SALIVARY SECRETORY IgA

INDIRECT ENZYME IMMUNOASSAY KIT

For Research Use Only
Not for use in Diagnostic Procedures

Item No. 1-1602, (Single) 96-Well Kit;
1-1602-5, (5-Pack) 480 Wells

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TABLE OF CONTENTS

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Intended Use

The Salimetrics® SIgA Indirect Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary SIgA. It is not intended for diagnostic use. This assay kit was designed and optimized for salivary research use in humans. Salimetrics has not validated this kit for serum, plasma or saliva samples from any other species.

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

Secretory Immunoglobulin A (SIgA) is the dominant immunoglobulin in external secretions that bathe mucosal surfaces (respiratory, intestinal, and reproductive), where it acts as a key component of the immune system's "first line of defense" against microbial invasion (1,2). Dimeric IgA secreted by mucosal plasma cells adjacent to the salivary glands is bound and transported through the salivary cells by a polymeric Ig receptor (pIgR). The IgA dimer, in complex with a fragment of the pIgR polypeptide, is then released into saliva as secretory IgA (SIgA) (2,3).

IgA-producing plasma cells are generally undetectable in the mucosae before 10 days of age, but they increase rapidly thereafter (1). SIgA levels are generally thought to be very low in newborn infants and to rise quickly during the first month of life (4,5,6). Levels of salivary SIgA continue to increase as children age, stabilizing within the adult range around 5-7 years (6,7,8). Reports of changes in SIgA secretion in old age are inconsistent, with both increases and decreases having been observed (9,10).

Relative numbers of IgA-producing plasma cells are higher in the submandibular and sublingual glands compared to the parotid glands, and even higher in certain minor glands, leading to differing levels of SIgA in the secretions from these glands. It is speculated that greater density of plasma cells in certain glands may be due to increased antigenic interactions in those parts of the mouth (3).

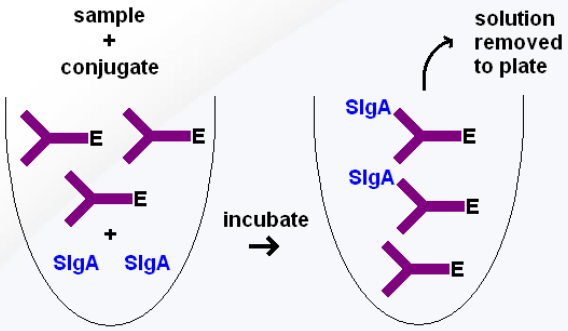
The active transport mechanism into saliva for SIgA serves as a rate-limiting factor, (2) and salivary levels of SIgA decrease as flow rates increase (7,10). The contributions to whole saliva from the various salivary glands in the mouth also vary greatly according to the rate of flow (3). The literature therefore recommends that variability in salivary flow rate should be taken into account when estimating salivary levels of SIgA and making comparisons between individuals (2,3,7).

Salivary SIgA levels vary in a complex fashion in response to stress, mood, and emotionality (2).

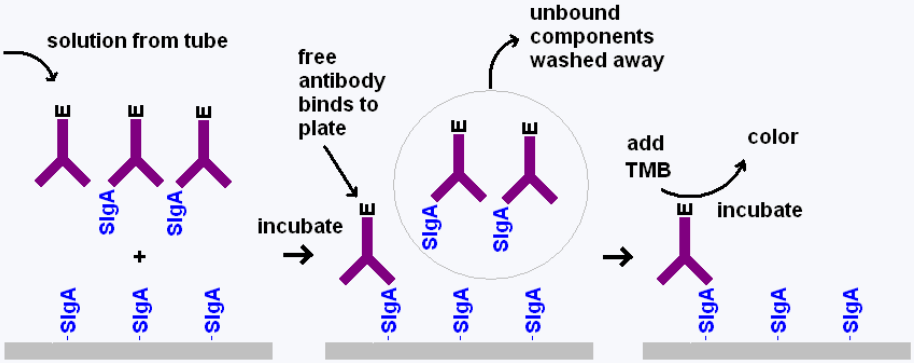
The present enzyme immunoassay protocol represents a significant advance over the traditional SIgA measurement approach to employing single radial immunodiffusion (SRID). This enzyme immunoassay is designed to capture the full range of salivary SIgA levels and uses only 25 µL of saliva per test, with minimal incubation times.

