SALIVARY Insulin

ELISA KIT

For Diagnostic In-Vitro Use

Item No. 1-4410, (Single) 96-Well Kit;
1-4410-5, (5-Pack) 480 Wells

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1.800.790.2258 • support@salimetrics.com • salimetrics.com

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Intended Use

The Salimetrics® Insulin ELISA Kit is an indirect sandwich ELISA specifically designed and validated for the quantitative measurement of salivary Insulin. Measurements obtained by this device may be used in the diagnosis of various metabolic disorders. Salimetrics has not validated this kit for use with serum/plasma samples.

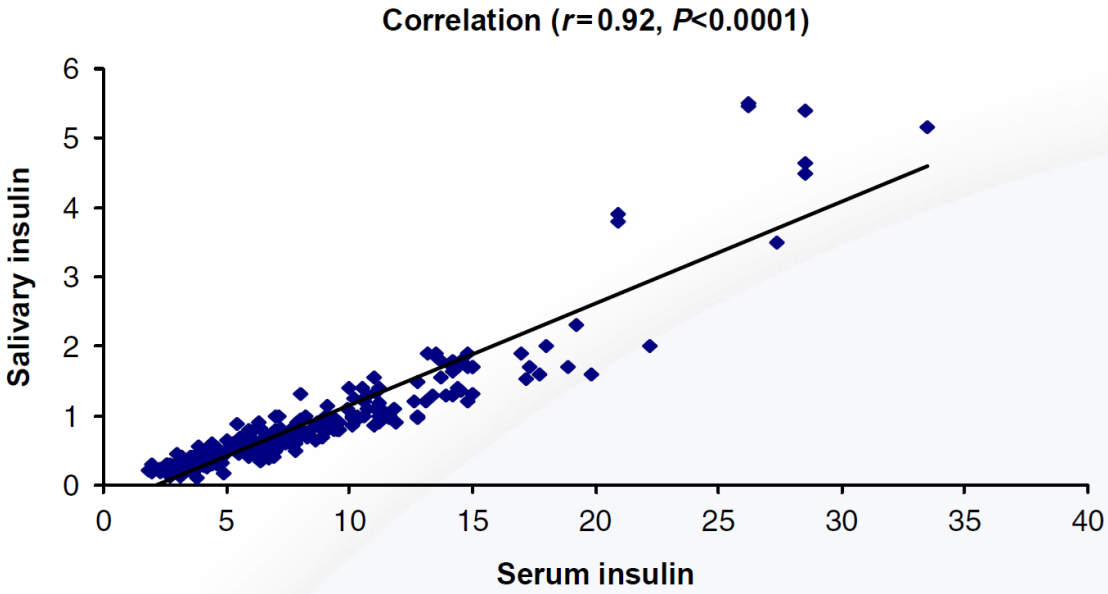
Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in unreliable values.

Introduction

Insulin, a critical peptide hormone produced by the pancreatic β-cells, regulates glucose homeostasis and energy metabolism by facilitating the uptake of glucose into cells and influencing carbohydrate, protein and lipid metabolism, as well as cell division and growth (1). The lack of Insulin production in Type I Diabetes Mellitus, caused by autoimmune islet destruction, results in pathogenic levels of glucose in the blood and requires exogeneous Insulin administration (2). Type II Diabetes Mellitus is caused by the lack of Insulin activity referred to as Insulin Resistance. Elevated fasting Insulin levels and/or the dysregulation of Insulin after ingestion of glucose over time are diagnostic hallmarks of Insulin resistance (3-6). In many cases, people are unaware of their pre-diabetic state since Insulin resistance can occur prior before systemic glucose levels become problematically high (7). Fortunately, Insulin Resistance is reversible by behavioral health changes which present an important opportunity for a pre-diabetic individual to avoid Type II Diabetes altogether (8).

