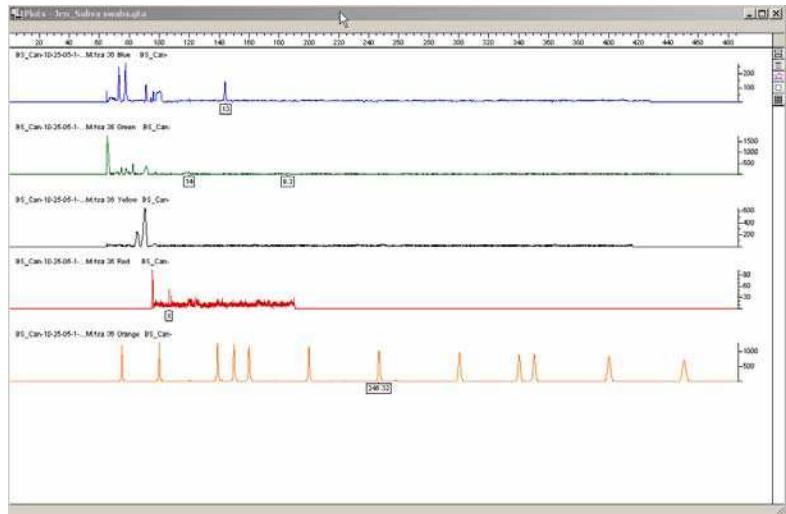
Identifiler STR profile of 'Soda Can' sample processed for stain ID with RSID™-Saliva and for DNA-STR analysis.

Multiple cutting protocol used for stain ID and DNA-STR analysis.



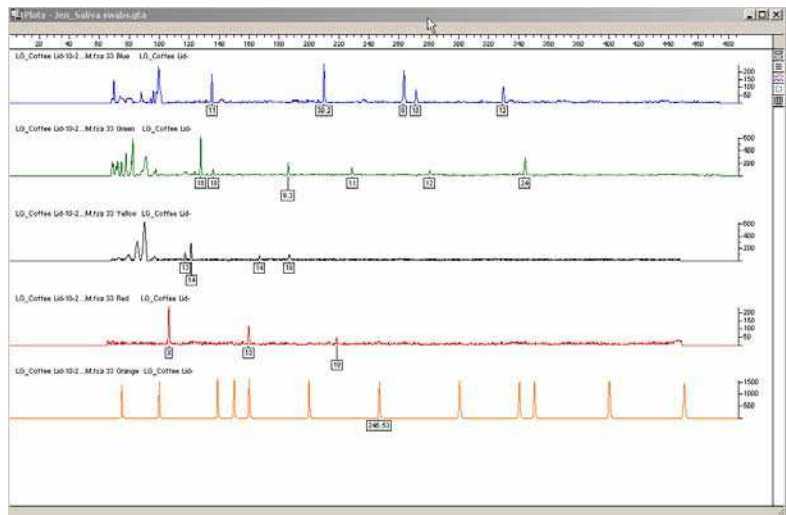
Identifiler STR profile of 'Soda Can (2)' sample processed for stain ID with RSID™-Saliva and for DNA-STR analysis.

Multiple cutting protocol used for stain ID and DNA-STR analysis.



Identifiler STR profile of 'plastic coffee lid(2)' sample processed for stain ID with RSID™-Saliva and for DNA-STR analysis.

Multiple cutting protocol used for stain ID and DNA-STR analysis.



Conclusion: RSID™-Saliva can detect saliva from swab cuttings derived from swabs used to sample cans and plastic lids.

Additional testing with RSID™-Saliva – Forensic-like Samples.

We sampled a variety of surfaces and materials in an effort to rigorously test RSID™-Saliva. The samples include envelopes, additional plastic bottles, and a different metal soda can.

Procedure: All samples were ‘sponged’ with a moistened sterile cotton swab and after air-drying in a protected environment, the swab batting was extracted in 400 µl of RSID™-Saliva extraction buffer in a microcentrifuge tube. 25 µl of each extract was tested with RSID™-Saliva. Positive control was an oral swab extracted in 1.0 ml of RSID™-Saliva extraction buffer, 5 µl of extract tested. Samples included:

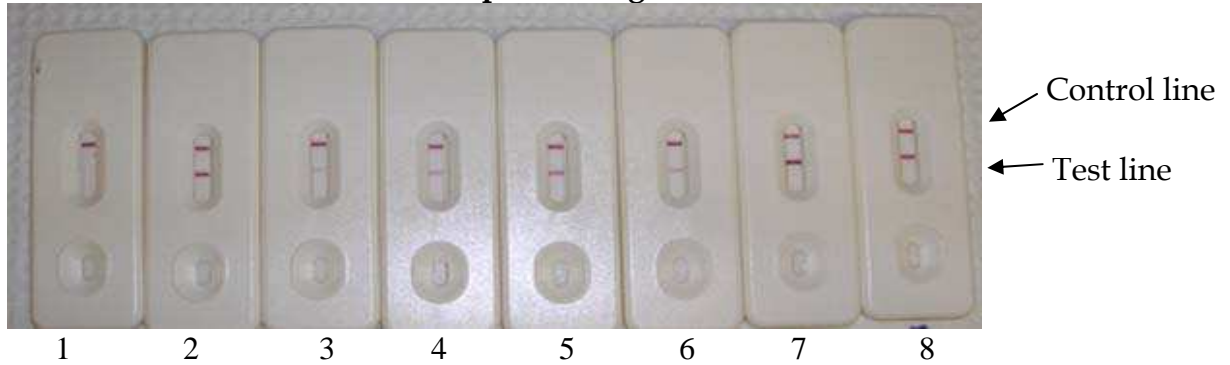
- 1) Negative Control
- 2) Positive Control
- 3) Envelope, licked, sealed, steamed open and upper flap sampled with swab technique
- 4) Envelope, licked, sealed, steamed open and lower flap sampled with swab technique
- 5) Plastic bottle, threads and cap tested.
- 6) Glass bottle, threads and cap tested
- 7) oral swab
- 8) metal soda can

Results: As was expected the amount of saliva in the above eight samples varied widely and this was reflected in the intensity scores of the test line (see figure below).

Sample

- (1) Neg
- (2) Pos
- (3) Envelope upper flap
- (4) Envelope lower flap
- (5) Plastic Bottle
- (6) Glass. Bottle
- (7) Oral Swab
- (8) Metal can

RSID™-Saliva, Additional Sample Testing



References Cited

Svensson, B. Regional distant sequence homology between amylases, alpha-glucosidases and transglucanases., FEBS Lett. 230(1-2):72-6, 1988

Acknowledgements

We gratefully acknowledge the contributions, help and advice of numerous scientists and organizations. In particular we thank, Jean DuBach, Brookfield Zoo, Brookfield Zoo, Brookfield IL, 60513; Mark Warneke, Brookfield Zoo, Brookfield IL, 60513; Margaret V. Root Kustritz, DVM, PhD, DACT, Department of Veterinary Clinical Sciences University of Minnesota College of Veterinary Medicine St. Paul, MN 55108; Scott Madill, BVSc, DVSc, DACT, Department of Veterinary Population Medicine University of Minnesota College of Veterinary Medicine, St. Paul, MN 55108; Juan Romano, DVM, PhD, DACT, Department of Veterinary Population Medicine University of Minnesota College of Veterinary Medicine, St. Paul, MN 55108; Select Breeders Service Inc., Coloma MD; 21907; The San Diego Sheriff's Regional Crime Laboratory, San Diego CA 92117 and Joan Wraxall, SERI, Richmond CA 94806.
