

SALIVARY BLOOD CONTAMINATION ENZYME IMMUNOASSAY KIT

Catalog No. 1-1302/1-1312, 96-Well Kit

Intended Use

This assay kit is a competitive immunoassay designed for the quantitative measurement of the presence of blood in saliva samples. It is intended for use as an analytical tool to screen saliva samples which should be excluded from assays for other salivary analytes because of blood component leakage into the oral mucosa. It is not intended for use with serum/plasma or for diagnostic use. It is intended only for research use with saliva. Please read the complete kit insert before performing this assay. For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction

The quantitative measurement of analytes in saliva is invalid if the physicochemical and physical barrier between the general circulation and the oral mucosa is compromised such that there is a "leakage" of blood or plasma into saliva (1). This is especially true when levels of the analyte of interest in blood are substantially higher than levels observed in saliva (i.e., ng/mL in serum vs. pg/mL in saliva). Screening samples and excluding those contaminated by blood may be critical if valid conclusions are to be drawn from salivary data (2,3).

A computerized literature search revealed that, with few exceptions (4), investigators working with salivary measurements rarely screen samples for this problem. This suggests that most researchers may be unaware that this phenomenon might be affecting their salivary data. Studies that report screening samples typically use a dipstick method designed for use with urine specimens. However, saliva contains peroxidases, which cause the same color change on the dipstick as does hemoglobin, leading to false positive results. Therefore, the dipstick method is not an accurate measurement of blood contamination in saliva (2,3).

To solve this problem, Salimetrics designed a simple, efficient, and inexpensive immunoassay for researchers to use as an analytical tool to screen samples for blood presence in saliva. Each 96 well plate screens 80 samples with only 1 hour of total incubation time and uses only 20 μ L of sample per test. We expect that the routine application of this screening tool will significantly improve the quality of the information learned in future studies.

The Salimetrics' Salivary Blood Contamination Enzyme Immunoassay quantitatively measures transferrin, a large protein (mol wt of 76,000) present in abundance in blood that is normally present in only trace amounts in saliva. Higher levels of transferrin measured in saliva by this assay indicate the presence of blood contamination and serve as a warning to investigators that samples should be excluded from subsequent quantitative assays for salivary analytes and statistical analyses.

As a general guideline, saliva samples with transferrin values 0.5 mg/dL or higher should be candidates for exclusion when assaying salivary samples for testosterone. (2)
Values greater than 1 mg/dL should be considered as candidates for exclusion in other salivary assays (2,3). The criterion score adopted will vary depending on the analyte under investigation and the protocol used to measure it. We recommend computing a regression between transferrin levels and the levels of the analyte of interest and establishing a cut score based on statistical analysis of this associative relationship.

Test Principle

Standards and unknowns are added to a 96-well microtiter plate along with rabbit antibodies to transferrin and transferrin linked to horseradish peroxidase (conjugate). The transferrin in standards and unknowns and the conjugate compete for the antibody binding sites. After incubation, unbound components are washed away. Bound conjugate is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with 2-molar sulfuric acid. Optical density is read on a standard plate reader at 450 nm. The amount of transferrin peroxidase detected is inversely proportional to the amount of transferrin present (5).

Precautions

1. Stop Solution is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.
2. This kit uses break-apart microtitre strips. Unused wells must be stored at 2 - 8°C in the sealed foil pouch and used in the frame provided.
3. Do not mix components from different lots of kits.
4. When using a multichannel pipette, reagents should be added to duplicate wells at the same time. Follow the same sequence when adding additional reagents so that incubation time with reagents is the same for all wells.
5. See 'Material Safety Data' at the end of procedure for biohazard and chemical hazard warnings.
6. Routine calibration of pipettes is critical for the best possible assay performance.
7. Pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate.
8. When running multiple plates, or multiple sets of strips, a standard curve should be run with each individual plate and/or set of strips.
10. The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68 - 74°F (20 - 23.3°C). Higher or lower temperatures will cause an increase or decrease in OD values, respectively. Salimetrics cannot guarantee test results outside of this temperature range.

Storage

All components of this kit are stable at 2 - 8°C until the kit's expiration date.

Reagents and Reagent Preparation

1. **Microtitre plate:** A 96-well high affinity binding plate.
2. **Antiserum:** 12 mL of a solution of rabbit anti-human transferrin antibody.
3. **Blood Contamination Standard:** 400 μ L of transferrin in a saliva-like matrix with a non-mercury preservative, at a concentration of 6.6 mg/dL.
4. **Wash Buffer:** 100 mL of a 10X phosphate buffered solution containing detergents and a non-mercury preservative. Dilute only the amount needed for current day's use. Discard any leftover reagent. Dilute the wash buffer concentrate 10-fold with room temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized H₂O). (**Note:** *If precipitate has formed in the concentrated wash buffer, it may be heated to 60°C for 15 minutes. Cool to room temperature before use in assay.*)
5. **Blood Contamination Diluent:** 63 mL of a phosphate buffered solution containing a pH indicator and a non-mercury preservative.
6. **Enzyme Conjugate:** 50 μ L of a solution of transferrin labeled with horseradish peroxidase. Dilute prior to use with Blood Contamination Diluent.
7. **Tetramethylbenzidine (TMB):** 25 mL of a non-toxic ready-to-use solution.
8. **Stop Solution:** 12.5 mL of a 2-molar solution of sulfuric acid (USA customers only). Stop solution is provided in powdered form to customers outside the USA. Reconstitute the powdered stop solution with 12.5 mL of deionized water. Let sit for 10 minutes before use.
9. **Positive Control:** 200 μ L of transferrin in a saliva-like diluent. See vial for ranges.

