BOCA RATON POLICE SERVICES DEPARTMENT
BIOLOGICAL PROCESSING LABORATORY

MEMORANDUM

TO: **Amy Flagler, FSM**

FROM: **Kristen Archuleta, BPL Analyst**
Cayleigh Shufelt, BPL Analyst
Caitlin Rogers, BPL Supervisor

DATE: **June 18, 2024**
Amended July 2, 2024 (updated Developmental Validation, Limitations, and References sections to reflect provided developmental validation report from Abacus Diagnostics)

SUBJECT: **Saliva Testing Internal Validation Report**

INTRODUCTION

The indication of saliva can yield important insights in forensic casework. There are several off-the-shelf products on the market for saliva testing. Most methods involve (or are believed to involve) the detection or action of salivary amylase. Human salivary amylase is an enzyme that is present in large quantities in human saliva. Its biological function is to aid in digestion by breaking down starches into sugars. Two commercially available products were evaluated for use at the Boca Raton Police Services Department (BRPD) Biological Processing Laboratory (BPL): SALIgAE® and RSID™-Saliva.

Abacus Diagnostics' SALIgAE® is a colorimetric test that yields a yellow color change in the presence of saliva and remains colorless if negative. The exact mechanism by which the SALIgAE® test works is proprietary; however, published studies indicate that the test detects salivary amylase. This is supported by the equation for calculating the concentration of salivary amylase in the Technical Information Sheet provided by the manufacturer for labs using a spectrophotometer to perform the SALIgAE® test quantitatively.

Independent Forensics' RSID™-Saliva tests for the presence of human salivary α-amylase, using an immunochromatographic assay. If human salivary amylase is present in the sample, it will bind to a mobile colloidal gold-labeled mouse monoclonal anti-α-amylase antibody in the sample well. The antigen-antibody complex will migrate across the test membrane by capillary action/bulk flow. The test (T) region contains immobilized mouse monoclonal anti-α-amylase antibody. As dye-labeled antigen-antibody complexes are captured by the immobilized anti-α-amylase antibody in the test region and accumulate, a line will form. If human salivary amylase is not present in the sample, the gold-conjugated antibody-antigen complexes are not formed and will not accumulate at the test line. Regardless of the development of a line in the test region, a red line should develop in the control (C) region of the test strip, demonstrating that the sample fluid traveled the length of the test strip and that the test strip is working properly.

OVERVIEW

Two saliva testing products were selected for evaluation for potential implementation at BPL: SALIgAE® and RSID™-Saliva. This internal validation study was designed and conducted to satisfy ANSI/ASB Standard 077 *Standard for the Developmental and Internal Validation of Forensic Serological Methods* to determine the most appropriate and cost-effective saliva test to be implemented at BPL for routine casework use. All currently employed analytical personnel at BPL were involved in sample preparation and the validation studies conducted as summarized herein.

PROCEDURE

SALIgAE® Overlay Testing Procedure

The manufacturer's Technical Information Sheet (Rev: 06/2019) provides an elution test protocol that requires the extraction of a portion of the sample in 50 µL water and the addition of the extract to the SALIgAE® test vial. The insert provides two alternative methods of testing, including mapping (referred to at BPL as "overlay"). Overlay is commonly employed at BPL for other test methods, such as phenolphthalein testing for blood and AP testing for seminal fluid; therefore, the overlay method suggested by the manufacturer was selected for validation purposes:

1. Place a few drops of deionized water onto a piece of filter paper.
2. Press the moistened filter paper against the stain or swab.
3. Using a disposable transfer pipette, apply sufficient SALIgAE® to the filter paper to cover the sample area.
4. Read the results at 10 minutes; alternatively, a positive result may be read within 10 minutes.
    a. Positive: yellow color change.
    b. Negative: no color change or a color change other than yellow.
    c. Inconclusive: uncertainty regarding the appearance of a yellow color change.

