



## **Saliva Collection and Handling Advice**

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## Introduction

Researchers are increasingly turning to saliva testing because it allows samples to be collected in a convenient, non-invasive manner, and on a repeated basis. Anyone planning to use saliva testing must be aware, however, that it is important to follow proper collection and handling procedures in order to insure that the highest quality data is obtained. We offer the following advice based on our extensive experience with saliva collection and testing.

Knowledge about saliva testing is rapidly growing and being revised, however, and there are still areas where knowledge is limited, or where differences of opinion have not been resolved. We try to assist our customers by providing them with up-to-date advice, but ultimately it is each customer's responsibility to make decisions about the best collection methods to use. We advise consulting the literature on the analytes to be measured, and when the available literature is thin we strongly recommend a pilot study.

For an additional discussion of this important topic, please visit the article *Saliva Collection Advice* on our website ([www.salimetrics.com](http://www.salimetrics.com)), under *All Things Saliva*.

## Preliminary Considerations

### Variability of Salivary Analytes

Levels of many analytes in saliva do not remain static. Many steroid hormones are released into the bloodstream in short bursts or pulses.(1) Because most steroids diffuse easily into saliva these variations are also reflected relatively quickly in salivary concentrations. Salivary levels of some steroids, and other analytes like  $\alpha$ -amylase, also vary according to regular diurnal cycles.(2,3) Additionally, levels of some salivary analytes are affected by stress or other stimuli.(4,5) It is therefore important to understand the underlying physiology so as to be able to develop the best strategies for sample collection. Several factors may be of importance depending on the analyte of interest and the nature of the study:

- The diurnal cycle of the analyte must be understood. In most cases, sample collection should be made at standardized times.
- The response and recovery characteristics of each analyte should be understood so that sample collections are timed to properly capture responses.
- For analytes with pulsatile behavior we recommend collecting a minimum of three samples over a two hour period. Equal volumes from each of the samples should be pooled to create one sample that physically averages the fluctuations over that time period.
- Studies have shown that concentrations of SIgA and DHEA-S are affected by saliva flow rates.(6,7) Ongoing research may eventually find other analytes that are also affected. For these analytes it is necessary to record the saliva flow rate in order to express the assay results as a function of time.

## **Effect of Mouth Location on Salivary Analyte Content**

Saliva is not a simple fluid with a consistent composition. Each of the major and minor salivary glands has a different cellular makeup, and consequently there can be some differences in the composition of saliva produced in each location. Levels of the steroid hormones measured by our kits are not affected by the type of saliva collected, but a few analytes, such as SIgA and  $\alpha$ -amylase, do show differences from one gland to another.(8,9) For these analytes, researchers must be aware of the potential that absorbent devices have to collect specific glandular saliva rather than whole, or mixed, saliva. Consistent placement of the absorbent devices is important, and we offer recommendations below.

## **Research Participant Preparation and Documentation**

In order to avoid the possibility of contaminating substances in the saliva that could interfere with the immunoassay, we recommend the following precautions for research participants who will be donating saliva:

- Avoid alcohol for 12 hours before sample collection.
- Do not eat a major meal within 60 minutes of sample collection.
- Avoid dairy products for 20 minutes before sample collection.
- Avoid foods with high sugar or acidity, or high caffeine content, immediately before sample collection, since they may compromise the assay by lowering saliva pH and increasing bacterial growth.(12,13)
- Rinse mouth with water to remove food residue before sample collection, and swallow to increase hydration.
- Wait at least 10 minutes after rinsing before collecting saliva to avoid sample dilution.
- Document consumption of alcohol, caffeine, nicotine, and prescription and over-the-counter medications. (14-16)
- It is also advisable to document the physical activity level of research participants and the presence of oral diseases. (17,18)

## **Sample Volume and Salivary Stimulants**

Modern immunoassays are generally designed to work with small sample volumes, and in most cases stimulants should not be required to collect adequate sample. We recommend against the use of oral stimulants when collecting saliva samples, due to the possibility of causing assay interference or alteration of levels of some analytes.(10) If stimulants are absolutely necessary they must be used sparingly and in a consistent manner throughout the study.(11) Customers are encouraged to contact us concerning the necessary sample volume based on the number and type of assays to be performed prior to sample collection.

