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

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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 samples 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 in use 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 the use of a new, lateral flow immunochromatographic strip test for human saliva detection and present 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 specifically in human saliva.

The enzyme's physiological role is to aid in the digestion of dietary starches. The RSID® test for saliva is specific for human saliva and has numerous advantages over current enzymatic detection methods for amylase detection, including increased sensitivity, specificity, speed and reduced cost. The RSID® System Test for 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® test for saliva as well as numerous experiments demonstrating the ability of the test to detect human saliva from a variety objects that are typically encountered in forensic laboratory case work.

The new **R**apid **S**tain **ID**entification System (RSID®) Test for saliva is designed for fast, easy, and reliable detection of human saliva; test development is complete within 10 minutes and the limit of detection of the assay is 1μl of human saliva. 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 test package insert.

Configuration of the salivary amylase lateral flow test

The RSID® test for Saliva is an immunochromatographic assay that uses two monoclonal antibodies specific for human salivary α-amylase. 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 buffer/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 anti-human

salivary α-amylase monoclonal antibodies. The diluent 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' creating a visible red 'bar' against the white membrane. Colloidal gold labeled mouse antibody will continue to progress along the membrane and be bound by anti-mouse antibody at the 'control line' again creating a visible signal. A visible line at the 'test' position indicates the presence of human saliva; a visible line at the 'control' position indicates that the test is working correctly.

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 were 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: intensity scores are provided to insure test-to-test consistency during validation and to reduce operator-introduced bias. A copy of the intensity score sheet can be found at the end of this document.

***Note: Tested volumes for RSID-Saliva were either 150 µl when strips were tested individually in test tubes *without* a cassette or 100 µl when strips were tested in a plastic housing.**

We do not recommend using 150 µl test volumes for cassette tests: the only approved final volume for cassettes is 100 µl.

Sensitivity Testing: Saliva Extract and Human Oral Swab Extract

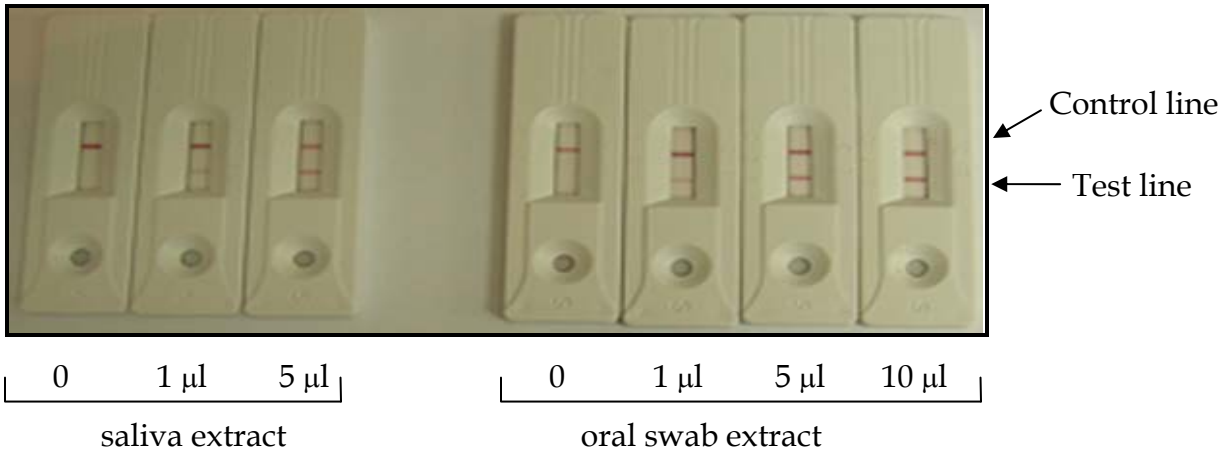
Methods: For sensitivity studies, we tested saliva extracts and human oral swab extracts. For saliva extracts, 50 μl of human saliva was deposited on a sterile cotton swab and allowed to air-dry. The end of the swab with the cotton batting was cut off using laboratory clean technique and placed in a 1.5 ml microcentrifuge tube. The swab head was extracted in 1 ml of PBS for 1 hour at room temperature. We calculate that the PBS 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 a individual's cheek for 10 seconds with a cotton swab, and extracting the swab in 1 ml PBS 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 in PBS.

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 diluent/running buffer and bringing the total volume to 150* μl. Final volume of ***all*** tested samples was always brought to 150* μl with TBS+ running buffer, usually in a 0.6 ml microcentrifuge tube. The full 150* 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 strip test window were scored after 10 minutes by comparison with the Intensity Score Sheet.

Results- Sensitivity of Saliva Extract and Oral Swab Extract

After 10 minutes, the test line of the 0, 1 and 5 μl saliva extracts were scored as intensity ~0, ~4 and ~8, respectively (see photo, left panel). These results indicate that the limit of detection for RSID®-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 scored similarly: band intensities of 0, ~5, ~8, and ~9 after 10 minutes for 0, 1, 5, and 10

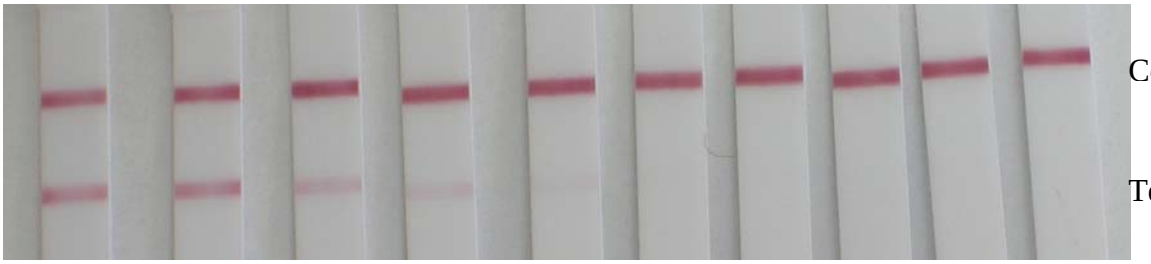
µl of oral extract, respectively. The intensity of the control lines for all tested samples were either ~8 or ~9 indicating consistent test performance.