Test Principle

This is an indirect competitive immunoassay kit. A constant amount of goat anti-human SIgA conjugated to horseradish peroxidase is added to tubes containing specific dilutions of standards or saliva. The antibody enzyme conjugate binds to the SIgA in the standard or saliva samples. The amount of free antibody enzyme conjugate remaining is inversely proportional to the amount of SIgA present in the sample.



After incubation and mixing, an equal solution from each tube is added in duplicate to the microtitre plate coated with human SIgA. The free or unbound antibody enzyme conjugate binds to the SIgA on the plate. After incubation, unbound components are washed away. Bound SIgA Antibody 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 SIgA Antibody Enzyme Conjugate detected is inversely proportional to the amount of SIgA present in the sample (11).



Safety Precautions

Read Safety Data Sheets before handling reagents.

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 two partial runs. The volumes of wash buffer, SIgA Diluent Concentrate and SIgA Antibody 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.
- We recommend saving all reagents until data analysis has confirmed a successful run to facilitate troubleshooting if necessary.
- Prior to sample addition, please label each strip to assure plate orientation and sample order when data is acquired on plate reader.

- 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.

Due to the differences in SIgA levels from specific salivary glands, (3) the preferred type of sample for general studies is to 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. Samples may be screened for possible blood contamination using our Blood Contamination EIA Kit (Item Nos. 1-1302/1-1302-5). Do not use dipsticks, which result in false positive values due to salivary enzymes.

Record the time and date of specimen collection.

Due to the influence that saliva flow rates have on SIgA levels, Salimetrics advises measuring the amount of time needed to collect the desired volume, then using this information to determine the flow rate. The measured concentration of SIgA (µg/mL) should then be multiplied by the flow rate (mL/min) to express the results as product measured per unit of time (µg/min).

If an absorbent device from the SalivaBio Oral Swab family (SOS, SCS, or SIS) is used to collect the saliva, the length of time the swab is in the mouth should be noted. The amount of saliva collected can be estimated by comparing the weight of the swab and storage tube before and after collecting saliva. The flow rate can then be estimated. To ensure a valid estimate, the swab must be removed from the mouth before it has absorbed its maximum volume (approximately 2 mL). Not more than one minute is recommended, unless donors have low saliva flow due to medications, oral disease, strenuous exercise, etc. A pilot study may be advisable to choose the proper time interval. Because the smaller SIS may reach saturation quickly, it may be difficult to estimate the flow rate reliably with this device.

Since concentrations of SIgA vary significantly depending on the location in the mouth, (3) consistency in collection location is important when collecting with a swab. We find that placing the swab underneath the tongue on the floor of the mouth yields results similar to those from whole saliva collected by passive drool. Under certain conditions, however, there is a possibility that the swab might collect specific glandular saliva. Researchers should be aware of this potential and decide on their collection strategy accordingly.

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 making dilutions. Pipette clear sample into appropriate dilution tubes. Re-freeze saliva samples as soon as possible after running assay. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles.

Saliva samples must be diluted for this assay. See Procedure for details.

Materials Supplied with Single Kit

	Item	Quantity/Size
1	**Microtitre Plate** Coated with highly purified human SIgA.	1/96 well
2	**SIgA Standard** 600 µg/mL, in a saliva-like matrix. Serially dilute before use according to Reagent Preparation. Contains: highly purified human SIgA, buffer, preservative.	1 vial / 75 µL
3	**SIgA Controls** High, Low, in a saliva-like matrix. Ready to use. Contains: human SIgA, buffer, preservative.	2 vials / 50 µL each
4	**SIgA Antibody Enzyme Conjugate** Concentrate. Dilute before use with 1X SIgA Diluent. (See step 6 of Procedure.) Contains: goat anti-human SIgA conjugated to HRP, preservative.	1 vial / 50 µL
5	**SIgA Diluent Concentrate (5X)** Dilute before use according to Reagent Preparation. Contains: phosphate buffer, preservative.	1 bottle / 50 mL
6	**Wash Buffer Concentrate (10X)** Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle / 100 mL
7	**TMB Substrate Solution** Non-toxic, ready to use.	1 bottle / 25 mL
8	**Stop Solution**	1 bottle / 12.5 mL
9	**Adhesive Plate Cover**	1