RSID™-Saliva Reduced Incubation Time Procedure

The manufacturer's Technical Information & Protocol Sheet (Rev. November 2016) provides a shortened incubation protocol in comparison to the originally published method. The reduced incubation time procedure as suggested by the manufacturer was selected for validation purposes:

1. For stains or swabs, place a small cutting into a microcentrifuge tube with 200µL RSID™-Universal Buffer.
2. Vortex for 10 seconds.
3. Take 20µL of the extract and it to 80µL of RSID™-Universal Buffer into new tube.
4. Vortex for 10 seconds.
5. Add the total 100µL to the sample well of the RSID™ card.
6. Read the results at 10 minutes; alternatively, a positive result may be read within 10 minutes.
    a. Positive: 2 lines (1 in the control region, 1 in the test region).
    b. Negative: 1 line in the control region.
    c. Invalid: No line in the control region. Test must be repeated.
    d. Inconclusive: 1 line in the control region, uncertainty about the presence or absence of a line in the test region.

DEVELOPMENTAL VALIDATION

Records of the published developmental validation studies were sought online. The RSID™-Saliva (Old et al., 2010) developmental validation was obtained online and reviewed. A validation study for SALIgAE® (Miller & Hodges, 2005) cited on the manufacturer's website was reviewed, but was found not to have been conducted by the manufacturer. The developmental validation was requested and provided by the manufacturer (Abacus Diagnostics, n.d.), but does not appear to have been previously published. The developmental validations predate the publication of the ANSI/ASB Standard 077 *Standard for the Developmental and Internal Validation of Forensic Serological Methods* and may not reflect all studies specified by Standard 077. Where possible, these elements were incorporated into the internal validation study. The developmental validation study for the selected test method, SALIgAE® will be retained at BPL, accessible to all laboratory personnel.

STUDY RESULTS

Control Study

Control studies were performed to establish the control samples needed for each procedure, the frequency with which the controls will be performed, and the performance expectations for each control. Both tests under evaluation are purchased and will be handled as a single "lot" from the manufacturer. The control study was repeated one week after the initial date of testing to ensure that there is consistency in test performance across a single manufacturer's lot. Positive controls were prepared by adding approximately 100 µL neat saliva to cotton swabs. Filter paper was used as the negative control for the for SALIgAE® overlay method while a cotton swab was used as the negative control for RSID™-Saliva.

Sample	SALIgAE®	RSID™-Saliva
Positive Control (Neat Saliva)	+	+
Negative Control (Filter Paper or Cotton Swab)	N	N*
Positive Control (Neat Saliva) – 1 week	+	+
Negative Control (Filter Paper or Cotton Swab) – 1 week	N	N

*Not confident in result until cassette was taken apart to evaluate test strip.

SALIgAE® and RSID™-Saliva showed no performance differences after one week when stored as recommended by the respective manufacturers. There was no performance difference across the lot detected during the control study.

Based on the results of the control study, positive and negative controls will be required to verify the lot's performance prior to implementation in casework. Positive and negative controls do not need to be repeated on the day of analysis once a lot has been verified.

Sensitivity Study

Sensitivity studies are performed to determine the upper and lower limits of a test to accurately detect the analyte of interest and are performed using serial dilutions. Since neat saliva was tested as the positive control and concentrated saliva samples are unlikely to be forensically relevant, the sensitivity study was performed to determine the lower limit of test performance (limit of detection). Dilutions

were prepared using saliva from a single donor using deionized water. Approximately 100 µL was applied to each swab and allowed to dry completely prior to testing. The study was performed by Scientist 1.

Sample	SALIgAE®	RSID™-Saliva
1:2 Saliva Dilution	+	+
1:5 Saliva Dilution	+	+
1:10 Saliva Dilution	+	+
1:50 Saliva Dilution	Very weak + / weak +*	+
1:100 Saliva Dilution	Very weak + / N*	+
1:500 Saliva Dilution	N	Inconclusive / N*
1:1000 Saliva Dilution	N	N

*Retested to clarify the limit of detection.