RSID®-Saliva Limit of detection Experiment.

In this experiment a broad range of saliva extracts 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 PBS, was used throughout. Equivalent saliva volumes are calculated assuming 100% extraction efficiency.

Strip	Extract Amount (µl)	TBS+ Amount (µl)	Equivalent Saliva (µl)
1	50	100*	2.5
2	25	125*	1.25
3	10	140*	0.5
4	5	145*	0.25
5	1	149*	0.05
6	1 from 1:2 dilution	149*	0.025
7	1 from 1:5 dilution	149*	0.01
8	1 from 1:10 dilution	149*	0.005
9	1 from 1:100 dilution	149*	0.0005
10	25, sham extraction	125*	0.0



Test strips have been removed from cassettes for clarity

Results: A clear signal at the test line can be observed for strips 1, 2, 3, 4 and 5.

Detection limit is therefore ~50 nl (0.05 μl) of saliva.

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

Methods: 50 μl of saliva, urine, semen, or blood were each deposited on separate sterile cotton swabs and allowed to air-dry. The tip of the swab with the cotton batting was cut off using laboratory clean technique and placed in a 1.5 ml microcentrifuge tube and extracted into 1 ml of PBS for 2 hours at room temperature. Extracts, 25 μl each or as mixtures, were combined with diluent/running buffer to a final volume of 150* μl and deposited in the sample well of test strips. Extracts from body fluids were combined with and without saliva to evaluate potential cross-reactivity and possible inhibition. Extracts were prepared such that the tested volume, 25 μl of body fluid extract, was equivalent to approximately 1.25 μl of blood, semen, urine or saliva (assuming 100% extraction efficiency).

Results: Extract from swabs of human blood, saliva, semen, and urine alone or as a mixture of body fluids do not cross react with or inhibit RSID®-Saliva. Digital images of three experiments testing cross-reactivity of blood, semen and urine with RSID-Saliva are shown below. In all three experiments, the sensitivity of RSID-Saliva with 25 μl saliva extract alone or in combination with blood, urine, and semen extract was the

same and was scored as ~9 after 10 minutes. There was no background seen with 25 µl urine, blood, or semen alone after 10 minutes.

Experiment #1

Bu Sa B Se U 4 3

Test strips have been removed from cassettes for clarity

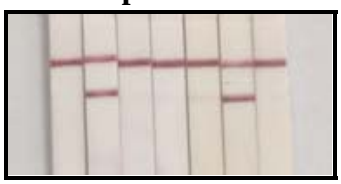
Bu =Buffer only
Sa =25 µl saliva extract
B =25 µl Blood extract
Se =25 µl Blood extract
U =25 µl Urine extract
4 =mixture of 25 µl each body fluid extract
3 =25 µl each of Blood, Semen, and Urine extract

Experiment #2

Bu Sa Se U B 4

Bu =Buffer only
Sa =25 µl saliva extract
B =25 µl Blood extract
Se =25 µl Blood extract
U =25 µl Urine extract
4 =mixture of 25 µl each body fluid extract
3 =25 µl each of Blood, Semen, and Urine extract

Experiment #3



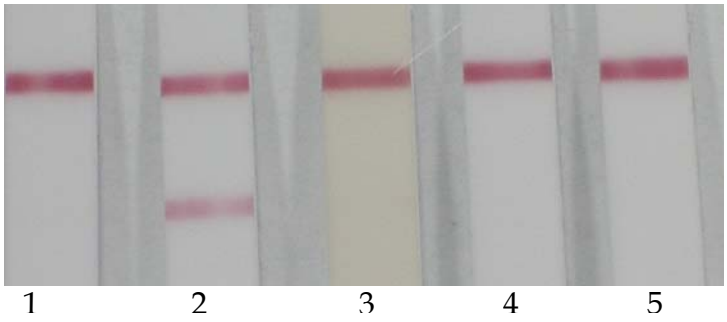
Bu Sa Se U B 4 3

Test strips have been removed from cassettes for clarity

Additional Body Fluid Specificity Testing.

Procedure: Extracts from human blood, semen, and urine were analyzed with RSID®-Saliva to test specificity of the assay. 25 µl of extract was combined with 125* µl TBS+ (8.5) running buffer.

Strip	Sample
1	25 µl sham extract
2	25 µl saliva extract
3	25 µl blood extract
4	25 µl semen extract
5	25 µl urine extract



Test strips have been removed from cassettes for clarity

As an additional test of body fluid specificity, extracts of saliva, blood, semen and urine where combined in various ratios (1:1, 1:3, and 3:1) and used with RSID®-Saliva. Indicated volumes of extracts were tested in a total volume of 150* µl of running buffer/diluent (see table below).

Strip	Saliva Extract (µl)	Other Fluid Extract / (µl)
1	24.0 (sham)	0.0
2	6.0	0.0
3	12.0	0.0
4	18.0	0.0
5	6.0	Blood/18.0
6	12.0	Blood/12.0
7	18.0	Blood/6.0
8	6.0	Semen/18.0
9	12.0	Semen/12.0
10	18.0	Semen/6.0
11	6.0	Urine/18.0
12	12.0	Urine/12.0
13	18.0	Urine/6.0

Results: RSID®-Saliva is not affected by mixtures of saliva and urine, saliva and semen, or saliva and blood.

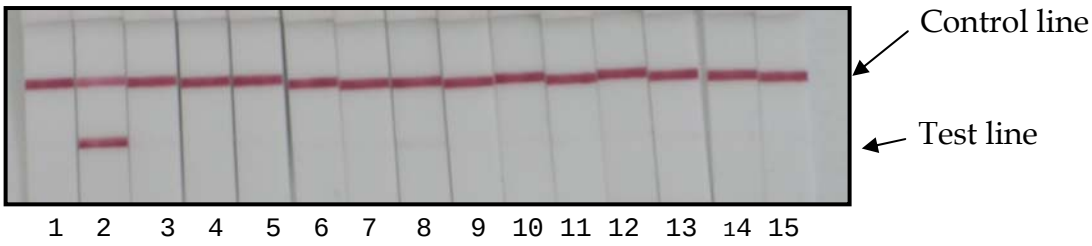
Conclusion: RSID®-Saliva shows no cross reactivity with extracts from urine, blood, or semen either alone or in combination.
In addition, extracts of blood, urine and semen do not inhibit RSID®-Saliva or reduce the apparent limit of detection of the strip test.

