Human Melatonin ELISA Kit

Enzyme Immunoassay for the quantitative determination of human Melatonin in saliva

Catalog number: ARG80864

For research use only. Not for use in diagnostic procedures.

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MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: No. 22, Ln. 227, Gongyuan Rd., Hsinchu City 300, Taiwan

Phone: +886 (3) 562 1738

Fax: +886 (3) 561 3008

Email: info@arigobio.com

INTRODUCTION

Melatonin plays an important role in the synchronisation and characterisation of sleep-wake cycles. Endogenous melatonin is a hormone produced in the pineal gland during the dark phase of the day-night cycle. Its secretion is controlled by an endogenous oscillator that is inhibited by light. Daytime melatonin levels are low or even undetectable and rise in the evening during decreasing light intensity.

Endogenous Clock

In humans, circadian Melatonin profiles in body fluids (saliva, serum, plasma) provide an excellent marker for the setting of the endogenous clock. Night-time levels peak at around 03:00 h am (acrophase) in most healthy individuals. As a general modulator of human and other vertebrate biorhythm, Melatonin is involved in the timing of functions such as sleep, mood, reproduction and immune system activities.

Onset of Melatonin Production

Both the intra-individual Melatonin onset and acrophase are very stable. Therefore, it will be sufficient to measure the onset of Melatonin production only, once a whole Melatonin profile has been established. The onset point of melatonin secretion under dim light conditions is called **DLMO (Dim Light Melatonin Onset)**. In contrast to other circadian markers like cortisol, melatonin is the preferred circadian marker because of its robustness even in the presence of various exogenous factors like mental pressure or others.

PRINCIPLE OF THE ASSAY

This assay employs the competitive enzyme immunoassay technique. A highly specific Melatonin antibody has been pre-coated onto a microtiter plate. Melatonin containing samples or standards and a Melatonin-Biotin conjugate are given into the wells of the microtiter plate. Biotin labeled and free Melatonin compete for the antibody binding sites. After washing, the enzyme label, streptavidin conjugated to horseradish peroxidase (HRP) is added. After incubation at room temperature, the wells are washed with diluted washing solution to remove unbound material. A substrate solution is added and incubated, resulting in the development of a blue color. The color development is inhibited by the addition of a stop solution, and the color turns yellow. The yellow color is measured at 450 nm. The concentration of Melatonin is indirectly proportional to the color intensity of the test sample.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated Microplate	12 strips x 8-well	4°C
Biotin-Melatonin Conjugate	5.5 ml (Ready-to-use)	4°C
HRP-Streptavidin Conjugate	11 ml (Ready-to-use)	4°C
Standards (A:0.6, B:1.5, C:4.0, D:10.0, E:25.0 pg/ml)	5 vials (Lyophilized)	4°C
Control Low (Target: 3.8 pg/ml) (Expect range: 1.5 - 6.0 pg/ml)	1 vial (Lyophilized)	4°C
Control High (Target 11.4 pg/ml) (Expect range: 4.6 – 18.3 pg/ml)	1 vial (Lyophilized)	4°C

Pretreatment Buffer	5 ml (Ready-to-use)	4°C
Neutralizing Solution	5 ml (Ready-to-use)	4°C
Blanking Reagent	1 vial (Lyophilized)	4°C
Incubation Buffer	12 ml (Ready-to-use)	4°C
10X Wash Buffer	100 ml	4°C
TMB Substrate	11 ml (Ready-to-use)	4°C (Protect from light)
STOP solution	11 ml (Ready-to-use)	4°C
Plate sealer	3	RT

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- Orbital microplate shaker: 3 mm (0.1118 in) 600 ± 10 rpm or 19 mm (0.75 in) 170 ± 10 rpm.
- Saliva Collection Devices
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- This kit is intended for professional use only. Users should have a thorough understanding of this protocol for the successful use of this kit. Only the test instruction provided with the kit is valid and has to be used to run the assay. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
- Wear protective gloves, clothing, eye, and face protection especially

while handling blood or body fluid samples.

- Store the kit at 4°C at all times.
- All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
- If crystals are observed in the 10X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials and devices are prepared ready at the appropriate time.
- The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.
- The blanking reagent contains a saturated melatonin solution. Avoid any contamination of other reagents of this kit. Change disposable tips after each pipetting step.
- Change pipette tips between the addition of different reagent or samples.
- Samples contain azide cannot be assayed.

SAMPLE COLLECTION & STORAGE INFORMATION

Collection:

- Collect saliva using the Saliva Collection Devices. The devices can absorb up to 3 ml of saliva. The procedure calls for 0.2 ml of saliva.
- The saliva samples absorbed in the cotton swab may be stored in the saliva collection device for up to 7 days at 2-8°C. If not assayed within one week after collection, samples should be frozen and may be stored for at least 6 months at ≤-20°C. Repeated freeze-thaw cycles should be avoided.
- If shipment is needed, the collected saliva s samples must be shipped to the laboratory within two days at 2-8°C.
- Do not use cotton swabs containing citrate acid.

Sport: Patients should perform the collection on an evening without sporting activities and any intense efforts.

Light: Bright light can suppress melatonin production. Therefore, it is important to avoid bright light during the test. Muted lighting from a reading lamp or from the television is preferable. When collecting saliva at night, a dim flash light or a ≤100 lux yellow light should be used in order to avoid a possible light influence on the melatonin profile.

Eating: Nothing should be eaten during the collection time. The last meal must be taken at least 30 minutes before starting the collection. Bananas and chocolate should not be eaten during the entire day before the collection. Do not stimulate saliva flow by chewing gums or eating lemons.

Drinking: Drinks containing artificial colorants, caffeine (coffee, black or green tea, iced tea, cola) or alcohol are not allowed on the evening of the

collection.

