SALIVARY
BLOOD CONTAMINATION
ENZYME IMMUNOASSAY KIT

For Research Use Only
Not for use in Diagnostic Procedures

Item No. 1-1302, (Single) 96-Well Kit;
1-1302-5, (5-Pack) 480 Wells

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Intended Use

The Salimetrics® Blood Contamination Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of the presence of blood in saliva samples. It is not intended for diagnostic use. It is intended only for research use in humans and some animals as an analytical tool to screen saliva samples that should be excluded from assays for other salivary analytes because of blood component leakage into the oral mucosa. Salimetrics has not validated this kit for serum or plasma samples.

Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in unreliable values.

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 physiochemical 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 µL 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

This is a competitive immunoassay kit. Transferrin in standards and samples compete with transferrin conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Blood Contamination Enzyme Conjugate is measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with an acidic solution. The optical density is read on a standard plate reader at 450 nm. The amount of Blood Contamination Enzyme Conjugate detected is inversely proportional to the amount of transferrin present in the sample (5).

Safety Precautions

Read Safety Data Sheets before handling reagents.

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

Liquid Stop Solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

Handling

Follow good laboratory practices when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using appropriate 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 call a physician.

The above information is believed to be accurate but is not all-inclusive. This information should be used only as a guide. Salimetrics will not be liable for accidents or damage resulting from misuse of product.

Safety Data Sheets are available by contacting Salimetrics at support@salimetrics.com (See www.salimetrics.com for alternative contact options).

General Kit Use Advice

- This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
- The quantity of reagent provided with a single kit is sufficient for three partial runs. The volumes of wash buffer and enzyme conjugate prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
- Do not mix components from different lots of kits.
- To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
- When using a multichannel pipette to add reagents, always follow the same sequence when adding all reagents so that the incubation time is the same for all wells.
- When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
- 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 may affect OD values.
- Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
- When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.

Storage

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

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

Collect whole saliva by unstimulated passive drool. Donors may tilt the head forward, allowing the saliva to pool on the floor of the mouth, then pass the saliva through the SalivaBio Collection Aid (SCA) into a polypropylene vial. Collection protocols/methods are available online at www.salimetrics.com or upon request.

Samples visibly contaminated with blood should be recollected.

Sample Handling and Preparation

After collection, it is important to keep samples cold in order to avoid bacterial growth in the specimen. Refrigerate sample within 30 minutes, and freeze at or below -20ºC within 4 hours of collection. (Samples may be stored at -20ºC for up to 6 months.) For long term storage, refer to the Salimetrics Collection and Handling Advice Booklet.

Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the immunoassay.

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Re-freeze saliva samples as soon as possible after adding to the assay plate. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.

Materials Supplied with Single Kit

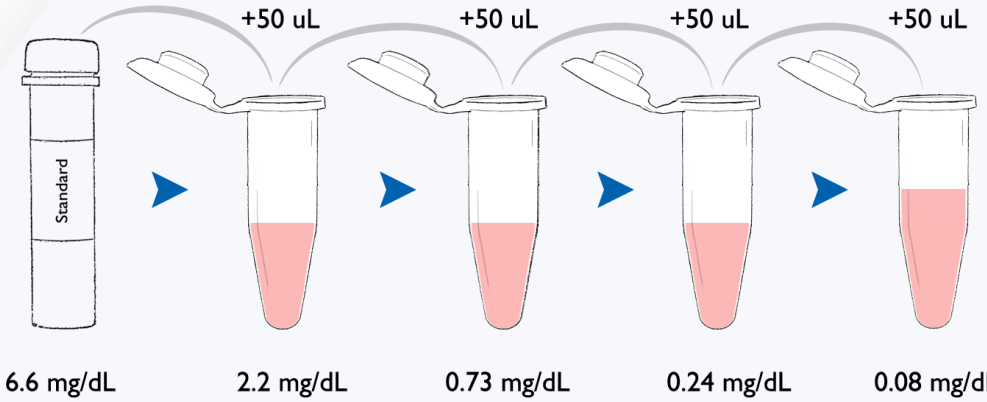
	Item	Quantity/Size
1	**Microtitre Plate**	1/96 well
2	**Blood Contamination Standard** 6.6 mg/dL, in a saliva-like matrix. Serially dilute before use according to Reagent Preparation. Contains: Transferrin, buffer, preservative.	1 vial / 400 µL
3	**Blood Contamination Positive Control** In a saliva-like matrix. Ready to use. Contains: Transferrin, buffer, preservative.	1 vial / 200 µL
4	**Blood Contamination Enzyme Conjugate** Concentrate. Dilute before use with Blood Contamination Diluent. (See step 5 of Procedure.) Contains: Transferrin conjugated to HRP, preservative.	1 vial / 50 µL
5	**Blood Contamination Diluent** Contains: phosphate buffer, preservative.	1 bottle / 60 mL
6	**Blood Contamination Antiserum** A solution of rabbit anti-human transferrin antibody.	1 bottle / 12 mL
7	**Wash Buffer Concentrate (10X)** Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle / 100 mL
8	**TMB Substrate Solution** Non-toxic, ready to use.	1 bottle / 25 mL
9	**Stop Solution**	1 bottle / 12.5 mL