Traditionally measured in serum, advancements now allow for the assessment of Insulin levels in saliva, providing a non-invasive, simpler alternative for research involving frequent sampling (9-12). In saliva, Insulin shares a nearly linear correlation to fasting blood levels ($r = 0.92$) and is a reliable surrogate for serum measurements as shown in the figure below (13). The detection of salivary Insulin has a crucial temporal aspect; there is an approximate 40-minute delay in salivary Insulin response compared to serum after a glucose challenge (14). Saliva collection reduces patient discomfort, risk of infection, and the need for specialized medical staff, making it particularly beneficial for vulnerable groups and suitable for large-scale studies or fieldwork. One significant benefit of measuring Insulin in saliva is the ability to easily and painlessly collect multiple samples after a glucose challenge, facilitating post-prandial studies without the need for repeated blood draws. These advantages seems to be particularly helpful for studies of childhood obesity or gestational diabetes in pregnant women.

This kit enables the measurement of both fasting and post-meal or glucose challenge Insulin levels in saliva, offering valuable insights into Insulin dynamics. By facilitating detailed, non-invasive assessments of metabolic responses to dietary or pharmaceutical interventions over short periods, this kit supports comprehensive research aimed at unraveling the complexities of metabolic regulation and disease progression.



Fabre, B., *et. al.* (2012). Measurement of fasting salivary Insulin and its relationship with serum Insulin in children, Endocrine Connections, 1, 58-61.

Test Principle

This is an indirect sandwich ELISA kit. A "sandwich" is formed when both a capture anti-Insulin antibody and a detection anti-Insulin antibody bind Insulin. The detection antibody is directly linked to horseradish peroxidase. After incubation, unbound components are washed away. Bound anti-Insulin detection antibody 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 anti-Insulin detection antibody is directly proportional to the amount of Insulin present in the sample.

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 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.

pH Indicator

Insulin values from samples with a pH $\leq$ 4.0 or $\geq$ 9.0 may be inaccurate. A pH indicator in the Assay Diluent alerts the user to samples with high or low pH values. Upon addition of the Assay Diluent, acidic samples will turn yellow and alkaline samples will turn purple. Dark yellow or purple wells indicate that a pH value for that sample should be obtained using pH strips. Samples with a pH $\leq$ 4.0 or $\geq$ 9.0 should be recollected (12).

Specimen Collection

Avoid sample collection within 30 minutes after eating a major meal. Properties such as pH of certain foods or liquids may interfere with the Insulin assay if present in oral fluids upon collection, so to minimize these effects, 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. Samples may be screened for possible blood contamination (13,14) 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.

It is important to record the time of specimen collection when samples are obtained relative to food or beverage consumption.

Sample Handling and Preparation

After collection, it is important to keep samples cold in order to avoid sample instability and 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