Conclusion: RSID®-Saliva shows no cross reactivity with extracts from urine, blood, or semen at mixed ratios.

Species Specificity Testing

Saliva swabs from different animal species were kindly provided by the Brookfield Zoo. Extracts were prepared by removing the swab heads of provided samples into a 1.5 ml microcentrifuge tube using clean laboratory technique and extracting in 1 ml of PBS for 1 hour at room temperature, 25 µl of each extract was added to 125* µl of diluent/running buffer and added to the sample well of an RSID®-Saliva cassette. Animals species tested in this experiment include: Negative control (lane 1), positive control (lane2), sheep (lane 3), Nubian goat (lane 4), llama (lane 5), domestic pig (lane 6), domestic rabbit (lane 7), dwarf mongoose (lane 8), grey gull (lane 9), domestic cat (lane 10), panther chameleon (lane 11), Fjord horse (lane 12), Callimico (Goeldi's marmoset) (lane 13) , and two different domestic mix-breed dogs (lanes 14 and 15).

Animal	Signal	Animal	Signal
Border Collie	NO	llama	NO
Opossum	NO	dwarf mongoose	NO
Guinea pig	NO	grey gull	NO
Woodchuck	NO	Panther Chameleon	NO
cow	NO	Fjord horse	NO
domestic cat	NO	marmoset	NO
domestic rabbit	NO	mixed breed dog	NO
sheep	NO	Nubian goat	NO

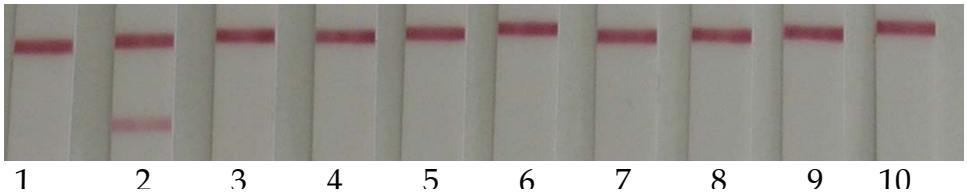


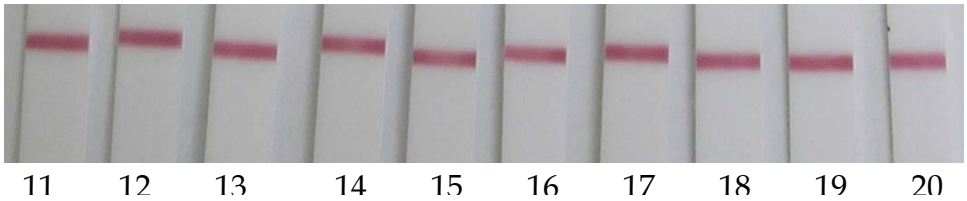
Test strips have been removed from cassettes for clarity

Additional Species Specificity Testing

Species specificity testing was repeated with a different production lot of RSID®-Saliva and with fresh extracts from a variety of animal species. Air dried cotton swabs containing saliva from various domestic and exotic animals were obtained from pet shops and the Brookfield Zoo. The swabs were extracted in 1.0 ml of PBS and 25 µl of the extract was added to 125* µl TBS+ running buffer and applied to saliva strip tests. Positive (25 µl human saliva extract, strip 2) and negative (25 µl sham extract, strip 1) controls were included for comparison.

Animals tested: tamarin (strip 3), opossum (strip 4), ferret (strip 5), 2 different mix breed dogs (strips 6 and 7), callimico (strip 8), horse (strip 9), chameleon (strip 10), llama (strip 11), goat (strip 12), sheep (strip 13), border collie (strip 14), marsh snake (strip 15), hedgehog (strip 16), domestic pig (strip 17), domestic rabbit (18), mongoose (19), and grey gull (strip 20).





Test strips have been removed from cassettes for clarity

Results: No cross-reactivity was observed with any species tested.

Conclusions: RSID®-Saliva shows no cross reactivity with any of the animal species tested and appears to be specific for human saliva.

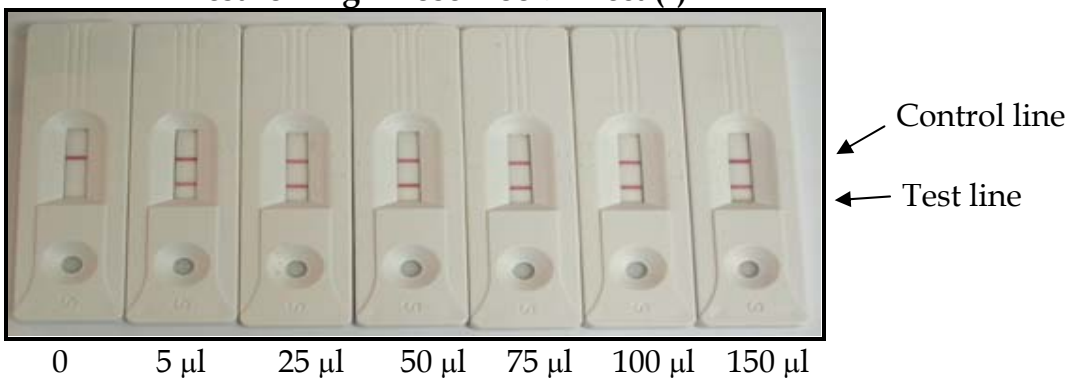
Testing for High Dose Hook Effect with RSID®-Saliva

A *high dose Hook effect* refers to the false negative result seen with immuno-chromatographic strip tests when very high levels of target are present in the tested sample. Under these conditions, unbound salivary α-amylase antigen could reach the test line *before* the colloidal gold-labeled antibody-bound antigen, resulting in a false negative result.