SALIgAE® could reproducibly detect 1:50 dilutions of saliva. Results were variable or negative at greater dilutions; therefore, 1:50 dilution of saliva will be considered the limit of detection for the SALIgAE® overlay method. RSID™-Saliva could reliably detect 1:100 dilutions of saliva. Results were variable or negative at greater dilutions of saliva; therefore, 1:100 dilution of saliva will be considered the limit of detection for the for RSID™-Saliva reduced incubation procedure. These results indicate that RSID™-Saliva is more sensitive than SALIgAE®.

Repeatability Study

Repeatability studies were performed to verify the results of the test by the same personnel. The scientist who performed the sensitivity study (Scientist 1) performed two additional tests of the 1:10 saliva dilution to assess the repeatability of their results. Additional swabs prepared concurrently with those used in the sensitivity study (same donor and prepared dilution solutions) were used for the repeatability study.

Sample	SALIgAE®	RSID™-Saliva
1:10 Saliva Dilution (Test 1, from Sensitivity Study)	+	+
1:10 Saliva Dilution (Test 2)	+	+
1:10 Saliva Dilution (Test 3)	+	+

The repeatability study demonstrated that the test results were repeatable when a sample less concentrated than the established limit of detection was retested under the same testing conditions.

Reproducibility Study

Reproducibility studies are performed to assess the ability to obtain the same test results when an experiment is repeated between different operators. A second scientist (Scientist 2) repeated a subset of the samples from the sensitivity study and their results were compared to the results obtained by Scientist 1. Additional swabs prepared concurrently with those used in the sensitivity study (same donor and prepared dilution solutions) were used for the reproducibility study.

Sample	SALIgAE®	RSID™-Saliva
1:5 Saliva Dilution (Scientist 2)	+	+
1:10 Saliva Dilution (Scientist 2)	+	+
1:50 Saliva Dilution (Scientist 2)	N / N*	+
1:100 Saliva Dilution (Scientist 2)	N / N*	+
1:500 Saliva Dilution (Scientist 2)	N	N / N*
1:1000 Saliva Dilution (Scientist 2)	N	N

*Tested in duplicate to mimic sensitivity study.

The reproducibility study indicated that two examiners testing similar samples under the same test conditions produce consistent results above the limit of detection. At the limit of detection, there was some variability noted (e.g. 1:50 for SALIgAE®), which is not unexpected.

Contamination Study

Contamination studies are performed to assess the risk that unintended material may be introduced into a sample from test components, instrumentation, the operator, or test procedures. No instrumentation is required for the performance of SALIgAE® or RSID™-Saliva. Positive and negative control samples were completed side-by-side for the control study on the first day of testing and when repeated one week later. No cross-contamination was observed from test components or the operator following the test procedures in the laboratory environment.

BPL assessed the risk of unintended material introduced into the sample prior to submission of evidence to the laboratory to see if sample contaminants or substrates may interfere with either test. Samples were prepared as follows:

- Cleaning solutions (e.g. reagent alcohol, bleach): approximately 100 µL applied to cotton swabs.
- Cleaning solutions applied to neat saliva: approximately 100 µL saliva applied to cotton swabs, dried, and subsequently saturated in approximately 100 µL solution.
- Saliva on substrates (e.g. fabric, dirt/soil): donor spit on substrate.
- Consumption samples: approximately 100 µL of saliva applied to cotton swabs.