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 with 0.08-0.17 inch orbit capable of 500 rpm
- Plate reader with 450 nm and 490 to 492 reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 10 mL
- Five small disposable polypropylene tubes for dilution of standard
- Pipette tips
- Serological pipette to deliver up to 10 mL
- Centrifuge capable of 1500 x g

Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is recommended for the 8 mL of Blood Contamination Diluent used in Step 5 (conjugate dilution) to come to room temperature.
- Bring Microtitre Plate to room temperature before use. ***It is important to keep the foil pouch with the plate strips closed until warmed to room temperature, as humidity may have an effect on the coated wells.***
- Prepare 1X wash buffer by diluting Wash Buffer Concentrate (10X) 10-fold with room-temperature deionized water (100 mL of Wash Buffer Concentrate (10X) to 900 mL of deionized water). ***Dilute only enough for current day's use and discard any leftover reagent.*** (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)
- Prepare serial dilutions of the Blood Contamination Standard as follows:
    - Label four polypropylene microcentrifuge tubes or other small tubes 2 through 5.
    - Pipette 100 µL of Blood Contamination Diluent into 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 are, respectively, 6.6 mg/dL, 2.2 mg/dL, 0.73 mg/dL, 0.24 mg/dL, and 0.08 mg/dL.



Procedure

Step 1: Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards and controls should be assayed in duplicate. For screening purposes we recommend assaying saliva samples singly and confirming positive results by repeat testing.)

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

*NSB = Non-specific binding wells. These may serve as blanks. Use is optional.

Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

Step 3: Pipette 8 mL of Blood Contamination Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 5.

Step 4:

- Pipette 20 µL of standards, controls, and saliva samples into appropriate wells.
- Pipette 20 µL of Blood Contamination Diluent into 2 wells to serve as the zero.
- If using NSB wells, pipette 70 µL of Blood Contamination Assay Diluent into each of those 2 wells.

Step 5: Dilute the Blood Contamination Enzyme Conjugate 1:400 by adding 20 µL of the conjugate to the 8 mL tube of Blood Contamination Diluent. (Scale down proportionally if not using the entire plate.) Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 50 µL to each well using a multichannel pipette.

Step 6: Pipette 50 µL of Blood Contamination Antiserum into all wells, except for the NSB wells (if used), using a multichannel pipette.

Step 7: Mix plate on a plate rotator for 5 minutes at 500 rpm and incubate at room temperature for a total of 45 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 discarding the liquid over 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 Substrate Solution to each well with a multichannel pipette.

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

Step 11: Add 100 µL of Stop Solution with a multichannel pipette.

Step 12:

- Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow.

  Caution: Spillage may occur if mixing speed exceeds 600 rpm.
- 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. (For best results, a secondary filter correction at 490 to 492 nm is recommended.)

Quality Control

The Salimetrics' Blood Contamination Positive Control should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Calculations

1. Compute the average optical density (OD) for all duplicate wells.
2. Subtract the average OD for the NSB wells (if used) from the OD of the zero, standards, controls, and saliva samples.
3. Calculate the percent bound (B/Bo) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (Bo). (The zero is not a point on the standard curve.)
4. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
5. Samples with transferrin values greater than 6.6 mg/dL may be diluted with Blood Contamination Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the assay results by the dilution factor.

