SALIVARY MELATONIN

ENZYME IMMUNOASSAY KIT

For Research Use Only
Not for use in Diagnostic Procedures

Item No. 1-3402, (Single) 96-Well Kit;
1-3402-5, (5-Pack) 480 Wells

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TABLE OF CONTENTS

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Intended Use

The Salimetrics® Melatonin Enzyme Immunoassay Kit is a competitive immunoassay specifically designed and validated for the quantitative measurement of salivary Melatonin. It is not intended for diagnostic use. It is intended only for research use in humans and some animals. 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

Melatonin (N-acetyl-5-methoxytryptamine) is a compound secreted mainly by the pineal gland, but, synthesized also in many other tissues and cells. In humans, nocturnally peaking oscillations of Melatonin are involved in sleep-wakefulness where Melatonin concentrations are lower during the day (1, 2). In recent years, the role of Melatonin and its metabolites have been identified as potent, broad acting antioxidants and free radical scavengers in addition to playing a role in the upregulation of antioxidant enzymes (reviewed in 3,4).
Melatonin levels in plasma are paralleled by corresponding variations in saliva where the saliva concentrations are about 30% of that found in plasma (5). Measurement of salivary Melatonin is advantageous, especially to avoid invasive venipuncture procedures (6).

Test Principle

This is a competitive immunoassay kit. Melatonin in standards and samples compete with Melatonin conjugated to horseradish peroxidase for the antibody binding sites on a microtitre plate. After incubation, unbound components are washed away. Bound Melatonin 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 Melatonin Enzyme Conjugate detected is inversely proportional to the amount of Melatonin present in the sample (7).

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 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.

It is important to record the time and date of specimen collection.

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** Coated with rabbit anti-Melatonin monoclonal antibodies#.	1/96 well
2	**Melatonin Standard** 50 pg/mL, in a saliva-like matrix. Serially dilute before use according to Reagent Preparation. Contains: Melatonin, buffer, preservative.	1 vial / 1.5mL
3	**Melatonin Controls** High and Low, in a saliva-like matrix. High control is ready to use. Low control formulated for stability when stored at 4°C. Prepare before use according to Reagent Preparation. Contains: Melatonin, buffer, preservative.	2 vials / 1 mL High 200 µL Low
4	**Melatonin Enzyme Conjugate** Concentrate. Dilute before use with Melatonin Assay Diluent. (See step 5 of Procedure). Contains: Melatonin conjugated to HRP, buffer, preservative.	1 vial / 75 µL
5	**Melatonin Assay Diluent** Contains: phosphate buffer, sodium chloride, protein, stabilizer and preservative.	1 bottle / 30 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 Covers**	2

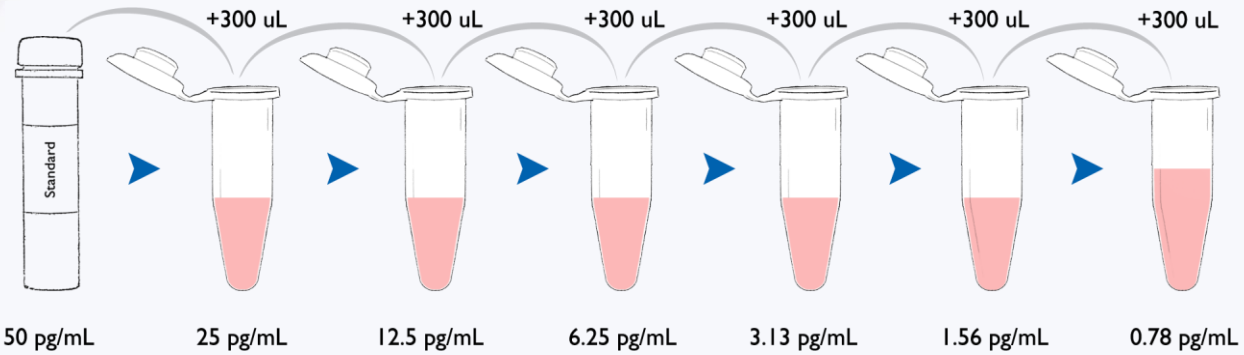
This product is covered by U.S. patents: 5,675,063; 7,429,487 and patent application 61/794,713