Methods: To assess the threshold of high-dose Hook effect with the RSID®-Saliva we tested increasing amounts of saliva extract made from depositing 50 µl of saliva on a sterile swab and extracting the entire swab batting in 1 ml PBS in a 1.5 ml microcentrifuge tube. The following volumes of extract were tested: 0, 5, 25, 50, 75, 100, and 150* µl (final volume loaded into the sample well was always 150* µl with running buffer/diluent added as required).

Results :The test line intensities did not decrease even at the lowest dilution, (highest concentration) of saliva extract. All extracts scored ~9 after 10 minutes (see digital photo below).

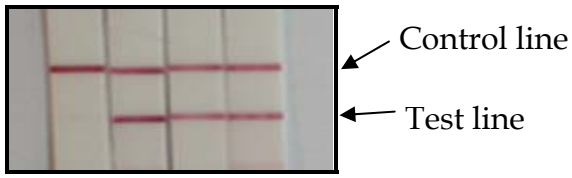
Test for High Dose Hook Effect (I)



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 PBS or into 200 µl PBS. From these concentrated saliva extracts, 50 µl and 150* µl of the 400 µl extraction and the entire volume from the 200 µl extract were tested on RSID®-Saliva.

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)



1 2 3 4

Test strips have been removed from cassettes for clarity

Strip 1– Buffer only

Strip 2– 50 µl saliva extract (50 µl saliva extracted in 400 µl PBS)

Strip 3– 150 µl saliva extract (50 µl saliva extracted in 400 µl PBS)

Strip 4 – 60 µl saliva extract (50 µl saliva extracted in 200 µl PBS)

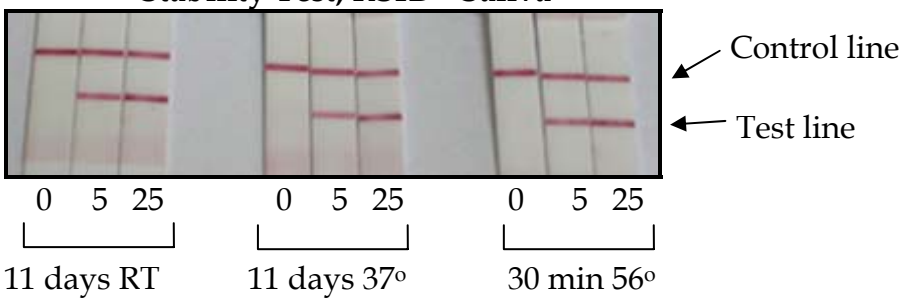
Conclusion: At all saliva extract volumes tested, no high dose Hook effect was observed with RSID-Saliva and users can expect to observe no false negative results due to high dose Hook reactions.

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.

PBS extracts from positive control swabs were prepared and 0, 5 and 25 µl of extract (equivalent to 0, 0.25 and 1.25 µl 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

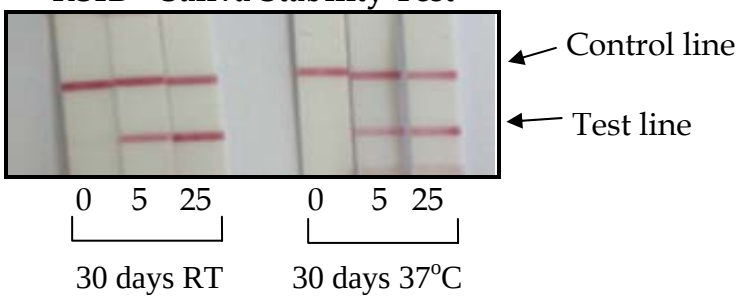


Test strips have been removed from cassettes for clarity

We performed an additional stability experiment meant to simulate 1 year storage of RSID®-Saliva. Here we tested 0, 5 µl, and 25 µl of positive control extract (equivalent to 0, 0.25 µl and 1.25 µl 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 µl, scored ~4 and ~5 (respectively) for test strips stored at 37°C for one month, while scores of ~4 and ~6 (respectively) were observed for test strips stored at room temperature for one month. Overall sensitivity of RSID®-Saliva was not significantly affected.

RSID®-Saliva Stability Test



Test strips have been removed from cassettes for clarity

Conclusions: RSID®-Saliva 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

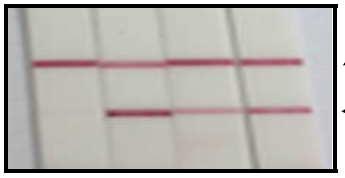
Test Sample 5: Cigarette Butts

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

Procedure: Sterile cotton swabs were moistened with ddH_20 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 PBS for 2 hours at room temperature. 25 µl of the PBS 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 at an intensity of ~6 and ~7, 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



