Version 26-04/25

User's Manual

Salivary Melatonin ELISA Kit

REF **DEIA-S10024**

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RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The Salimetrics Salivary Melatonin 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 false 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.

General Description

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. 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. Melatonin levels in plasma are paralleled by corresponding variations in saliva where the saliva concentrations are about 30% of that found in plasma. Measurement of salivary melatonin is advantageous, especially to avoid invasive venipuncture procedures.

Principles of Testing

A microtitre plate is coated with rabbit monoclonal antibodies to melatonin. Melatonin in standards, controls and unknowns compete with melatonin linked to horseradish peroxidase for the antibody binding sites on the microplate. After incubation, unbound components are washed away. Bound melatonin peroxidase is measured by the reaction of the horseradish peroxidase enzyme with the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with 2-molar acid solution. Optical density is read on a standard microplate reader at 450 nm. The amount of melatonin-tagged peroxidase detected, as measured by the intensity of color, is inversely proportional to the amount of melatonin present in the sample.

Reagents And Materials Provided

1. Microtitre Plate Coated with rabbit antimelatonin monoclonal antibodies. 1/96-well.
2. Melatonin Standard in a Trizma buffered solution with stabilizer protein and a nonmercury preservative. Serially dilute before use according to Reagent Preparation. Higher order melatonin CRM: 50 pg/mL. 1 vial, 1.5 mL.
3. Melatonin Controls. High, Low, in a Trizma buffered solution with stabilizer protein and a non-mercury preservative. Refer to control insert for ranges. 2 vials, 1 mL each.
4. Wash Buffer Concentrate (10X). Dilute before use according to Reagent Preparation. Contains: phosphate

buffer, detergent, preservative. 1 bottle/ 100 mL.

5. 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.
6. Melatonin Assay Diluent. Ready to use. Contains a Trizma buffer with protein stabilizer and preservative. 1 bottle/ 30 mL.
7. TMB Substrate Solution Non-toxic, ready to use. 1 bottle/25 mL.
8. 2 M Stop Solution Contains: sulfuric acid. 1 bottle/ 12.5 mL.

Materials Required But Not Supplied

1. Precision pipettes to deliver 10 µL to 300 µL.
2. Precision multichannel pipettes to deliver 50 µL to 100 µL.
3. Vortex.
4. Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm.
5. Plate reader with 450 nm and 620-630 nm reference filters.
6. Computer software for data reduction.
7. Deionized water.
8. Reagent reservoirs.
9. Disposable polypropylene tubes to hold at least 8 mL.
10. Small disposable polypropylene tubes for dilution of standards.
11. Pipette tips.
12. Serological pipette to deliver up to 8 mL.
13. Centrifuge capable of 1500 x g (@3000 rpm).

Storage

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

Specimen Collection And Preparation

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.

The preferred method for collecting whole saliva is by unstimulated passive drool. Donors may collect whole saliva by tilting the head forward, allowing the saliva to pool on the floor of the mouth, and then passing the saliva through the Saliva Collection Aid (SCA), into a polypropylene vial. Samples from adults and from children ages 6 and above may also be collected using the SalivaBio Oral Swab (SOS). Samples from children under the age of 6 may be collected with the SalivaBio Children's Swab (SCS). The SalivaBio Infant's Swab (SIS), is available for use with children under the age of 6 months.

Do not use Salivettes, sorbettes, cotton, or polyester materials to collect samples. Salimetrics has not validated these substances for salivary Melatonin collection. Samples visibly contaminated with blood should be recollected. Samples may be screened for possible blood contamination using our Blood Contamination ELISA Kit. Do not use dipsticks, which result in false positive values due to salivary enzymes.

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 -20°C within 4 hours of collection. (Samples may be stored at -20°C for up to 6 months. For long term storage, > 6 months, store at -60°C or lower.) Melatonin levels will decrease ≥ 20% after 4 days at 2 - 8°C.

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

Freezing saliva samples will precipitate mucins. On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding, leading to falsely elevated 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. Centrifuge/re-centrifuge saliva samples each time that they are thawed. Avoid more than 2 freeze-thaw cycles after the initial freeze/thaw.

Reagent Preparation

1. 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.
2. 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.
3. Prepare 1X wash buffer by diluting wash buffer concentrate 10-fold with room-temperature deionized water (100 mL of wash buffer concentrate (10X) to 900 mL of deionized H2O). 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.)
4. Prepare serial dilutions of the Melatonin standard as follows:

Label six 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 (by vortexing ensuring no unmixed dilution sample is trapped in the cap of the vial.) 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, respectively, are 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. Melatonin Assay Diluent is used for the Zero Standard. Conversion: 1 pg/mL = 4.3 pmol/L.