Materials Needed But Not Supplied

- Precision pipette to deliver 10 µL to 300 µL
- Precision multichannel pipette to deliver 50 µL to 100 µL
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of operating at 500 rpm & 2-8°C.
- Plate reader with 450 nm and 620 to 630 nm reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 8 mL
- Six small disposable polypropylene tubes for dilution of standard
- Pipette tips
- Serological pipette to deliver up to 8 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 Melatonin Assay 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.)
- **Dilute Melatonin Low Control by pipetting 1000 µL of the Melatonin Assay Diluent directly into the Melatonin Low Control vial.**
- Prepare serial dilutions of the Melatonin Standard as follows:
    - Label six polypropylene microcentrifuge tubes or other small tubes 2 through 7.
    - Pipette 300 µL of Melatonin Assay Diluent into tubes 2 through 7.
    - Serially dilute the standard 2X by adding 300 µL of the 50 pg/mL standard (tube 1) to tube 2. Mix well.
    - After changing pipette tips, transfer 300 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, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.25 pg/mL, 3.13 pg/mL, 1.56 pg/mL, and 0.78 pg/mL.
    - Conversion: 1 pg/mL = 4.3 pmol/L
    - Melatonin Assay Diluent is used as the Zero Standard.



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	50 Std	50 Std	Ctrl-H	Ctrl-H								
B	25 Std	25 Std	Ctrl-L	Ctrl-L								
C	12.5 Std	12.5 Std	SMP-1	SMP-1								
D	6.25 Std	6.25 Std	SMP-2	SMP-2								
E	3.13 Std	3.13 Std	SMP-3	SMP-3								
F	1.56 Std	1.56 Std	SMP-4	SMP-4								
G	0.78 Std	0.78 Std	SMP-5	SMP-5								
H	0 Std	0 Std	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 8 mL of Melatonin Assay Diluent into a disposable tube. (Scale down proportionally if not using a full plate). Set aside for Step 5.

Step 4:

- Pipette 100 µL of standards, high control, diluted low control (see page 9), and saliva samples into appropriate wells.
- Pipette 100 µL of Melatonin Assay Diluent into 2 wells to serve as the Zero Standard.

Step 5: Dilute the Enzyme Conjugate 1:500 by adding 16 µL of the conjugate to the 8 mL of Melatonin Assay 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: Place adhesive cover provided over plate. Mix plate on a plate rotator ***continuously*** at 500 rpm for 3 hours at **2-8°C**.

Step 7: 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. If using a plate washer, blotting is still recommended after the last wash.

Step 8: Add 100 µL of TMB Substrate Solution to each well with a multichannel pipette.

Step 9: Mix plate on a plate rotator ***continuously*** at 500 rpm while incubating the plate in the dark (covered) at room temperature for 30 minutes.

Step 10: Add 50 µL of Stop Solution with a multichannel pipette.

Step 11:

- 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 to 630 nm is recommended.)

Quality Control

The Salimetrics' High and Low Melatonin 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. 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).
3. 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.
4. Samples with Melatonin values greater than 50 pg/mL should be diluted with Melatonin Assay Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the results by the dilution factor. Dilution of a sample by more than 2-fold is not recommended.

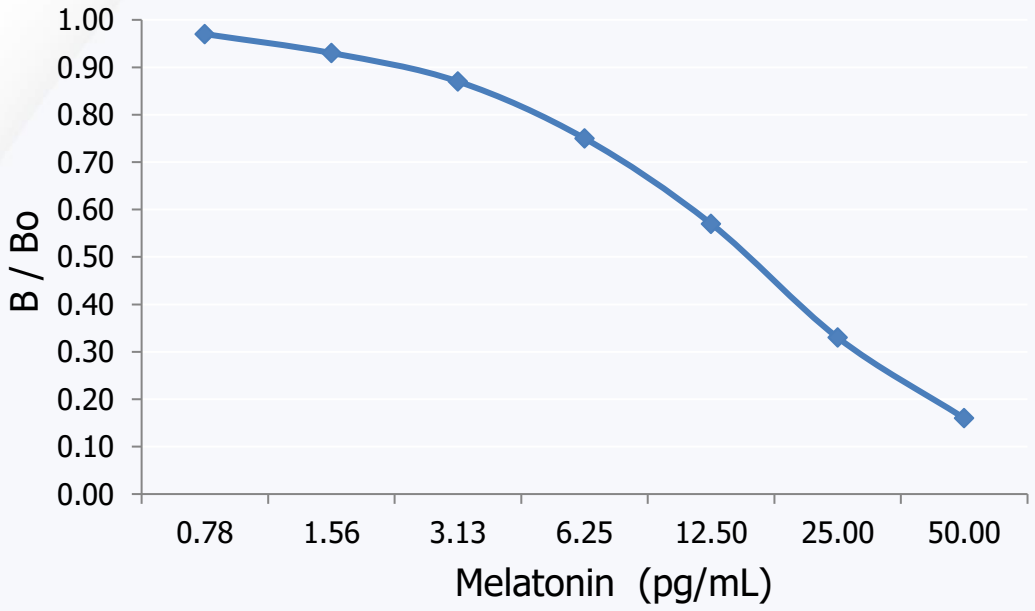
A new Standard Curve must be run with each full or partial plate.