## **Blood Contamination in Saliva**

Contamination of saliva samples with blood can also be a concern because the levels of most analytes are higher in the general circulation than in saliva. Blood can leak into saliva under certain conditions,

including poor oral health, abrasive brushing, or injury, and *even an invisible amount of blood contamination has the potential to falsely elevate salivary analyte levels.* (19-21) We recommend the following:

- Research participants should not brush their teeth within 45 minutes prior to sample collection.
- Dental work should not be performed within 48 hours prior to sample collection.
- Research participants should be screened for oral health problems or injuries.
- Saliva samples visibly contaminated with blood should be discarded and recollected.
- Samples collected from populations that have little or no dental care, or known oral health problems, may be screened with our Blood Contamination Assay Kit (Salimetrics Item No. 1-1302; 1-1302-5).

### **Saliva Pipetting Advice**

Saliva contains mucus, which can make accurate pipetting difficult. Salimetrics advises freezing all saliva samples once before performing the assay, followed by vortexing after the sample is thawed. This procedure helps break up the mucus, and it can then be centrifuged into the bottom of the tube. Any other cellular debris or food particles that were present are also removed in this step. Remove the sample for testing from the clear solution, avoiding the pellet in the bottom of the tube. Due to the viscosity of saliva, greater accuracy in sample volume is obtained by aspirating slowly, so as to avoid the formation of bubbles.

## **Collection Methods and Devices: Adults and Older Children**

The selection of a collection method and device will depend on the analyte(s) of interest and the age of the research participants. The following options are available:

### **Passive Drool**

A very cost-effective method often used by our customers is the collection of whole saliva by passive drool into a small vial. *Passive drool is highly recommended because it is approved for use with almost all analytes, unlike absorbent devices, which can sometimes cause interference in immunoassays.* It is important to use high-quality polypropylene vials, since other vials can lead to problems with analyte retention or the introduction of contaminants that can interfere with the immunoassay. The vials used must also seal tightly and be able to withstand temperatures as low as -80°C. We sell 2 ml cryovials that meet these requirements (Salimetrics Item No. 5002.01).

### **Materials required**

Plastic drinking straws; Scissors; Cryovials (polypropylene, 2 mL capacity); Labels

### **Prior to Saliva Collection**

1. Have research participants rinse their mouth with water 10 minutes prior to collection. Consult the Research Participant Preparation and Documentation section above for additional advice.
2. Cut plastic drinking straws into 2-inch (5 cm) pieces.
3. Give each research participant one straw piece and one cryovial.

### **Instructions for Collecting Saliva**

1. Instruct research participants to allow saliva to pool in the mouth. Some find it helpful to imagine eating their favorite food.
2. With head tilted forward, research participants should drool down the straw and collect saliva in the cryovial. (It is normal for saliva to foam, so we advise using a vial with twice the capacity of the desired sample volume.)
3. Repeat as often as necessary until sufficient sample is collected. One mL (excluding foam) is adequate for most tests. Collection of samples to be analyzed for more than one analyte may require larger vials.

*Note: Secretory IgA and DHEA-S concentrations in saliva are affected by saliva flow rates. We recommend recording the amount of time necessary to collect a given volume of saliva so as to express the analyte measured as a function of time. Contact Salimetrics for details.*

4. Keep samples cold after collection (4°C) and freeze (-20° to -80°C) as soon as possible.

*Note: Freeze-thaw cycles should be minimized for some analytes. Contact Salimetrics for further details.*

### **The Salimetrics Oral Swab (SOS)**

Some research participants are not willing or able to drool saliva into a vial. If the saliva samples are to be analyzed for cortisol, testosterone,  $\alpha$ -amylase, cotinine, C-reactive protein, or SIgA, the Salimetrics Oral Swab (SOS) (Item No. 5001.02) is an excellent alternative to passive drool because of its ease of use. The SOS is made of a non-toxic, inert polymer shaped into a 30 x 10 mm cylinder. It is not recommended for children under the age of six, however, due to the possibility of a choking hazard.