Sample	SALIgAE®	RSID™-Saliva
Reagent Alcohol	N	N
Reagent Alcohol Applied to Neat Saliva	+	+
Bleach	N	N
Bleach Applied to Neat Saliva	N	Inconclusive
Hydrogen Peroxide	N	N
Hydrogen Peroxide Applied to Neat Saliva	+	+
Windex	N	Inconclusive
Windex Applied to Neat Saliva	+	N
Neat Saliva on Denim	+	+
Neat Saliva on Yellow Fabric	+	+

Neat Saliva on Pink or Red Fabric	+	+
Neat Saliva in Dirt/Soil	+	+
Saliva Collected Immediately After Soda Consumption	+	+
Saliva Collected 1 Hour After Soda Consumption	+	+
Saliva Collected Immediately After Coffee Consumption	+	+
Saliva Collected 1 Hour After Coffee Consumption	+	+
Saliva Collected Immediately After Alcohol Consumption*	+	+
Saliva Collected 1 Hour After Alcohol Consumption*	+	+

*Samples were collected voluntarily on a non-working day.

Commonly encountered cleaning products and first aid supplies (alcohol, bleach, hydrogen peroxide, and Windex) did not cause false positive results with either test method. When applied to neat saliva, alcohol and hydrogen peroxide did not interfere with the ability to detect saliva using either method. Bleach applied to saliva interfered with the ability to detect saliva using SALIgAE® (false negative) and RSID™-Saliva (inconclusive). Windex applied to saliva interfered with the ability to detect saliva using RSID™-Saliva (false negative), but did not interfere with the ability to detect saliva using SALIgAE®.

Saliva was detectable using both methods when deposited on various fabric substrates and colors. Saliva was also detectable using both methods when recovered from dirt/soil.

Donors consumed various beverages prior to sample donation to see if contamination of the oral cavity impacted the ability of either test to detect saliva. Samples collected from donors immediately after and approximately 1 hour after consuming soda, coffee, and alcohol still tested positive with both tests, indicating no impact to test performance.

Specificity Study

Specificity studies are performed to assess the ability of the system to provide reliable results for targeted analytes in the presence of cross-reactive substances. Our specificity study assessed the possible cross-reactivity of forensically relevant human body fluids as well as saliva from regularly encountered household pets. Samples were prepared as follows:

- Vaginal and nasal secretions: saturated (approximately 100 µL) swab.
- Menstrual blood: transferred fluid from used tampon to dry cotton swab.
- Fecal: dry swabs used to swab toilet paper after use.
- Non-human saliva: swabs used to swab used water bowls.
- Perspiration: dry swabs used to swab sweat on skin.
- All other fluids: approximately 100 µL applied to each swab.

Sample	SALIgAE®	RSID™-Saliva
Semen	N	N
Blood	N	N
Urine	N	N

Perspiration	N	N
Fecal	+	+
Breast Milk	+	+
Vaginal Secretions	N	N
Menstrual Blood	N	N
Nasal Secretions	Weak +	N
Dog saliva	N	Inconclusive
Cat saliva	N	Inconclusive

Fecal material and breast milk demonstrated cross-reactivity with both tests. Nasal secretions resulted in a cross reaction using the SALIgAE® test, but not with RSID™-Saliva, indicating that it may be more specific. Dog and cat saliva did not cross-react with SALIgAE®; however, there was uncertainty regarding the presence or absence of a test line when these were tested with RSID™-Saliva (inconclusive).

Mixture Study

Mixture studies are performed to assess the performance of the test method when samples containing mixtures of similar or different body fluids are tested. Samples were prepared as follows:

- Saliva/vaginal secretions: a previously prepared vaginal swab (see specificity study) was saturated with approximately 100 µL saliva.
- Saliva/menstrual blood: previously prepared menstrual blood swab (see specificity study) was saturated with approximately 100 µL saliva.
- Saliva/fecal: previously prepared fecal swab (see specificity study) was saturated with approximately 100 µL saliva.
- Saliva/urine: previously prepared urine swab (see specificity study) was saturated with approximately 100 µL saliva.
- All other mixtures: prepared in a 50/50 ratio and approximately 100 µL of the mixture was applied to each swab.

Saliva/perspiration samples were not tested in the mixture study, as multiple saliva on skin samples were tested in the mock casework study.