Test strips have been removed from cassettes for clarity

Strip 1 – Buffer only

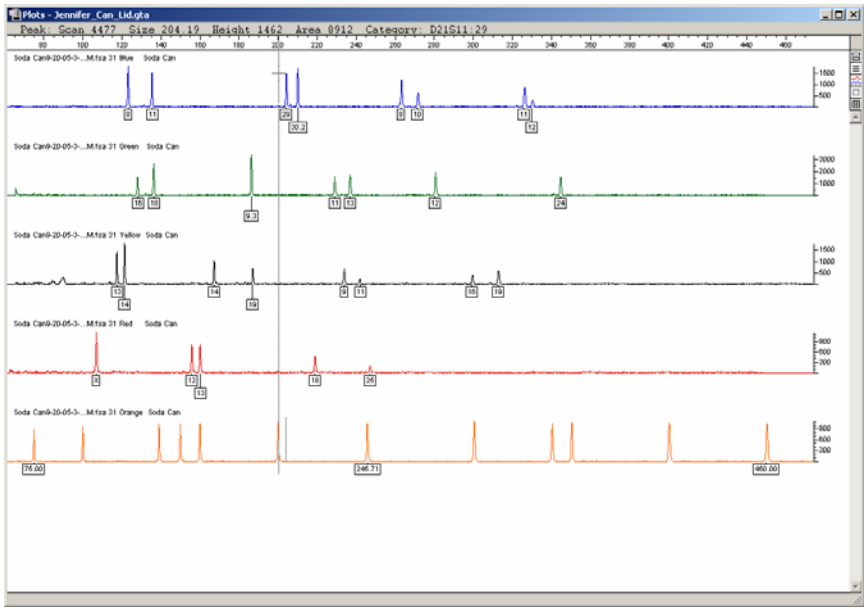
Strip 2 – 25 µl oral swab extract

Strip 3 – 25 µl plastic lid extract

Strip 4 – 25 µl Coke can extract

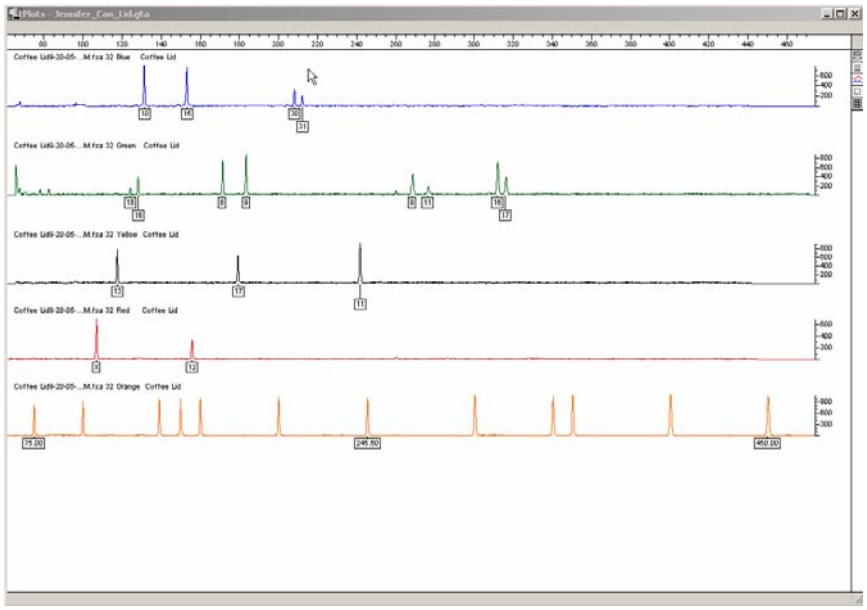
Identifiler STR profile of 'Coke can' sample, processed for stain ID with RSID®-Saliva and for DNA-STR analysis.

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

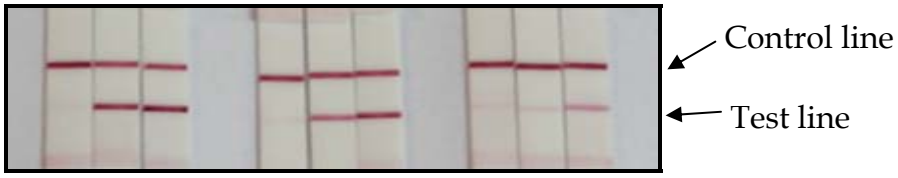
Test Sample 4: Glass water bottle (Perrier)

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

Results: Saliva from Sample 3, the plastic water bottle was readily detected and RSID®-Saliva was scored at ~7 and ~9 (5 and 25 µl extract, respectively). Saliva from glass bottle scored positive in this test, however intensity scores were less than for the plastic bottle; scores of ~2 and ~5, for 5 and 25 µl of extract, respectively.

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.