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/Bo	Melatonin (pg/mL)
A1,A2	S1	0.282	0.16	50.0
B1,B2	S2	0.573	0.33	25.0
C1,C2	S3	0.975	0.57	12.5
D1,D2	S4	1.287	0.75	6.25
E1,E2	S5	1.499	0.87	3.13
F1,F2	S6	1.597	0.93	1.56
G1,G2	S7	1.662	0.97	0.78
H1,H2	Zero	1.720	1.00	0.0

Example: Melatonin 4-Parameter Curve Fit



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Limitations

- Samples with Melatonin values greater than 50 pg/mL should be diluted with Melatonin Assay Diluent and rerun for accurate results. To obtain the final Melatonin concentration, multiply the concentration of the diluted sample by the dilution factor. Dilution of a sample by more than 2-fold is not recommended.
- 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.
- Avoid more than 2 freeze-thaw cycles after the initial freeze/thaw.
- Any quantitative results indicating abnormal Melatonin levels should be followed by additional testing and evaluation.

Salivary Melatonin Example Ranges and Method Comparison*

Saliva samples were collected just before bedtime (PM), just after arising from sleep (AM) and at mid-day (Noon). The samples were tested with the Salimetrics Salivary Melatonin EIA Kit and a second salivary Melatonin kit supplied by another commercial source that has been commonly used in the sleep field to determine dim light Melatonin onset (DLMO). The results for salivary range determination are listed in the tables below. Q1 is equivalent to the 1st quartile where 25% of the results were less than or equal to this value (Q1). Q3 is equivalent to the 3rd quartile where 25% of the results were greater than or equal to this value (Q3). The correlation between the two methods was 0.87. No significant difference was found in comparison of AM, Noon or PM samples (Salimetrics vs. Other) (T-Test, $P > 0.05$).

Salimetrics Melatonin EIA				
Time	**N**	**Median (pg/mL)**	**Q1 (pg/mL)**	**Q3 (pg/mL)**
AM	29	8.3	5.2	11.9
Noon	32	4.2	2.3	5.6
PM	25	12.7	7.3	21
Other Melatonin ELISA Assay				
Time	**N**	**Median (pg/mL)**	**Q1 (pg/mL)**	**Q3 (pg/mL)**
AM	47	11.4	6.7	17.1
Noon	59	2.8	1.6	5.7
PM	46	15.7	8.1	22

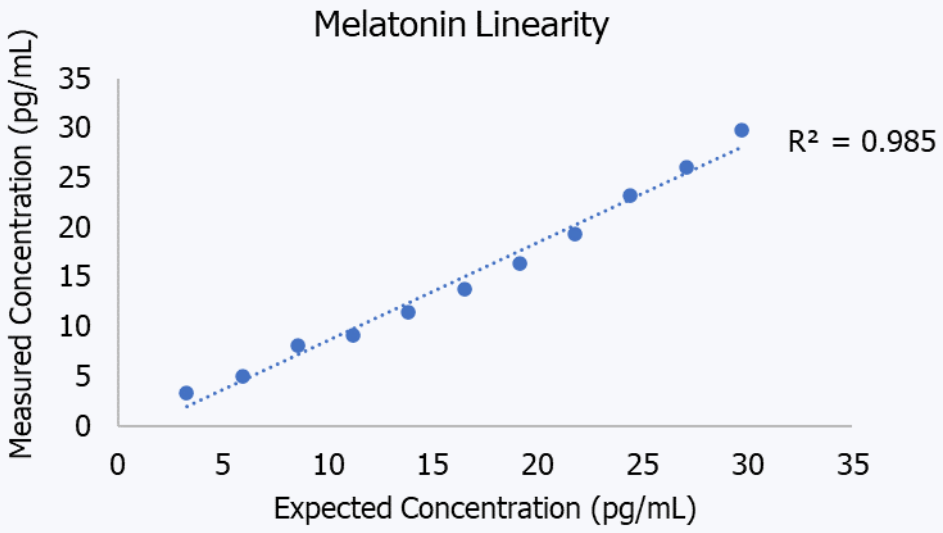
*To be used as a guide only. Each laboratory should establish its own range.