Sample	SALIgAE®	RSID™-Saliva
Saliva/Semen	+	+
1:10 Saliva Dilution/Semen	+	+
Saliva/Blood	+	+
1:10 Saliva Dilution/Blood	+	+
Saliva/Fecal	+	+
Saliva/Urine	+	+
Saliva/Vaginal Secretions	+	+
Saliva/Menstrual Blood	+	+

When mixed with other body fluids, SALIgAE® and RSID™-Saliva were able to detect saliva. Further, dilute saliva mixed with blood and semen was still detectable. This indicates that other human body fluids do not interfere with the ability to detect saliva using either test method.

Mock Casework Study

Mock casework studies are performed on samples that mimic or simulate a range of casework sample types. This may include laboratory-created or proficiency test samples. BPL does not plan to test items that can reasonably be expected to contain saliva (e.g. mouth of bottle/can, cigarette); therefore, these items were not directly evaluated. Saliva deposited on various fabrics, but not laundered or exposed to heat or the environment, were previously tested in the contamination study.

Samples were prepared as follows:

- Saliva on fabric (laundered): donor spit on a towel. The sample was allowed to dry, and subjected to a wash and dry cycle in residential laundry machines.
- Exposure samples were prepared by depositing approximately 100 µL neat saliva onto cotton swabs.
    - The heat/sun exposure sample was placed on the dashboard of a vehicle on a summer day in Boca Raton, FL and left for 24 hours.
    - The environmental exposure sample was affixed to a tree on a non-rainy summer day in Boca Raton, FL and left for 24 hours.
- Simulated sexual assault kit samples were prepared by licking, biting, or kissing an area of skin, allowing the area to dry, and using two moistened cotton swabs to swab the affected area.

Sample	SALIgAE®	RSID™-Saliva
Neat Saliva on Fabric (Laundered Post Deposition/Drying)	N	N
Neat Saliva Left in Car for 24 Hours (Heat/Sun Exposure)	+	+
Neat Saliva Left Outside for 24 Hours (Environmental Exposure)	+	+
Swabs of Licked Skin	+	+
Swabs of Bitemark on Skin	+	+
Swabs of Kissed Area on Skin	+	+

SALIgAE® and RSID™-Saliva could detect saliva when exposed to heat and sun as well as to the elements. Saliva could not be detected using either method when it was washed (hot cycle with Tide laundry detergent) and dried (with a laundry sheet) in home laundry machines. Both tests could detect saliva on licked, kissed, and bitten skin.

PRODUCT IMPRESSIONS

SALIgAE®

The SALIgAE tests are provided from the manufacturer in boxes of 10 vials each that must be stored refrigerated. The product was shipped by the vendor in a cooler with ice packs. The proximal packaging is clearly marked with the manufacturer's lot number and expiration date. The interior packaging (each

vial) is marked with a blank label that can be used to record the date opened, if the contents of the vial are not fully used.

The overlay method was quick and easy to perform and allows for a representative sample of the swab, stain, or substance of interest. Results from dilute samples can be difficult to read due to the pale yellow color that was developed. Supplemental white light (e.g. flashlight) may aid in reading results.

RSID™-Saliva

The RSID™-Saliva tests were provided from the manufacturer in boxes of 25 test cassettes, each with one bottle of RSID™ Universal buffer. The test cassettes are marked with a manufacturer lot number and expiration date and must be retained at room temperature. The buffer is marked with a different manufacturer lot number and expiration date and must be retained refrigerated. However, the product was shipped from the vendor without refrigeration (i.e. no cooler or ice packs). Disposable transfer pipettes and microcentrifuge tubes are not included with the test kits.

The reduced incubation time procedure was relatively quick and easy to conduct, but required four pipetting steps, two sample tubes, and two vortexing steps for each sample. All analysts involved in the BPL internal validation experienced hesitancy recording results from the test at various times during the validation. The test cassettes appear to have deep sample wells which resulted in possible "shadow bands" in the test area, making it difficult to read results. Multiple samples were recorded as inconclusive during the validation study (consensus between analysts) due to the uncertainty reading the test results. Analysts attempted to deconstruct the test cassettes to better visualize the test strips; however, they are very difficult to disassemble to do so.