*When saliva is collected by placing the SOS underneath the tongue on the floor of the mouth, we find that assay results are similar to those from whole saliva collected by passive drool. Under certain conditions, however, there is a possibility that the SOS might collect specific glandular saliva, rather than whole saliva. This could affect assay results for analytes such as  $\alpha$ -amylase, CRP, and SIgA. Researchers should be aware of this potential and decide on their collection strategy accordingly. The SOS may also be used intentionally to collect samples from the parotid duct openings in the cheeks, as directed below.*

The SOS should be ordered with a Swab Storage Tube (Item No. 5001.01), which consists of a capped, conical centrifuge tube with a separate insert that snaps into place inside the tube. The insert has a small hole in its bottom, which allows the saliva to be centrifuged out of the swab into the bottom of the conical tube. If centrifugation is not available, saliva from the swab may be expressed into a cryovial (Item No. 5002.01) using a needle-less 5 cc plastic syringe.

### Instructions for Use

*Note: We recommend placing the SOS into the tube insert shortly before distribution to the research participants.*

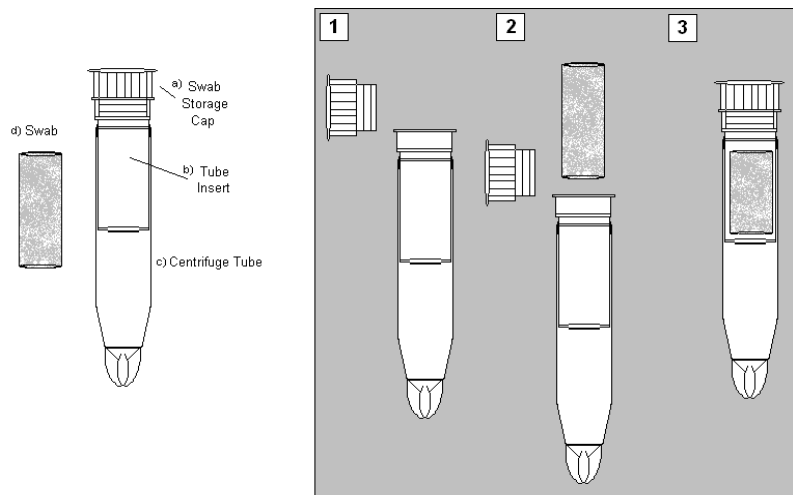
1. Remove SOS from tube leaving tube insert in place. Place into mouth as directed below. Keep in place for 1-2 minutes. (If collecting from the parotid glands in the cheek saliva flow will be lower, and collection time should be extended for up to 5 minutes to ensure adequate volume.)

| Recommended SOS Placement                |   |
|--|---|
| cortisol, cotinine, testosterone         | Under tongue                                      |
| $\alpha$ -amylase* (with other analytes) | Under tongue                                      |
| $\alpha$ -amylase* (alone)               | Between cheek and gum (near upper 2nd molar)      |
| SIgA†, CRP†                              | Placement may vary depending on focus of research |

\* Saliva from the parotid glands has higher concentrations of  $\alpha$ -amylase than pooled whole saliva from under the tongue

†Concentrations may vary depending on location in the mouth

2. Return SOS into tube insert.
3. Replace cap and snap securely onto tube.



4. Label the exterior of the tube using computer-generated, bar-coded labels provided by Salimetrics, or waterproof pen. Position label so that the barcode lies horizontally along the length of the swab storage tube.

*Note: Use labels recommended for freezing (cryolabels), not ordinary paper labels, which will fall off.*



5. If samples cannot be frozen immediately, refrigerate or keep cool using insulated container with ice packs. If swab storage box is used, place tubes in storage box cap side up.

6. We recommend freezing samples at or below  $-20^{\circ}\text{C}$  within 1-2 hours of collection.

*Note: Freeze-thaw cycles should be minimized for some analytes. Contact Salimetrics for further details.*

7. On the day samples are to be assayed, bring them to room temperature and then centrifuge for 15 minutes at approximately 3,000 RPM (1500 x g). After centrifugation the tube insert and swab may be discarded, but keep the cap. Assays should be performed using only clear saliva, avoiding any sediment that may have accumulated.