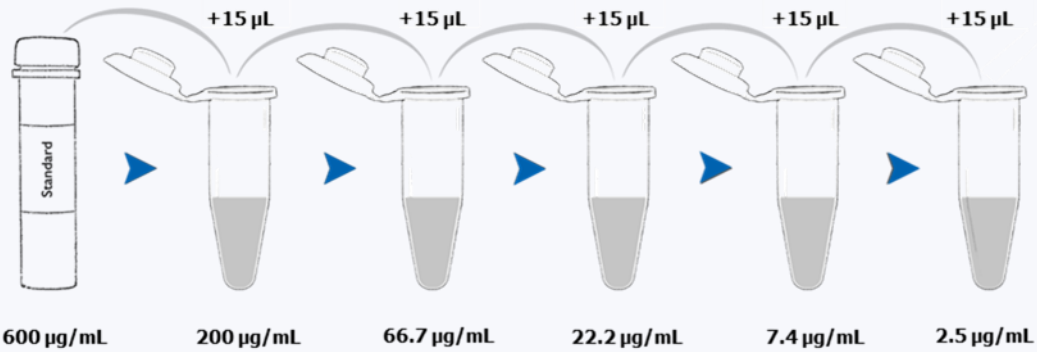
Materials Needed But Not Supplied

- Precision pipette to deliver 10 µL, 15 µL, 25 µL, 30µL, 50 µL, and 100 µL
- Precision repeater pipette to deliver 50 µL and 100 µL
- Precision multichannel pipette to deliver 50 µL
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of 400 and 500 rpm
- Plate reader with 450 nm and 490 to 492 nm reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- Polypropylene tubes with caps to hold up to 5 mL
- Small disposable polypropylene tubes for dilution of standard and samples
- Pipette tips
- Repeater and/or serological pipette to deliver 3 mL, 4 mL and 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 50 mL of SIgA Diluent Concentrate (5X) 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 1X SIgA Diluent by diluting SIgA Diluent Concentrate (5X) 5-fold with room-temperature deionized water (50 mL of SIgA Diluent Concentrate (5X) to 200 mL of deionized water). Dilute only the amount needed for current day's use and discard any leftover reagent.

  NOTE: The quantity of SIgA Diluent Concentrate (5X) included is sufficient to run samples <u>in duplicate only!</u> Please contact Salimetrics to purchase additional SIgA Diluent Concentrate (5X) if needed.
- Prepare serial dilutions of the SIgA Standard as follows:
  - Label five polypropylene microcentrifuge tubes or other small tubes 2 through 6.
  - Pipette 30 µL of 1X SIgA Diluent into tubes 2 through 6.
  - Serially dilute the standard 3X by adding 15 µL of the 600 µg/mL standard (tube 1) to tube 2. Mix well.
  - After changing pipette tips, remove 15 µL from tube 2 to tube 3. Mix well.
  - Continue for tubes 4, 5, and 6.
  - The final concentrations of standards for tubes 1 through 6 are, respectively, 600 µg/mL, 200 µg/mL, 66.7 µg/mL, 22.2 µg/mL, 7.4 µg/mL, and 2.5 µg/mL.



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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, controls, and saliva samples should be assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	600 Std	600 Std	Ctrl-H	Ctrl-H								
B	200 Std	200 Std	Ctrl-L	Ctrl-L								
C	66.7 Std	66.7 Std	SMP-1	SMP-1								
D	22.2 Std	22.2 Std	SMP-2	SMP-2								
E	7.4 Std	7.4 Std	SMP-3	SMP-3								
F	2.5 Std	2.5 Std	SMP-4	SMP-4								
G	0 Std	0 Std	SMP-5	SMP-5								
H	Blank	Blank	SMP-6	SMP-6								

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 3 mL of 1X SIgA Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 6.

Step 4: Sample Preparation, Part 1 (1:5 Saliva Dilution)

Note: *Skip this step if repeating samples straight. Go directly to Step 5.*

Dilute only saliva samples. Do not pre-dilute controls.