SAFETY/DISPOSAL CONSIDERATIONS

SALIgAE® and RSID™-Saliva do not require additional personal protective equipment or laboratory safety equipment beyond what is routinely used at BPL.

Refer to the SDS for SALIgAE® and RSID™ Universal Buffer for disposal considerations.

COST ANALYSIS

The cost per test was evaluated for SALIgAE® and RSID™-Saliva.

Sample	SALIgAE®	RSID™
Cost Per Package	$86	$151
Number of Tests Per Package	10 vials	25 cassettes
Cost Per Test	$8.60 vial / $2.87 overlay test	$6.04

SALIgAE® costs $2.54 more per test vial than RSID™-Saliva does per cassette test; however, approximately 3 overlay tests could be performed using each test vial. Therefore, SALIgAE® costs $3.17 less per test when performed using the overlay method as validated.

LIMITATIONS

SALIgAE®

- A negative result may be observed if saliva is present at a level below the detection ability of the test.
    - The demonstrated limit of detection using the SALIgAE® overlay method was a 1:50 saliva dilution.
    - Miller & Hodges (2005) demonstrated that the SALIgAE® test yielded positive results for saliva dilutions up to 1:1000 using the elution method, but results were difficult to read in dilutions past 1:400. Abacus Diagnostics (n.d.) demonstrated consistent positive results up to a 1:500 dilution of saliva using the elution method and variable results at greater dilutions. Lim et al. (2007) demonstrated a detection limit of 1:600 saliva dilution using a modified immersion method.
- Cross-reactivity was observed with human breast milk, fecal material, and nasal secretions.
- Exposure to bleach and/or laundering may diminish the ability to detect saliva with SALIgAE®.
- SALIgAE® is a presumptive test method for human saliva.

RSID™-Saliva

- A negative result may be observed if saliva is present at a level below the detection ability of the test.
    - The demonstrated limit of detection using the RSID™-Saliva reduced incubation time procedure was a 1:100 saliva dilution.
    - The Independent Forensics developmental validation indicated the limit of detection is ~50 nL (0.05 µL) saliva.
- Cross-reactivity was observed with human breast milk and fecal material.
- Exposure to bleach, Windex, and/or laundering may diminish the ability to detect saliva with RSID™-Saliva.
- RSID™-Saliva is a presumptive test method for human saliva.

SUMMARY

This validation study demonstrated the quality and robustness of the results obtained using the tests evaluated and the limitations of each method. Based on the totality of the results obtained, analyst comfort with reading results, the SALIgAE® overlay method was selected for saliva testing in casework at BPL.

COMPETENCY

All currently employed analytical employees at BPL were directly involved in sample preparation and validation testing under observation using each test method evaluated. Therefore, no additional competency testing will be required. All BPL employees will complete a supplementary saliva training module, consisting of reading and answering questions prior to being authorized to conduct saliva testing in forensic casework. Once authorized to conduct saliva testing, employee proficiency will be regularly evaluated through the proficiency testing program. This training module will be incorporated into the BPL training program for future new employees.

REFERENCES

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APPROVAL

Respectfully submitted for approval,

Kristen V. Archuleta — Jul 2, 2024

Kristen Archuleta, Biological Processing Laboratory Analyst — Date

Cayleigh Shufelt — Jul 2, 2024

Cayleigh Shufelt, Biological Processing Laboratory Analyst — Date

Caitlin Rogers — 07/02/24

Caitlin Rogers, Biological Processing Laboratory Supervisor — Date

Amy Flagler — Jul 2, 2024

Amy Flagler (Jul 2, 2024 15:04 EDT)

Amy Flagler, Forensic Services Manager — Date

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