8. Re-centrifuge tubes following each freeze-thaw cycle since additional precipitates may develop upon refreezing.

9. Store unused swabs in the closed zip-lock bag under dry conditions (30-60% humidity) at room temperature.

#### **Cautions:**

- *Do not use the SOS for children under the age of 6.*
- *Investigators using saliva samples collected with the SOS for biomarkers not approved by Salimetrics do so at their own risk.*
- *Consult the section on Research Participant Preparation and Documentation above, and contact us with any questions.*
- *The SOS may cause temporary dryness of mucosal membranes or oral cavity.*
- *Use only as directed.*

*Note: The SOS is made from an inert material that should theoretically pose no problem to specimens stored frozen in the device. Studies of long-term storage at temperatures of  $-20$  degrees C or colder have shown no change in analyte stability over a period of two years. Nevertheless, before storage for periods longer than two years we recommend that the specimen be removed from the SOS by centrifugation or compression.*

## Collection Methods and Devices: Infants and Small Children

Collecting saliva from infants and children under the age of six requires special consideration due to the potential for choking when collection devices are placed in the mouth. A number of collection methods listed below have been successfully employed, but it should be noted that these techniques are not appropriate for use with all analytes.

### Sorbettes

The Sorbette (Salimetrics Item No. 5029.00) is a small, arrowhead-shaped absorbent device attached to a plastic shaft. We have approved it for collection of samples to be assayed for cortisol,  $\alpha$ -amylase, cotinine, and SIgA.

*Note: SIgA levels are influenced by the saliva flow rate, which may be difficult to estimate when collecting with this device.*

The Sorbette is especially recommended when collecting samples from infants,(22,23) and it may also be of use for older children under the age of six, or for bed-ridden elderly research participants. Sorbettes are used in conjunction with conical tubes (Salimetrics Item No. 5001.04).

### Instructions for Use

Supplies Needed: Sorbettes, conical tubes, sample ID labels.

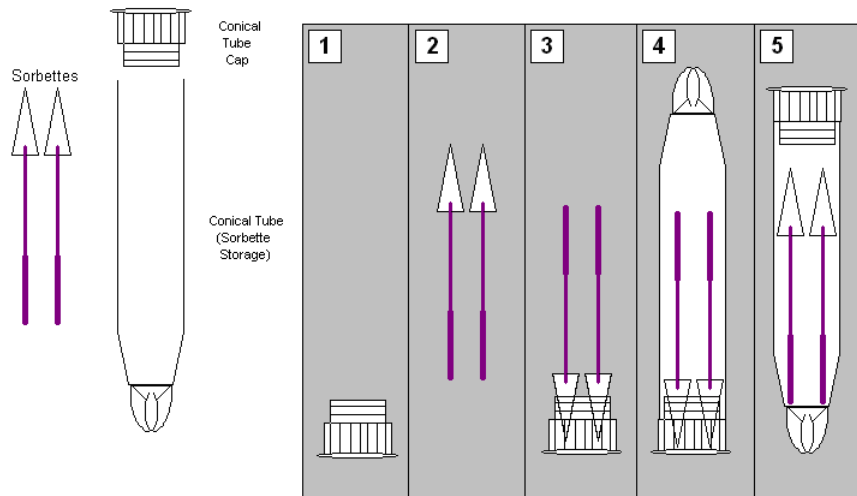
1. Remove one Sorbette from envelope. Close envelope immediately to protect the remaining Sorbettes from contact with moisture.
2. Put Sorbette ***under the tongue*** and allow it to absorb the whole saliva that pools there. Do not stop collecting when the Sorbette begins to puff up. Leave it in place for at least 60 seconds, or preferably for 90 seconds to ensure the device is saturated.