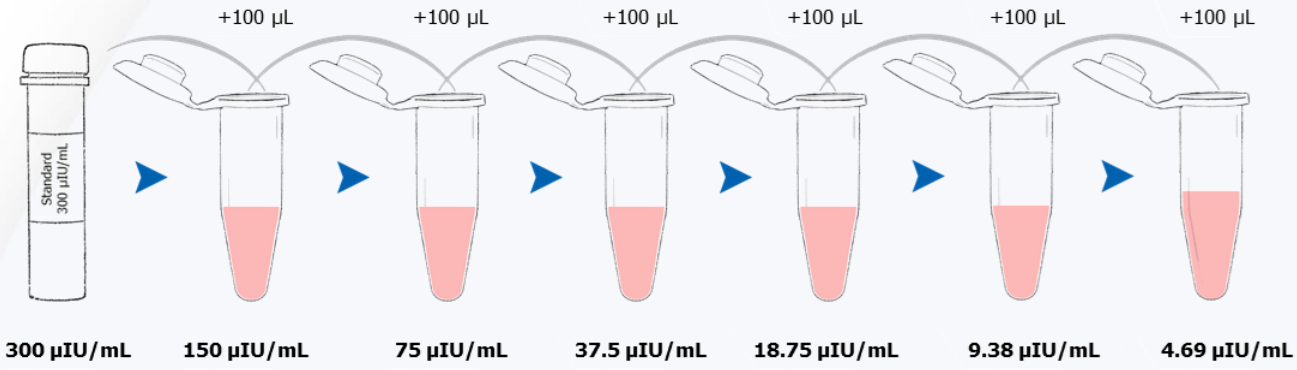
	Item	Quantity/Size
1	**Microtitre Plate** Coated with Streptavidin.	1 / 96 well
2	**Insulin Standard** 300 µIU/mL, in a saliva-like matrix. Serially dilute before use according to Reagent Preparation. Contains: Insulin, buffer, preservative.	1 vial / 500 µL
3	**Insulin Controls** High, Low, in a saliva-like matrix. Ready to use. Contain: Insulin, buffer, preservative.	2 vials / 500 µL each
4	**Insulin Capture/Detection Antibody Solution** Working Solution. Ready to use. Contains: anti-Insulin antibodies, preservative.	1 bottle / 12 mL
5	**Assay Diluent** Contains: phosphate buffer, pH indicator, preservative.	1 bottle / 60 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 25 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
- Either filter-based or muti-mode (monochromator) plate reader with the capability to read samples at 450 nm and 630 nm for reference subtraction.
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- Six small disposable polypropylene tubes for dilution of standard
- Pipette tips
- 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 60 mL of Assay Diluent used to dilute the standard curve 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 H_2O). ***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 Insulin Standard as follows:
    - Label six polypropylene microcentrifuge tubes or other small tubes 2 through 7.
    - Pipette 100 µL of Assay Diluent into tubes 2 through 6.
    - Serially dilute the Standard 1X by adding 100 µL of the 300 µIU/mL Standard (tube 1) to tube 2. Mix well.
    - After changing pipette tips, remove 100 µL from tube 2 to tube 3. Mix well.
    - Continue for tubes 4, 5, 6, and 7.
    - The final concentrations of standards for tubes 1 through 7 are, respectively, 300 µIU/mL, 150 µIU/mL, 75 µIU/mL, 37.5 µIU/mL, 18.75 µIU/mL, 9.38 µIU/mL, and 4.69 µIU/mL. Standard concentrations in pmol/L are 2,083, 1,042, 521, 260, 130, 65, and 32.5 pmol/L respectively.



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	300 Std	300 Std	Ctrl-H	Ctrl-H								
B	150 Std	150 Std	Ctrl-L	Ctrl-L								
C	750 Std	750 Std	SMP-1	SMP-1								
D	37.5 Std	37.5 Std	SMP-2	SMP-2								
E	18.75 Std	18.75 Std	SMP-3	SMP-3								
F	9.38 Std	9.38 Std	SMP-4	SMP-4								
G	4.69 Std	4.69 Std	SMP-5	SMP-5								
H	Zero	Zero	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.

Caution: Do not insert wells from one plate into a different plate.

Step 3:

- Pipette 25 µL of Standards, Controls, and saliva samples into appropriate wells.
- Pipette 25 µL of Assay Diluent into 2 wells to serve as the zero.

Step 4: Add 100 µL of the Conjugate Solution to each well using a multichannel pipette.

Step 5: Place adhesive cover provided over plate. Mix plate on a plate rotator for 30 seconds at 500 rpm and incubate at room temperature for a total of 1 hour.

Step 6: 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 7: Add 100 µL of TMB Substrate Solution to each well with a multichannel pipette.

Step 8: Mix on a plate rotator for 30 seconds at 500 rpm and incubate the plate in the dark (covered) at room temperature for a total of 15 minutes.

Step 9: Add 50 µL of Stop Solution with a multichannel pipette.

Step 10:

- 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 620 - 630 nm is recommended.)

Quality Control

The Salimetrics' High and Low Insulin 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. 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.
3. Samples with Insulin values greater than 300 µIU/mL should be diluted with Assay Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the assay results by the dilution factor.