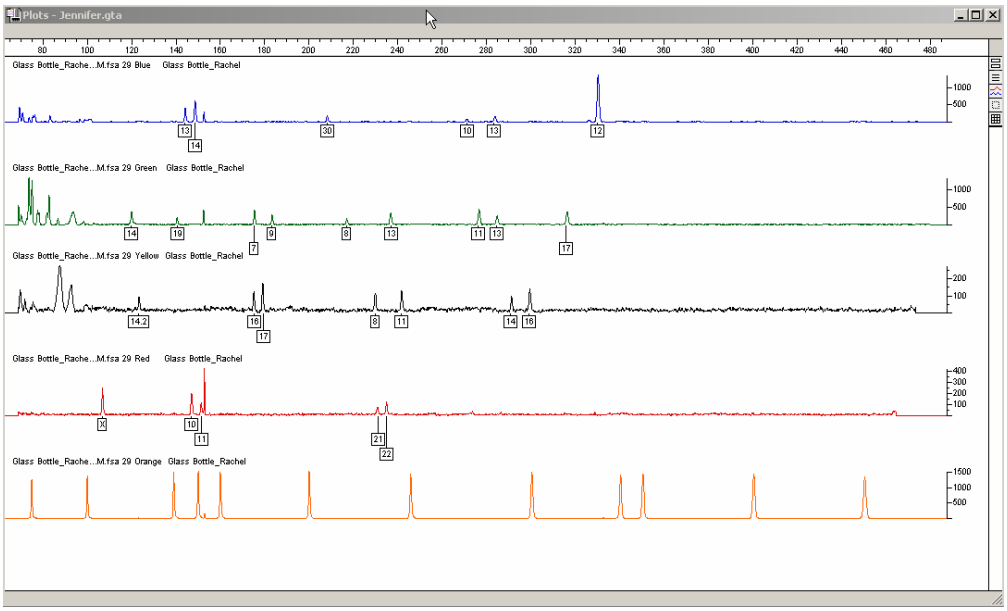
RSID®-Saliva,
Plastic and Glass Bottle



Test strips have been removed from cassettes for clarity

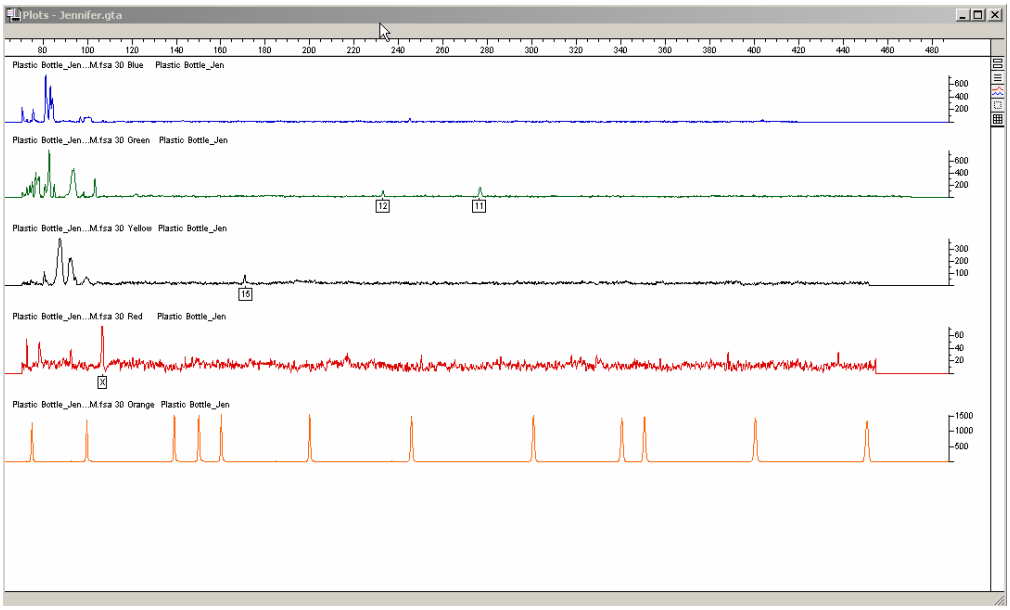
Identifiler STR profile of 'Glass bottle' sample, processed for stain ID with RSID®-Saliva and for DNA-STR analysis.

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.

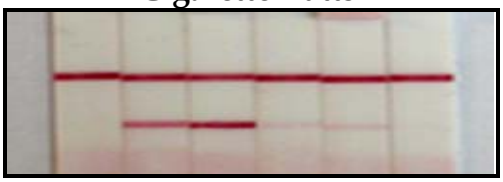


Test Sample 5: Saliva detection from Cigarette butts.

Procedure: Two received cigarette butts were sampled using moistened sterile cotton swabs which where subsequently extracted in 200 µl of PBS; an aliquot of the PBS extraction was used for RSID®-Saliva (25 µl) while the majority of the extract was processed for DNA-STR analysis.

Results: Positive control saliva extracts gave normal band intensities, ~6 and ~8 for 5 and 25 µl of saliva extract (respectively), 25 µl of samples 5a and 5b gave intensity scores of ~2 and ~3, low but clearly above background levels. This sample was analyzed for Y-STRs and provided clear data for 14 loci (see below).

RSID®-Saliva, Cigarette Butts



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

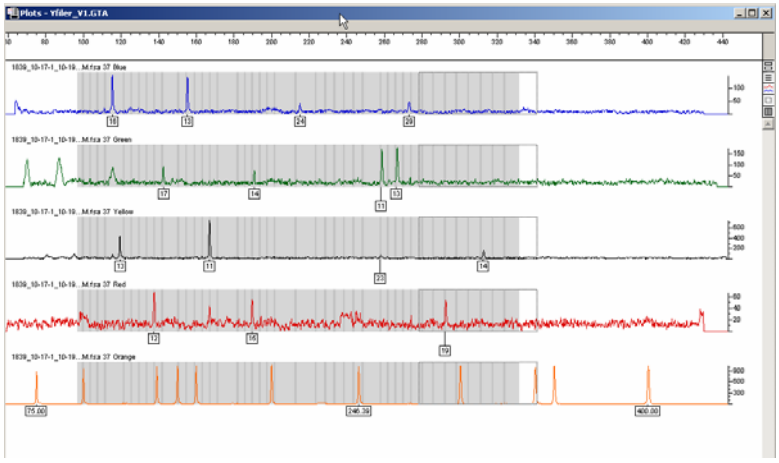
Strip 4 – 25 µl extract Cig Butt #5a

Strip 5 – 25 µl extract Cig Butt #5b

Strip 6 – 25 µl extract Cig Butt neg

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

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



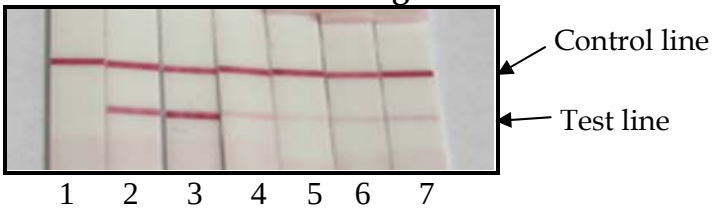
Conclusion: RSID-Saliva detects saliva from cigarette butts.

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) and less chance of contamination (fewer procedural steps). 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 0.6 ml microcentrifuge tubes. These cuttings were extracted in 50 µl PBS for 1 hour at room temperature at which time ~30 µl (all the volume available in the tube) was used for analysis with RSID®-Saliva.

Results: Clipping from the positive control scored at the expected intensity, ~7 and ~8 for 5 and 25 µl saliva extract, respectively. Extracts from the swab cuttings, ~30 µl each, scored ~2 and ~3, low but clearly above background signal. 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

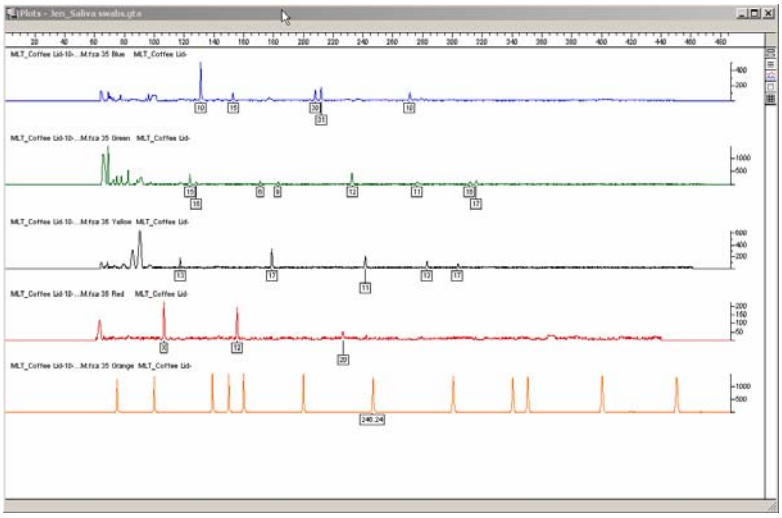


Test strips have been removed from cassettes for clarity

Strip 1 – Buffer only
Strip 2 – 5 µl saliva extract
Strip 3 – 25 µl saliva extract
Strip 4 – 30 µl 1st plastic lid clipping
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