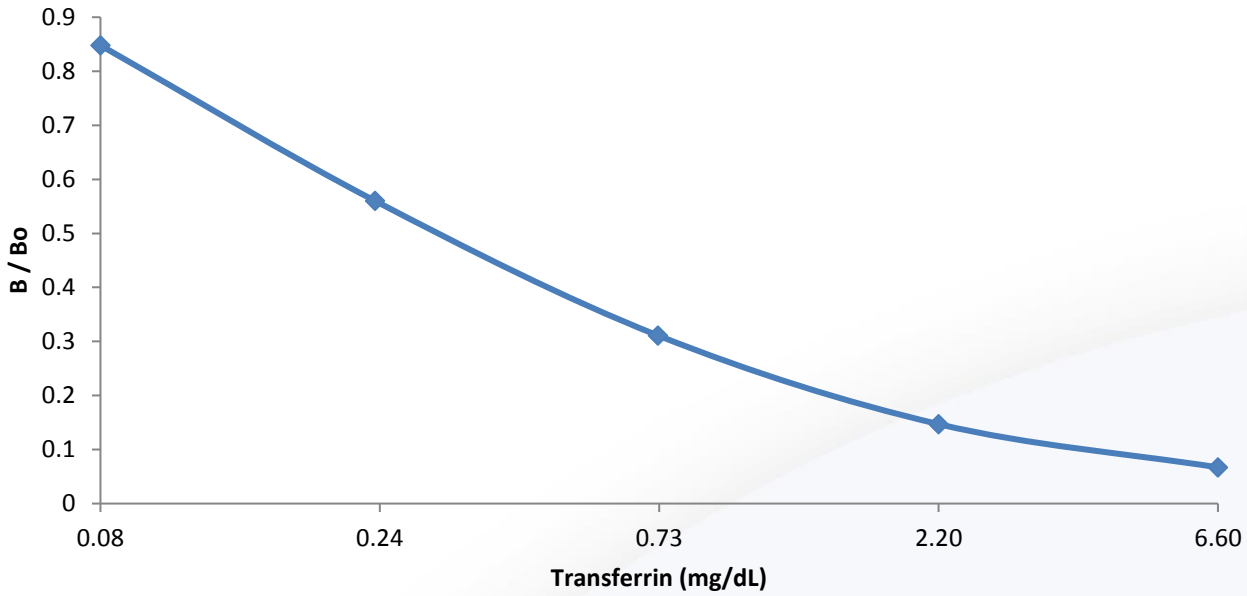
A new Standard Curve must be run with each full or partial plate.

Typical Results

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

Well	Standard	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

Example: Blood Contamination 4-Parameter Curve Fit



Limitations

- Samples with transferrin values greater than 6.6 mg/dL may be diluted with Blood Contamination Diluent and rerun for accurate results. To obtain the final transferrin concentration, multiply the concentration of the diluted sample by the dilution factor.
- See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.
- Samples collected with sodium azide are unsuitable for this assay.

Salivary Blood Contamination EIA Kit Performance Characteristics

Precision

The intra-assay precision was determined from the mean of 12 replicates each.

Saliva Sample	N	Mean (mg/dL)	Coefficient of Variation (%)
H	12	3.88	10.2
L	12	0.42	4.9

The inter-assay precision was determined from the mean of average duplicates for 10 separate runs.

Saliva Sample	N	Mean (mg/dL)	Coefficient of Variation (%)
H	10	4.93	7.2
L	10	1.02	7.1

Recovery

Four saliva samples containing different levels of an endogenous transferrin were spiked with known quantities of transferrin and assayed. The average recovery was 104.1%, range 96.0% to 117.7 %.

Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates at the 0 mg/dL level. The minimal concentration of transferrin that can be distinguished from 0 is 0.08 mg/dL.

Sample Dilution Recovery

Saliva samples were spiked with exogenous transferrin and serially diluted (1:2,4,8) with Blood Contamination Diluent. The average recovery was 96.0%, range 91.9% to 101.5%.

Antibody Specificity

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary Blood Contamination EIA
Cortisol	≥ 50	ND
DHEA	≥ 50	ND
17β-Estradiol	≥ 50	ND
Estriol	≥ 50	ND
Lactoferrin	≥ 50	ND
Melatonin	≥ 50	ND
Progesterone	≥ 50	ND
SIgA	≥ 50	ND
Testosterone	≥ 50	ND

ND = None detected (<0.004)

References

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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.
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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.

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."

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