Medicines: On the collection day, if possible, no aspirin and medicines that contain ibuprofen (Algiofor, Brufen, Dysmenol, Dolocyl, Ecoprofen) should be taken. If your sleep or sleep-wake rhythm is treated with melatonin, this must be discontinued at least one week before the collection.

Brushing teeth: Patients should avoid brushing their teeth, with or without toothpaste, during sampling periods. It is likely that patients with gingivitis will contaminate the saliva with plasma or even whole blood leading to unknown consequences.

REAGENT PREPARATION

- 1X Wash buffer: Dilute 10X Wash buffer into distilled water to yield 1X Wash buffer. The diluted wash buffer can be stored at 2-8°C up to 6 months.
- Blanking Reagent: Dissolve in 1 ml Incubation Buffer. The reconstituted Blanking Reagent can be stored at 2-8°C up to 4 months.
- Standards and Controls: Reconstitute with 1ml Incubation Buffer. The reconstituted Standards and Controls can be stored at 2-8°C up to 4 months.
- Sample recovery from saliva collection devices: Centrifuge the collection devices for around 5 min at 3000 rpm (~1500x g). Discard the suspended insert with the swap and store the tube at 2-8°C or-20°C.
- Pretreatment of Samples and Controls: Pipet 200 µl of controls and saliva samples, respectively, into correspondingly marked, clean polypropylene, tubes. Add 25 µl of Pretreatment Buffer to each tube using a

multipipettor device. Vortex for 5 seconds and keep the tubes at 18-28°C for 10 minutes. Add 25 µl of neutralizing solution to each tube using a multipipettor device. Vortex for 5 seconds. Centrifuge the pre-treated samples at 10,000 rpm for 5 minutes.

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 18-28°C) before use. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame. Return unused strips immediately to the plastic pouch containing the desiccant pack and reseal along the entire edge of zip-seal. Store at 2-8°C for up to 2 months.
2. Aspirate each well and wash, repeating the process one times for a **total 2 washes**. Wash by filling each well with **1X Wash Buffer (350 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
3. Add **100 µl Blanking Reagent (blank), Standards, controls** in duplicate into the appropriate wells. Add **100 µl Incubation Buffer** in duplicate into another wells as **Zero Calibrator**.
4. Add **100 µl of pretreated samples** into the appropriate wells.
5. Cover wells with adhesive foil and incubate for **16-20 hours at 2-8°C**.
6. Remove and discard plate sealer. Add **50 µl Biotin-Melatonin** into each well mix thoroughly. Cover the plate with a plate sealer and shake the plate on a microplate shaker at ~**600rpm for 1 min**. Then incubate for **3 hours at 2-**

8°C.

7. Remove and discard the plate sealer. Aspirate each well and wash, repeating the process 3 times for a **total 4 washes**. Wash by filling each well with **1X Wash Buffer (350 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
8. Add **100 µl** of **HRP-Streptavidin** to each well mix thoroughly. Cover the plate with a new plate sealer. Incubate the plate on a microplate shaker at ~600rpm for **60 minutes at RT.**
9. Aspirate and **wash wells as step 7**.
10. Add **100 µl** of **TMB Substrate (should be equilibrated to room temperature (RT, 18-28°C) before use)** to each well mix thoroughly or incubate the plate on a microplate shaker at 600 rpm. Incubate for **25-35 minutes at room temperature** in dark.
11. Add **100 µl** of **Stop Solution** to each well. Remove air bubbles by pricking them with a pipette tip. Gently tap the plate to ensure thorough mixing.
12. Read the OD with a microplate reader at **450 nm** within 30 min after adding Stop solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using lin/log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Average the duplicate values, subtract the average of the blank wells and record averages (=corrected average absorbance).

4. Using the corrected average absorbance value for each sample determine the corresponding concentration from the standard curve.

5. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results. The diluted samples must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

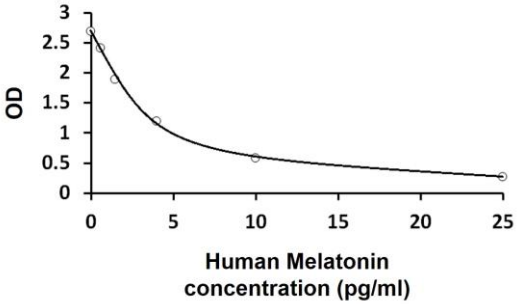
6. Calculate the binding (B) of each pair of calibrator wells as a percent of incubation buffer (B0), with the blank-corrected absorbance of the incubation buffer taken as 100 %.

B/ BO (%) = percent bound =

(net absorbance / net absorbance of incubation buffer)*100

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

LoB (detection limit): The analytical sensitivity was calculated from the mean plus two standard deviations of twenty (20) replicate analyses of Standard 0 is 0.5 pg/ml.

LoQ (Limit of Quantification): 1.6-20.5 pg/ml

Specificity

The following substances were tested for cross reactivity of the assay:

Substance	Cross reactivity (%)
Melatonin	100
Serotonin	< 0.001
6-sulfatoxymelatonin	< 0.001
N-acetylserotonin	0.045
5-hydroxy-indole acetic acid	< 0.001
5-methoxytryptamine	0.007
5-methoxytryptophane	< 0.001

2-methyl-5-hydroxytryptamine	< 0.001
5-methoxypsoralen	< 0.001
5-methoxytryptophol	0.002
Chloramelatonin	1.3
caffeine	< 0.001
caffeine acid	< 0.001
soluble coffee	< 0.001
soluble coffee decaffeinated	< 0.001

Intra-assay and inter-assay precision

The CV value of intra-assay precision was 12.6% and the CV value of inter-assay precision was 22.9%.

Recovery

72-112.2%