Salivary Melatonin EIA Kit Performance Characteristics

Linearity

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

Saliva Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1		29.73	29.73	100%
	1:9	27.09	26.03	96%
	2:8	24.44	23.22	95%
	3:7	21.80	19.26	88%
	4:6	19.15	16.35	85%
	5:5	16.51	13.80	84%
	6:4	13.87	11.38	82%
	7:3	11.22	9.15	82%
	8:2	8.58	8.06	94%
	9:1	5.94	4.97	84%
2		3.29	3.29	100%



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Spike and Recovery

Two saliva samples containing different levels of endogenous Melatonin were spiked with known quantities of Melatonin and assayed.

Saliva Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	9.5	2.37	10.22	10.72	105%
		2.03	9.88	9.85	100%
		0.00	7.85	8.00	102%
2	4.9	2.37	5.95	6.59	111%
		2.03	5.61	5.61	100%
		0.00	3.58	3.67	103%

Sample Dilution Recovery

Three saliva samples containing different levels of endogenous Melatonin were diluted in assay diluent and assayed.

Saliva Sample	Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	Neat		39.67	
	1:1	19.84	15.53	78%
2	Neat		21.06	
	1:1	10.53	7.56	72%
3	Neat		17.17	
	1:1	8.59	6.18	72%

Precision

The intra-assay precision was determined from the mean of 19-20 replicates each.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
1	20	33.69	1.53	4%
2	19	13.07	0.79	5%
3	20	12.02	0.73	5%
4	20	7.99	0.81	4%
5	19	3.17	0.49	13%

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

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
1	20	40.66	3.09	8%
2	20	19.77	1.91	10%
3	20	10.78	1.31	12%
4	20	10.43	1.18	11%
5	20	5.22	1.11	21%

Sensitivity

Analytical Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 30 sets of duplicates at the 0 pg/mL level. The minimal concentration of Melatonin that can be distinguished from zero is 1.35 pg/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 Melatonin EIA Kit is 3.31 pg/mL.

Comparative Saliva Sample Precision

The precision of the Salimetrics Melatonin EIA Kit was directly compared to the precision of a leading commercial assay. The Coefficient of Variation (CV) of the Mean, Standard Deviation, Minimum and Maximum values from the paired samples (total sample number was 36) in the example range study were compared between the two assays. Note: One of the samples collected was not tested in the Salimetrics assay due to insufficient volume.

Assay Kit	N	Mean (%CV)	Standard Deviation (%CV)	Min (%CV)	Max (%CV)
Salimetrics	35	5.9	4.5	0.2	17%
Other	36	6.5	7.5	0	33%

Antibody Specificity

Chemicals with structural similarities to Melatonin were spiked into saliva up to 500 pg/mL and tested as samples. The % Cross Reactivity was determined by dividing the standard curve 50% binding concentration (EC50) by the 50% concentration (EC50) of the cross reactant and then multiplied by 100.

Compound	Spiked Concentration (pg/mL)	% Cross-reactivity in Salivary Melatonin EIA
Serotonin hydrochloride	7.8 - 500	< 0.16
N-Acetyl-5-hydroxytryptamine	7.8 - 500	< 0.16
5-Methoxytryptamine	7.8 - 500	< 0.16
6-Hydroxymelatonin	7.8 - 500	< 0.16
L-Tryptophan	7.8 - 500	< 0.16
6-Chloromelatonin*	7.8 - 500	9.00
5-Methoxytryptophol	7.8 - 500	< 0.16
Caffeine	7.8 - 500	< 0.16
AFMK**	7.8 - 500	9.70

* Presents in urine only

** AFMK = N1-acetyl-N2-formyl-5-methoxykynuramine

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"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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