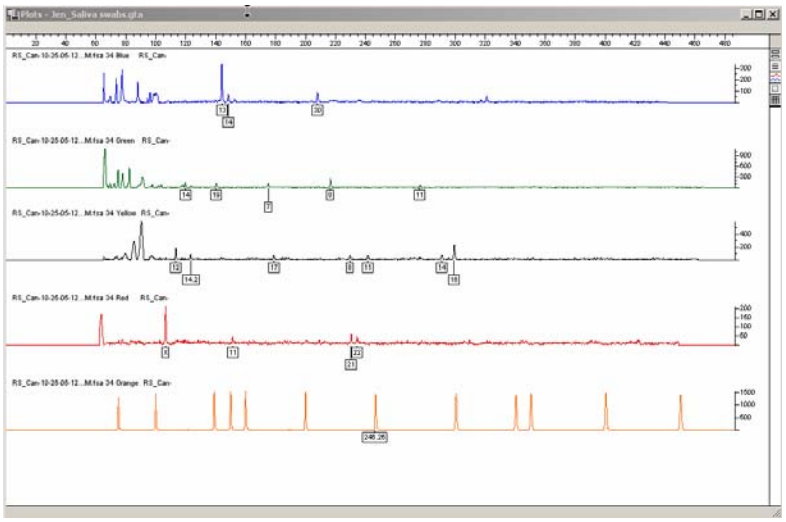
Identifiler STR profile of 'plastic coffee lid' sample processed for stain ID with RSID®-Saliva and for DNA-STR analysis.

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



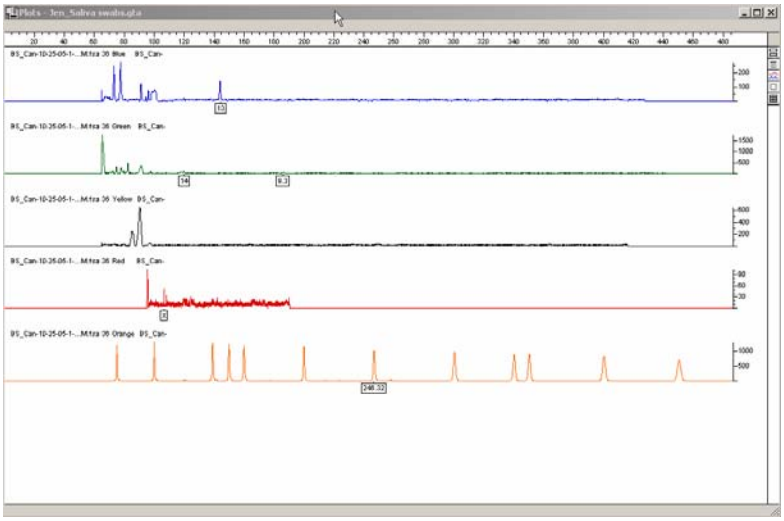
Identifiler STR profile of 'Soda Can' sample processed for stain ID with RSID®-Saliva and for DNA-STR analysis.

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



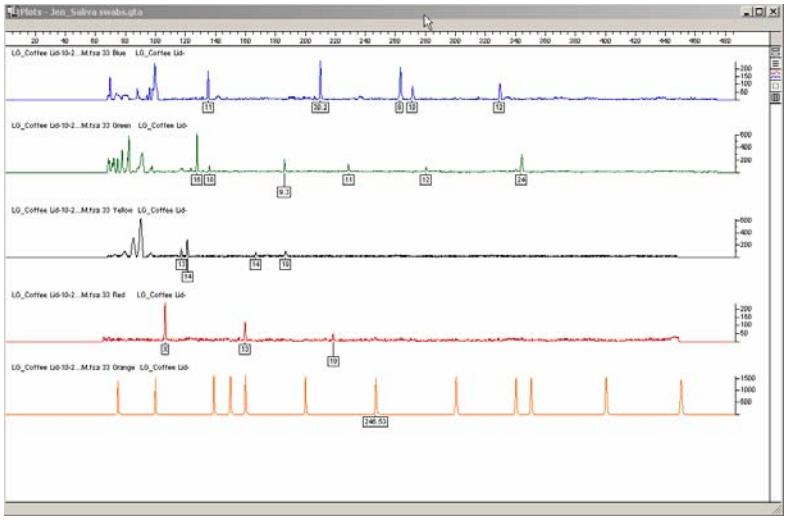
Identifiler STR profile of 'Soda Can (2)' sample processed for stain ID with RSID®-Saliva and for DNA-STR analysis.

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 PBS in a 0.6 ml microcentrifuge tube. 25 µl of each extract was tested with RSID®-Saliva. Positive control was an oral swab extracted in 1.0 ml of PBS, 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.

Sample	Score
(1) Neg	0
(2) Pos	8
(3) Env. upper flap	3
(4) Env. lower flap	5
(5) Plas Btl	6
(6) Gls. Btl	3
(7) oral Sw.	9
(8) Mt can	8

RSID®-Saliva, Additional Sample Testing

Additional Testing of RSID®-Saliva with Forensic-Type samples.

The performance of RSID®-Saliva on a variety of samples and materials was evaluated by testing for saliva from a water bottle, plastic mug, ceramic mug and a cigarette butt. Procedure: Sample exhibits were collected and swabbed with a moistened cotton swab. Swabs were air dried overnight and extracted in 200 µl of PBS for 1 hour. Finally, 25 µl each PBS extract was analyzed with RSID-Saliva. Positive (5 µl saliva extract) and negative (25 µl sham extract) controls were included for comparison. All extracts were brought up to 150* µl with diluent/running buffer. Cigarette butts A and C appeared to be freshly discarded while cigarette butt B appeared old and weathered. Cigarette butt C was extracted using an alternative method in which the paper surrounding the

filter was removed with a razor blade and extracted directly in 200 µl PBS (no swabbing was performed).