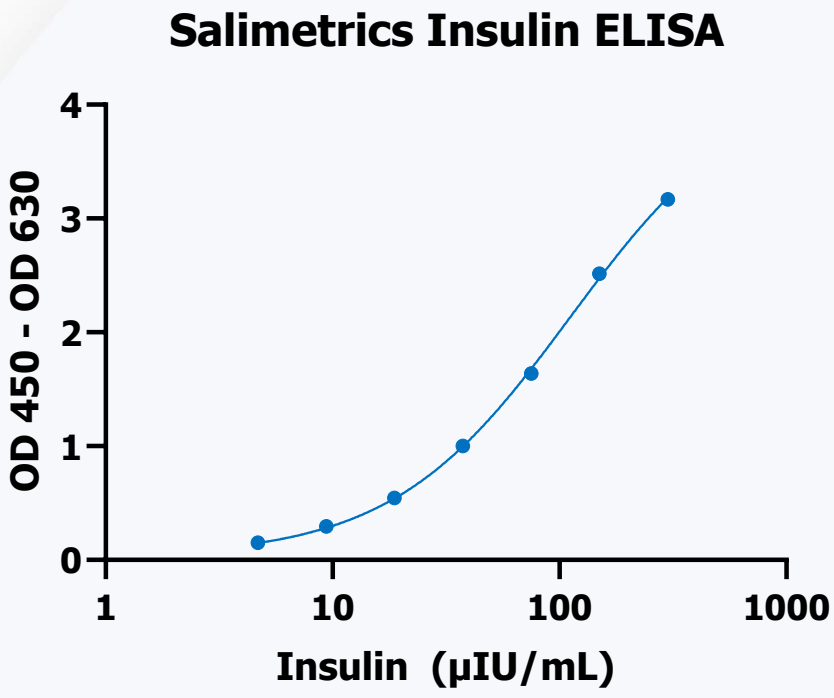
A new Standard Curve must be run with each full or partial plate.

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	Insulin (µIU/mL)
A1,A2	S1	3.1685	300
B1,B2	S2	2.5165	150
C1,C2	S3	1.6380	75
D1,D2	S4	1.0030	37.5
E1,E2	S5	0.5445	18.75
F1,F2	S6	0.2965	9.38
G1,G2	S7	0.1515	4.69
H1,H2	Zero	0.0120	0

Example: Insulin 4-Parameter Curve Fit



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Limitations

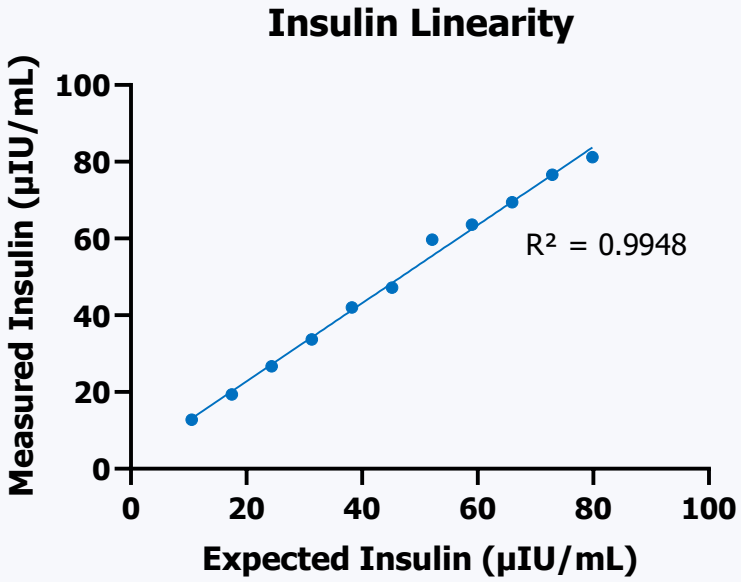
- Samples with Insulin values greater than 300 µIU/mL should be diluted with Assay Diluent and rerun for accurate results. To obtain the final Insulin concentration, multiply the concentration of the diluted sample by the dilution factor.
- A pH value should be obtained on samples that appear yellow or purple after the diluted conjugate solution is added and the plate is mixed (Step 6). Samples with pH values $\leq$ 4.0 or $\geq$ 9.0 should be recollected.
- 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 Insulin levels should be followed by additional testing and evaluation.