- Label one small microcentrifuge tube with the identity of each saliva sample.
- Using a repeater pipette, add 100 µL of 1X SIgA Diluent into each tube.
- Pipette 25 µL of saliva into the appropriate tube. (If saliva sample is less than 25 µL, use 10 µL of saliva to 40 µL of diluent.) Lightly vortex to mix.

Step 5: Sample Preparation, Part 2

- Label one 5 mL capped tube for each standard, control, saliva sample, and one for the zero value.
- Using a repeater pipette, add 4 mL of 1X SIgA Diluent to each tube.

- Pipette 10 µL of standards, controls, and diluted saliva samples into appropriate tubes and mix.
- Pipette 10 µL of 1X SIgA Diluent into the zero tube and mix.

Step 6: Incubation with the Antibody Enzyme Conjugate

- Dilute the Antibody Enzyme Conjugate 1:120 by adding 25 µL of the conjugate to the 3 mL of 1X SIgA Diluent. (Scale down proportionally if not using the entire plate.) Antibody Enzyme Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted Antibody Enzyme Conjugate solution and add 50 µL to each labeled tube using a repeater pipette.
- Gently mix each tube by inversion and incubate at room temperature for 90 minutes.

Step 7: Incubated Samples Transferred to Assay Plate

- Mix each tube again by gentle inversion. Pipette 50 µL from each labeled tube into the appropriate wells on the Microtitre Plate.
- Pipette 50 µL of 1X SIgA Diluent into each Blank well.
- Place adhesive cover (provided) over plate. Mix plate on a plate rotator ***continuously*** at 400 rpm for 90 minutes at room temperature.

Step 8: Wash the plate 6 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 50 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 40 minutes.

Step 11:

- Add 50 µL of Stop Solution with a multichannel pipette.
- 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 plate 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' High and Low SIgA Controls 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 Blank wells from the OD of the zero, standards, controls, and saliva samples.
3. Calculate the percent bound (B/B_0) for each standard, control, and saliva sample by dividing the OD of each well (B) by the average OD for the zero (B_0).
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. Multiply the calculated concentrations of the saliva samples by the dilution factor of 5 to obtain final SIgA concentrations in µg/mL.
6. Samples (diluted 5X) with SIgA values greater than 600 µg/mL (or >3000 µg/mL after multiplying by the dilution factor of 5) should be diluted further with 1X SIgA Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the results by the additional dilution factor.
7. If results are below the low limit of sensitivity, samples should be repeated straight (eliminating the 5X dilution). In this case, do not multiply the final results by 5.

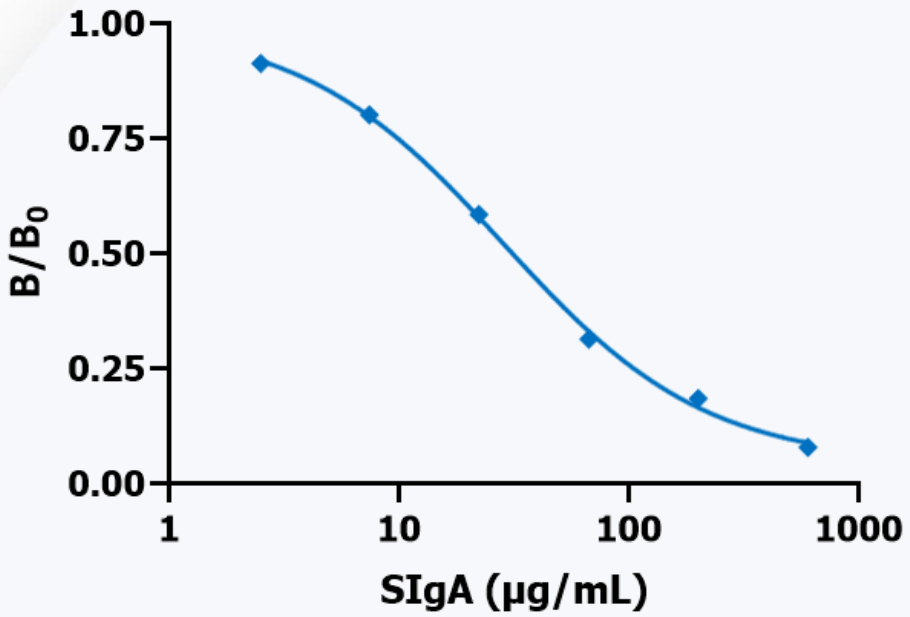
A new Standard Curve must be run with each full or partial plate.