*Note: Due to its small size, the Sorbette has the potential to collect localized secretions from specific areas of the mouth, which could affect results for analytes such as SIgA and alpha-amylase. We therefore generally recommend that the Sorbette not be moved around in the mouth.*

3. Under ideal circumstances, collection for 60-90 seconds yields approximately 200-300 uL of saliva. If a greater volume is needed for testing, two Sorbettes may be held together by their shafts at the same time. If taping the shafts together is necessary, be advised that the tape residue could ***potentially*** affect assay results. We advise using a low-tack, removable tape. A better procedure would be to cut off the contaminated portion of each shaft before placing the Sorbettes in the conical tube (see diagram below).
4. Sample collection from infants: The Sorbette can be placed ***under the tongue*** for 15-30 seconds at a time and re-introduced as needed until the Sorbette is saturated (at least 60-90 seconds total time).

5. Place Sorbettes into the conical tube cap with the tips facing the cap end of the tube, as seen in the following diagram. *The Sorbette position is critical for recovering absorbed saliva during centrifugation.*

### SORBETTE SAMPLE PREPARATION



- 1) Place cap on a flat surface as shown.
- 2) Saturate one to three Sorbettes per research participant.
- 3) Insert Sorbettes *tip down* into cap.
- 4) Slide conical storage tube over purple sticks and snap down securely onto cap.
- 5) If conical tube storage box is used, place tubes in box with cap side up.

6. Label the exterior of the tube using computer-generated, bar-coded labels provided by Salimetrics, or waterproof pen. Position label so that the barcode lies horizontally along the length of the swab storage tube.

*Note: Use labels recommended for freezing (cryolabels), not ordinary paper labels, which will fall off.*



7. If processing samples in-house, centrifuge the conical tube or cryovial for 15 minutes at 3000-3500 rpm to extract the saliva. Remove the Sorbette with tweezers, then recap and proceed with testing.

## Notes:

- It is crucial that the saliva collected via Sorbette does not evaporate. Make sure the cap of the tube is snug and sample is frozen **within two hours** after collection.

*Note: Freeze-thaw cycles should be minimized for some analytes. Contact Salimetrics for further details.*

- It is best to centrifuge the saliva out of the Sorbette **before** freezing to lessen the chance of evaporation.
- Sorbettes are currently offered for research use only.
- Data from Sorbettes stored for 48 hours at -60°C, 4°C, and at room temperature show no statistically significant differences in mean or CV (expressed as a proportion) from samples collected with a cotton collection device.
- We discourage the Sorbette collection method for adults unless passive drool options prove impractical (e.g., dementia patients).

## Cotton rope

Cotton in various forms has frequently been used for saliva collection. Unfortunately, its use may be problematic due to an unpleasant taste, the difficulty of recovering the sample and/or analyte from the cotton, and the fact that it causes interference with certain biomarkers, including testosterone, SIgA, estradiol, DHEA, and progesterone.(23,24) Still, for cortisol,  $\alpha$ -amylase, or cotinine testing with infants, 3/8" (0.95 cm) diameter braided cotton rope (Salimetrics Item No. 5016.00) is an option.

*Note: the cotton must be thoroughly saturated for accurate results.(23)*

## Instructions

1. Cut rope into 6" (15 cm ) lengths.
2. Hold one end of the rope firmly and place the other end into the infant's mouth. Let the cotton rope absorb saliva for approximately two or more minutes. Remove the rope and cut off the dry portion of the rope. Place the wet portion into a needle-less 5cc syringe and squeeze the saliva into a cryovial.
3. Alternatively, you may place the wet cotton into the Saliva Storage Tube (Item No. 5001.01) normally used for the SOS swab and centrifuge the saliva out of the cotton.