Salivary Insulin ELISA Kit Performance Characteristics

Linearity

Two saliva samples were diluted with each other proportionately and assayed.

Saliva Sample	Dilution	Expected (µIU/mL)	Observed (µIU/mL)	Recovery (%)
1		79.86	79.86	100%
	1:9	72.92	73.43	101%
	2:8	65.99	63.90	97%
	3:7	59.05	56.01	95%
	4:6	52.12	49.23	94%
	5:5	45.18	48.82	108%
	6:4	38.25	41.26	108%
	7:3	31.32	33.38	107%
	8:2	24.38	25.48	104%
	9:1	17.45	17.93	103%
2		10.51	10.51	100%



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Spike and Recovery

Three saliva samples containing different levels of endogenous Insulin were spiked with known quantities of Insulin and assayed.

Saliva Sample	Endogenous (µIU/mL)	Added (µIU/mL)	Expected (µIU/mL)	Observed (µIU/mL)	Recovery (%)
1	57.84	30.96	77.81	84.98	109%
		19.82	66.68	71.68	108%
		12.97	59.83	64.78	108%
2	22.61	30.96	49.27	57.84	117%
		19.82	38.14	42.88	112%
		12.97	31.29	34.17	109%
3	10.71	30.96	40.11	45.68	114%
		19.82	28.98	35.09	121%
		12.97	22.13	23.41	106%

Sample Dilution Recovery

Three saliva samples containing different levels of endogenous Insulin were diluted in Assay Diluent and assayed.

Saliva Sample	Dilution	Expected (µIU/mL)	Observed (µIU/mL)	Recovery (%)
1	Neat		13.87	
	x2	6.94	6.24	90%
	x4	3.47	3.16	91%
	x8	1.73	1.44	83%
2	Neat		75.93	
	x2	37.97	37.01	97%
	x4	18.98	18.94	100%
	x8	9.49	9.45	100%
3	Neat		26.65	
	x2	13.33	12.72	95%
	x4	6.66	6.60	99%
	x8	3.33	3.30	99%

Precision

The intra-assay precision was determined from the mean of 20 replicates each.

Saliva Sample	N	Mean (µIU/mL)	Standard Deviation (µIU/mL)	Coefficient of Variation (%)
1	20	109.47	7.13	5.6%
2	20	68.11	3.90	3.9%
3	20	38.73	2.34	3.4%
4	20	32.60	2.02	5.9%
5	20	11.75	0.75	3.7%

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

Saliva Sample	N	Mean (µIU/mL)	Standard Deviation (µIU/mL)	Coefficient of Variation (%)
1	20	92.42	3.97	4.3%
2	20	51.19	4.19	8.2%
3	20	24.44	3.61	14.8%
4	20	32.20	2.26	7.0%
5	20	13.30	1.00	7.5%

Sensitivity

Analytical 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 µIU/mL level. The minimal concentration of Insulin that can be distinguished from 0 is 0.39 µIU/mL.

Functional Sensitivity

The functional sensitivity was determined by assaying 60 saliva samples at a concentration level resulting in a CV of approximately 20%. The functional sensitivity of the salivary Insulin ELISA is 0.41 µIU/mL.

Antibody Specificity

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary Insulin EIA
Pro Insulin	100 ng/mL	ND
Glucagon	100 ng/mL	ND
C-Peptide	75 ng/mL	ND

ND = None detected

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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."

Salimetrics, LLC
5962 La Place Court, Suite 275
Carlsbad, CA 92008, USA
(T) 760.448.5397
(F) 814.234.1608
800-790-2258 (USA & Canada only)
www.salimetrics.com
support@salimetrics.com

Salimetrics, LLC
101 Innovation Blvd., Suite 302
State College, PA 16803, USA
(T) 814.234.2617
(F) 814.234.1608
800-790-2258 (USA & Canada only)
www.salimetrics.com
support@salimetrics.com

Effective: June 28, 2024