Assay Procedure

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

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 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:

1. Pipette 100 µL of standards, controls, and unknown samples into appropriate wells. Standards, controls, and unknown samples should be assayed in duplicate.
2. Pipette 100 µL of Melatonin Assay Diluent into 2 wells for the 0 Standard.

Step 5: Dilute the enzyme conjugate 1:500 by adding 16 µL of the conjugate to the 8 mL of melatonin assay diluent prepared in Step 3. (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 constantly on a plate rotator at 500 rpm and incubate at 2 - 8°C for a total of 3 hours.

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 by inverting the plate 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 8: Add 100 µL of TMB substrate solution to each well with a multichannel pipette.

Step 9: Incubate the plate in the dark at room temperature for 30 minutes mixing constantly on a plate rotator at 500 rpm.

Step 10: Add 50 µL of 2M stop solution with a multichannel pipette.

Step 11:

1. 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.
2. Caution: Spillage may occur if mixing speed exceeds 600 rpm.
3. Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.
4. Read in a plate reader at 450 nm with a secondary filter at 620 to 630 nm. Performance characteristics are not known without secondary or reference filter. Read the plate within 10 minutes of adding 2M stop solution.

Quality Control

The Salimetrics' High and Low salivary 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

Calculation

1. Compute the average optical density (OD) for all duplicate wells.
2. Calculate the percent bound (B/Bo) for each standard, control and unknown by dividing the OD of each well (B) by the average OD for the 0 (Bo).

3. Determine the concentrations of the controls and unknowns 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 further 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.

When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.

Typical Standard Curve

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

Well	Sample	Average OD	B/Bo	Melatonin (pg/mL)
A1,A2	S1	0.348	0.14	50.0
B1,B2	S2	0.754	0.31	25.0
C1,C2	S3	1.363	0.56	12.5
D1,D2	S4	1.868	0.77	6.25
E1,E2	S5	2.122	0.87	3.13
F1,F2	S6	2.238	0.92	1.56
G1,G2	S7	2.336	0.96	0.78
H1,H2	0	2.427	1.00	0.0

Precision

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

Saliva Sample	N	Mean pg/mL	Standard Deviation pg/mL	% CV
1	20	44.3	1.2	2.7
2	20	25.9	1.3	5.0
3	20	13.5	0.5	3.5
4	20	8.0	0.4	5.4
5	20	4.0	0.4	9.7

The inter-assay precision was determined from the average of 20 replicates across 10 runs, replicates of 2

Saliva Sample	N	Mean pg/mL	Standard Deviation pg/mL	% CV
1	20	42.9	3.2	7.6
2	20	24.2	1.4	5.9
3	20	13.4	1.1	8.4
4	20	6.8	0.7	10.6
5	20	3.8	0.6	14.6

per run.

Sensitivity

Analytical Sensitivity: The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 10 sets of duplicates randomized across the plate within one run at the 0 pg/mL level. The minimal concentration of Melatonin that can be distinguished from 0 is 0.58 pg/mL.

Functional Sensitivity: The functional sensitivity was determined as the concentration of 11 saliva samples, tested as 20 replicates each, resulting in a CV of 30% or less. The functional sensitivity of the Salivary Melatonin ELSIA is 1.9 pg/mL.

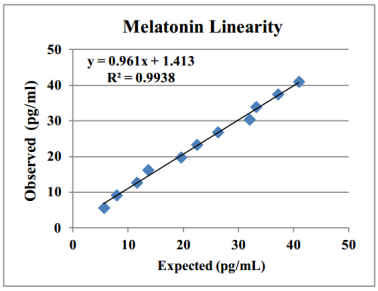
Specificity

Chemicals with structural similarities to melatonin were spiked into saliva to up to 2000 pg/mL and tested as samples. For cross reactants, the concentration of Melatonin at the EC50 (B/Bo=0.5) was divided by the concentration of the cross reactant at its

Linearity

Two saliva samples were diluted with each other proportionately and assayed in replicates of two.

Sample Mixtures				
Low	High	Observed pg/mL	Expected pg/mL	Recovery (%)
100%	0%	5.6	5.6	NA
90%	10%	7.9	9.1	86
80%	20%	11.5	12.6	91
70%	30%	13.6	16.2	84
60%	40%	19.5	19.7	99
50%	50%	22.4	23.2	96
40%	60%	26.2	26.8	98
30%	70%	32.0	30.3	105
20%	80%	33.2	33.9	98
10%	90%	37.1	37.4	99
0%	100%	40.9	40.9	NA
			Average	95



Recovery

Three saliva samples were spiked with different levels of Melatonin and assayed. The recovery is based on the prediction of the spike (minus endogenous), not the total concentration.