Materials Needed But Not Supplied

- Precision pipette to deliver 20 µL, 50 µL, and 100 µL
- Precision multichannel pipette to deliver 50 µL and 100 µL
- Vortex
- Plate rotator (if unavailable, tap to mix)
- Plate reader with a 450 nm filter
- Log-linear graph paper or computer software for data reduction
- Deionized water
- Reagent reservoirs
- One 10 mL disposable tube
- Five small disposable tubes
- Pipette tips
- 10 mL serological pipette

Specimen Collection

The preferred saliva collection method (6) is to use plain (non-citric acid) cotton Salivettes, P/N 5001. Samples may also be collected using Sorbettes, P/N 5029 (for infants) or cotton ropes, P/N 5016 or by passive drool. Samples may also be collected using Sorbettes (for infants) or cotton ropes, or by passive drool. **Do not** add sodium azide to saliva samples as a preservative. Freeze at -20°C or lower for long-term storage. Contact the technical service team at Salimetrics for more detailed information on specimen collection.

Saliva samples should be frozen prior to assay to precipitate the mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Pipette clear sample into appropriate wells. Particulate matter may interfere with antibody binding, leading to falsely elevated results.

Procedure

Bring all reagents to room temperature.

Step 1: Determine your plate layout. Here is a suggested layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	6.6 Std	6.6 Std	Unk 1									
B	2.2 Std	2.2 Std	Unk 2									
C	0.73 Std	0.73 Std	Unk 3									
D	0.24 Std	0.24 Std	Unk 4									
E	0.08 Std	0.08 Std	Unk 5									
F	Zero	Zero	Unk 6									
G	NSB	NSB	Unk 7									
H	Pos Ctrl	Pos Ctrl	Unk 8									

Step 2: Keep the desired number of wells in the strip holder and place the remaining strips back in the foil pouch. Reseal the zip-lock and refrigerate at 2 - 8°C.

Step 3:

- Label four microcentrifuge tubes or other small tubes 2 through 5.
- Pipette 100 µL of diluent in tubes 2 through 5. Serially dilute the standard 3X by adding 50 µL of the 6.6 mg/dL standard (tube 1) to tube 2. Mix well. After changing pipette tips, remove 50 µL from tube 2 to tube 3. Mix well. Continue for tubes 4 and 5. The final concentrations of standards for tubes 1 through 5 respectively are 6.6 mg/dL, 2.2 mg/dL, 0.73 mg/dL, 0.24 mg/dL, and 0.08 mg/dL.
- Pipette 8 mL of diluent into a disposable tube. Set aside for Step 6.

Step 4:

- Pipette 20 µL of standards, controls and unknowns into appropriate wells. Standards and controls should be assayed in duplicate. For screening purposes we recommend assaying unknowns singly and confirming positive results by repeat testing.
- Pipette 20 µL of diluent into 2 wells to serve as the zero.
- Pipette 70 µL of diluent into 2 wells to serve as the non-specific binding.

Step 5: Dilute the enzyme conjugate 1:400 by adding 20 µL of the conjugate to the 8 mL of diluent prepared in Step 3 (for full plate only). Immediately mix the diluted conjugate solution and add 50 µL to each well using a multichannel pipette.

Step 6: Pipette 50 µL of antiserum into all wells, except the non-specific binding wells, using a multichannel pipette.

Step 7: Mix plate on rotator for 5 minutes at 500 rpm (or tap to mix) and incubate at room temperature for 40 additional minutes.

Step 8: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle or by pipetting 300 µL of wash buffer into each well and then flipping the liquid into a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 9: Add 100 µL of TMB solution to each well with a multichannel pipette.

Step 10: Mix on a plate rotator for 5 minutes at 500 rpm (or tap to mix) and incubate the plate in the dark at room temperature for an additional 10 minutes.

Step 11:

- Add 100 µL of stop solution with a multichannel pipette.
- Mix on a plate rotator for 3 minutes at 500 rpm (or tap to mix). Be sure all wells have turned yellow. If green color remains, continue mixing until green color turns to yellow.
- Wipe off bottom of plate with a water-moistened lint-free cloth and wipe dry.
- Read in a plate reader at 450 nm. Read plate within 10 minutes of adding stop solution (correction at 492 to 620 is desirable).