*Note: Freeze-thaw cycles should be minimized for some analytes. Contact Salimetrics for further details.*

## Collection Methods and Devices: Non-Human Species

Analysis of hormones and other biomarkers in saliva is increasingly being used in an effort to monitor the health and well-being of animals kept in confined conditions such as farms and zoos, or as household pets. Salimetrics has provided EIA kits or testing services for studies that measure salivary cortisol in a wide range of animals. These studies most often use cotton swabs or ropes to collect the saliva. Some species, such as deer(25) and Guinea pigs,(26) have allowed plain cotton devices to be placed in their mouths. Studies involving primates have often enticed the animals to lick or chew cotton pads or ropes (attached to poles) by treating them with drink crystals, sugar solutions, or other flavorings.(27,28). Highly trained police dogs have allowed their handlers to collect saliva with plain cotton swabs,(29) but normal pet dogs have sometimes been encouraged to chew on cotton ropes by treating them with beef flavor. One recent study using pet dogs examines the effects that such flavorings, or the use of citric acid to stimulate saliva flow, may have on immunoassay results.(30)

A few papers have also experimented with other saliva collection devices for use with animals. The study of pet dogs already cited reports that hydrocellulose microsponges (Sorbettes) appear to be useful, offering a possible alternative to cotton. A study on CRP in pig saliva has reported that the swine readily chew on larger sponges attached to poles. (31) Saliva has even been collected from large and dangerous animals such as the rhinoceros by using a plastic spoon to scoop up several milliliters at a time from the lower lip.(32)

The literature also contains numerous descriptions of techniques for saliva collection from mice and rats. These include capillary tubes, filter paper strips, plastic pipettes, and more sophisticated suction devices. These techniques are often used in conjunction with anesthesia, and they frequently use pilocarpine or other chemicals to stimulate saliva flow. Salimetrics does not have direct experience with such methods and cannot advise on their use.

## Sample Handling and Storage

We encourage researchers to refrigerate or freeze samples as soon as possible after collection. Many analytes are not stable at room temperature, and keeping samples cold after collection is important. When samples remain at room temperature for periods of time longer than a few hours there is also opportunity for bacterial growth, which can compromise assay validity.(33) We advocate a conservative approach and advise that *all* samples should be maintained at 4°C for no longer than several hours before freezing them at or below -20°C (temperature of a regular household freezer). However, freeze-thaw cycles should be minimized *for some analytes*. It is critical that storage conditions are researched prior to initiation of sample collection. Contact Salimetrics for details.

On the day samples are to be assayed, bring them to room temperature, vortex, and then centrifuge for 15 minutes at approximately 3,000 RPM (1500 x g). Assays should be performed using only clear saliva, avoiding any sediment present in the bottom of the tube. Re-centrifuge tubes following each freeze-thaw cycle as additional precipitates may develop upon refreezing.

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| <b>Approved Saliva Collection Methods<br/>by Analyte</b> |   |            |               |   |                        |
|--|---|------------|---------------|---|------------------------|
|  | <b>Adults &amp;<br/>Children 6 or older</b> |            |               | <b>Infants &amp;<br/>Children under 6</b> |                        |
| <b>Analyte</b>   | <b>Passive<br/>Drool</b>                    | <b>SOS</b> | <b>Cotton</b> | <b>Sorbette</b>                           | <b>Cotton<br/>Rope</b> |
| Alpha-amylase  |   |            |               |   |                        |
| Androstenedione  |   |            |               |   |                        |
| CgA  |   |            |               |   |                        |
| Cortisol   |   |            |               |   |                        |
| Cotinine   |   |            |               |   |                        |
| C-Reactive Protein                                       |   |            |               |   |                        |
| Dexamethasone†   |   |            |               |   |                        |
| DHEA   |   |            |               |   |                        |
| DHEA-S*  |   |            |               |   |                        |
| Estradiol  |   |            |               |   |                        |
| Estriol  |   |            |               |   |                        |
| Estrone  |   |            |               |   |                        |
| IL-6   |   |            |               |   |                        |
| Melatonin  |   |            |               |   |                        |
| Neopterin  |   |            |               |   |                        |
| 17-OH-Progesterone                                       |   |            |               |   |                        |
| Progesterone   |   |            |               |   |                        |
| Secretory IgA*   |   |            |               |   |                        |
| Testosterone   |   |            |               |   |                        |
| Total Protein  |   |            |               |   |                        |
| Transferrin<br>(Blood Contamination)                     |   |            |               |   |                        |

(Shaded cells indicate approved methods)

\*Note: concentrations are affected by saliva flow rate

†Assay validated only with cotton collection