Strip	Sample	Extract/Buffer (µl)
1	Negative control	25/125*
2	Positive control	5/145*
3	Plastic bottle A	25/125*
4	Plastic mug	25/125*
5	Plastic bottle B	25/125*
6	Ceramic mug	25/125*
7	Cigarette butt A	25/125*
8	Cigarette butt B	25/125*
9	Cigarette butt C	25/125*

Test strips have been removed from cassettes for clarity

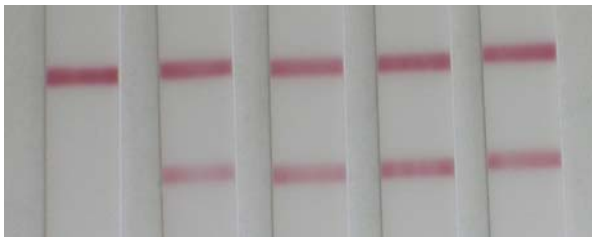
Results: Saliva was detected on all samples tested except for cigarette butt B.

Conclusions: RSID®-Saliva can detect saliva from a variety of samples, materials and surfaces, including envelopes, plastic, glass, metal cans, ceramic mug and cigarette butts.

RSID®-Saliva, Sensitivity to Extraction Method.

In order to determine if RSID®-Saliva detection is sensitive to the extraction method, positive control sterile cotton swabs (50µl of saliva extracted in 1.0 ml of PBS) were extracted in ddH_2O, TE, and TBS+ running buffer. Swabs were incubated in the indicated extraction solution for one hour and vortexed. Subsequently, 25 µl of the extraction was added to 125* µl TBS+ running buffer and applied to strips. A negative control (25 µl sham extract, extracted with PBS) was included for comparison.

Strip	Sample	Extraction Buffer
1	25 µl sham extract	PBS
2	25 µl saliva extract	PBS
3	25 µl saliva extract	Water
4	25 µl saliva extract	TE
5	25 µl saliva extract	TBS+



Test strips have been removed from cassettes for clarity

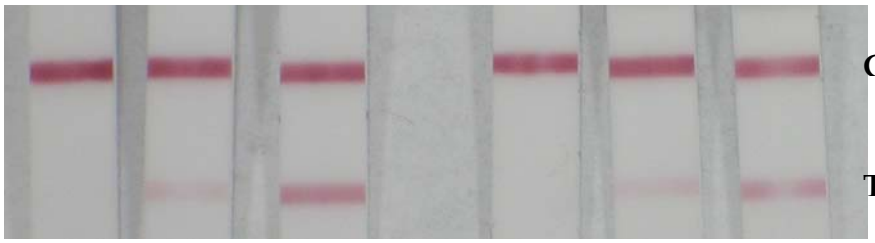
Results: RSID®-Saliva is insensitive to the extraction buffer used – a strong positive signal was obtained with PBS, TE, TBS^+ or ddH_2O as the extraction solution.

Conclusions: PBS, TBS^+, TE or ddH_2O are equally effective extraction buffers for RSID®-Saliva testing.

Stability of extracts in RSID®-Saliva diluent/running buffer.

Procedure: In order to determine if saliva extracts are stable in the diluent/running buffer used with RSID®-Saliva (provided TBS+ (pH 8.5)), extracts were prepared and incubated overnight in TBS+ (pH 8.5) and then used with RSID®-Saliva. Extracts prepared immediately prior to testing are included for comparison.

Strip	Sample	Incubation
1	25 µl sham extract	Overnight
2	5 µl saliva extract	Overnight
3	25 µl saliva extract	Overnight
4	25 µl sham extract	None
5	5 µl saliva extract	None
6	25 µl saliva extract	None



Results: No effect of overnight incubation of extracts was observed.

Conclusion: Extracts prepared in RSID®-Saliva diluent/running buffer are stable to overnight incubation.

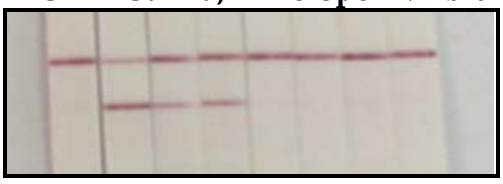
Casework Example: sample testing prior to DNA-STR analysis.

In the course of a forensic case, we received two envelopes (exhibits A and B) with a request for DNA-STR analysis. The case involved threatening letters received through the mail and therefore the identity of the sender was sought. Evidence in the case consisted of a series of envelopes and we therefore tested these exhibits for the presence of saliva before proceeding to DNA-STR processing. Pre-screening for saliva on these exhibits would greatly reduce the cost and effort of attempting DNA-STR analysis as samples that did not test positive for saliva, would fail to produce STR profiles and would be not be tested further.

Procedure: The upper and lower flaps of envelopes A and B were swabbed with a moistened sterile cotton swab and extracted in 400 µl PBS. A positive control envelope, sealed with saliva, was processed and analyzed in parallel. 25 µl of PBS extract was used with RSID®-Saliva.

Results: Positive controls, both oral swab and saliva sealed envelopes were clearly positive for saliva using RSID®-Saliva. Exhibits A and B tested negative: no human saliva detected and therefore DNA analysis was not attempted with these samples.

RSID®-Saliva, Envelope Exhibit



Test strips have been removed from cassettes for clarity

1 Buffer only
2 saliva extract
3 + control, upper
4 + control, lower
5 Envelope A, upper
6 Envelope A, lower
7 Envelope B, upper
8 Envelope B, lower

References Cited

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

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***Note: Tested volumes for RSID-Saliva were either 150 µl when strips were tested individually in test tubes *without* a cassette or 100 µl when strips were tested in a plastic housing.**

We do not recommend using 150 µl test volumes for cassette tests: the only approved final volume for cassettes is 100 µl.

Test Line Intensity Comparison Chart

Test Line: Intensity

Test Line: Score