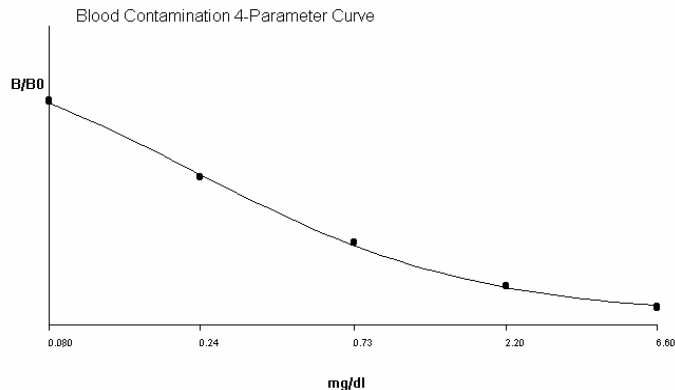
Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells from the average OD of the zero, standards, controls and unknowns.
3. Calculate the percent bound (B/Bo) for each standard, control and unknown by dividing the average OD (B) by the average OD for the zero (Bo).
4. Determine the concentrations of the controls and unknowns by interpolation using software capable of logistics. We recommend using a 4-parameter sigmoid minus curve fit.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Sample	Average OD	B	B/Bo	Transferrin (mg/dL)
A1,A2	S1	0.191	0.174	0.067	6.6
B1,B2	S2	0.397	0.380	0.147	2.2
C1,C2	S3	0.820	0.803	0.311	0.73
D1,D2	S4	1.462	1.445	0.560	0.24
E1,E2	S5	2.206	2.189	0.848	0.08
F1,F2	Bo	2.599	2.582	NA	NA
G1,G2	NSB	0.017	NA	NA	NA



Material Safety Data*

Potential Biohazardous Material

Kit products containing transferrin were derived from human blood sources and should be handled at Biosafety Level 2 (see CDC/NIH manual entitled *Biosafety in Microbiological and Biomedical Laboratories*). These products were tested for HIV antibody, hepatitis B surface antigen, hepatitis C antibody, HIV 1 antigen(s), antibody to HTLV-1/11, and syphilis using FDA guidelines. However, no test method can provide absolute assurance that infectious agents are absent.

Hazardous Ingredients

Stop Solution is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.

We recommend the procedures listed below for all kit reagents.

Handling

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing, give oxygen and call a physician.

*The above information is believed to be accurate but is not all-inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from contact with reagents.

Performance Characteristics

1. **Recovery** - Four saliva samples containing different levels of endogenous transferrin were spiked with known quantities of transferrin and assayed. The average recovery was 104.1%, range 96.0% to 117.7 %.
2. **Intra-assay precision** was determined from the mean of 12 replicates at high (3.88 mg/dL) and low (0.42 mg/dL) transferrin levels. The average intra-assay coefficients of variation were 10.2% and 4.9%, respectively.
3. **Inter-assay precision** was determined from the mean of averaged duplicates across 5 separate assay runs. The average intra-assay coefficients of variation were 9.0% for low (0.81 mg/dL) and 4.1% for high (5.03 mg/dL) transferrin levels.
4. **Linearity of Dilution**- Saliva samples were spiked with exogenous transferrin and serially diluted (1:2,4,8,) with assay buffer. The average recovery was 96.0%, range 91.9% to 101.5%.
5. **Sensitivity**- The lower limit of sensitivity was determined by interpolating the mean minus 2 SD for 10 sets of duplicates for the 0 mg/dL standard. The minimal concentration of transferrin that can be distinguished from zero is 0.08 mg/dL.
6. **Specificity**- The following analytes were tested in the Salivary Blood Contamination Assay at a concentration of 50 ng/mL or greater, with no detectable cross-reactivity measured: cortisol, testosterone, DHEA, progesterone, melatonin, estradiol, estriol, secretory IgA, and lactoferrin.

References

1. Malamud, D., & Tabak, L. (Eds.). (1993). *Saliva as a diagnostic fluid. Annals of the New York Academy of Sciences*, (Vol. 694). New York, NY.
2. Kivlighan, K. T., Granger, D. A., Schwartz, E. B., Nelson, V., & Curran, M. (2004). Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Hormones and Behavior*, 46, 39-46.
3. Schwartz, E., & Granger, D. A. (2004). Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clinical Chemistry*, 50, 654-656.

4. Lac G, Lac N, & Robert A. (1993). Steroid assays in saliva: a method to detect plasmatic contaminations. *Arch Int Physiol Biochem Biophys*, 101, 257-62.
5. Chard, T. (1990). *An introduction to radioimmunoassay and related techniques*. Amsterdam: Elsevier.
6. Schwartz, E.B., Granger, D.A., Susman, E.J., Gunnar, M.R., & Laird, B. (1998). Assessing salivary cortisol in Studies of Child Development. *Child Development*, 69, 1503-1513.

Seller's Limited Warranty

"Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller's satisfaction to be defective. All claims should be submitted in written form. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability, in all cases, will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller's product or out of the breach of any express or implied warranties."