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/B_0	SIgA (µg/mL)
A1,A2	S1	0.140	0.130	0.079	600.0
B1,B2	S2	0.313	0.303	0.185	200.0
C1,C2	S3	0.526	0.516	0.314	66.7
D1,D2	S4	0.970	0.960	0.585	22.2
E1,E2	S5	1.326	1.316	0.801	7.4
F1,F2	S6	1.509	1.499	0.913	2.5
G1,G2	Zero	1.652	1.642	1	0
H1,H2	Blank	0.010	NA	NA	NA

Example: SIgA 4-Parameter Curve Fit



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Limitations

- Samples (diluted 5X) with SIgA values greater than 600 µg/mL (or >3000 µg/mL after multiplying by the dilution factor of 5) should be diluted further with 1X SIgA Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the results by the additional 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.
- Any quantitative results indicating abnormal SIgA levels should be followed by additional testing and evaluation.

Salivary SIgA Example Ranges*

Group	N	Range (µg/mL)	Mean (µg/mL)	Standard Deviation (µg/mL)
Adults	21	93.2 - 974.03	379.39	261.47

*To be used as a guide only. Each laboratory should establish its own range.

Salivary SIgA EIA Kit Performance Characteristics

Spike and Recovery

Six saliva samples containing different levels of an endogenous SIgA were spiked with known quantities of SIgA and assayed.

Saliva Sample	Endogenous (µg/mL)	Added (µg/mL)	Expected (µg/mL)	Observed (µg/mL)	Recovery (%)
1	88.77	250.00	338.77	377.80	111.5
2	79.42	90.00	169.42	176.96	104.5
3	84.10	20.00	104.10	120.70	115.9
4	275.96	250.00	525.96	529.70	100.7
5	285.60	90.00	375.60	394.68	105.1
6	302.40	20.00	322.40	358.73	111.3

Sample Dilution Recovery

Three samples were serially diluted with 1X SIgA Diluent and assayed.

Saliva Sample	Dilution Factor	Expected (µg/mL)	Observed (µg/mL)	Recovery (%)
1			364.60	
	1:2	182.30	198.40	108.8
	1:4	91.15	87.76	96.3
	1:8	45.58	39.80	87.3
	1:16	22.79	19.92	87.4
2			456.70	
	1:2	228.35	262.35	114.9
	1:4	114.18	129.45	113.4
	1:8	57.09	51.45	90.1
	1:16	28.54	32.00	112.1
3			389.36	
	1:2	194.68	207.04	106.3
	1:4	97.34	95.04	97.6
	1:8	48.67	50.32	103.4
	1:16	24.34	23.84	97.9

Precision

The intra-assay precision was determined from the mean of 10 replicates each.

Saliva Sample	N	Mean (µg/mL)	Standard Deviation (µg/mL)	Coefficient of Variation (%)
H	10	805.38	56.32	6.99
M	10	336.03	17.89	5.32
L	10	91.08	4.09	4.49

The inter-assay precision was determined from the mean of average duplicates for 8 separate runs.

Saliva Sample	N	Mean (µg/mL)	Standard Deviation (µg/mL)	Coefficient of Variation (%)
H	8	204.10	17.65	8.65
L	8	25.33	2.26	8.93

Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 18 sets of duplicates at the 0 µg/mL level. The minimal concentration of SIgA that can be distinguished from 0 is 2.5 µg/mL.

Method Comparison

Inter-method correlations for SIgA levels from saliva samples (n = 21) assayed using the present EIA protocol and a radial immunodiffusion assay, and the present protocol and a commercially available SIgA ELISA, were r (19) = 0.94 and 0.91, $p < 0.0001$, respectively. The SIgA levels returned by the present EIA protocol (M = 379.39 µg/mL; St Dev = 261.47) and the comparison ELISA (M = 365.81 µg/mL; St Dev = 311.53) were not statistically distinct. SIgA levels returned by radial immunodiffusion were significantly higher (M = 675.21 µg/mL; St Dev = 467.94) than both immunoassay protocols.

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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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