Human Melatonin ELISA Kit

Enzyme Immunoassay for the quantification of human Melatonin in serum and plasma.

Catalog number: ARG80461

Package: 96 wells

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INTRODUCTION

The pineal gland ("corpus pineale") has been called a neuroendocrine transducer because of its important role in photoperiodism. The major hormone of the pineal gland is N-acetyl-5-methoxy-tryptamine or melatonin which is synthezised from the amino acid tryptophane. Melatonin has its highest levels in plasma during nighttime. Its characteristic nocturnal surge appears to encode temporal information such as length of night. Regulation of the melatonin secretion is under neural control.

Sympathetic innervation seems to play a major role via its release of noradrenaline. Altered patterns and/or levels of melatonin secretion have been reported to coincide with sleep disorders, “jet lag“, depression, stress, schizophrenia, hypothalamic amenorrhea, pregnancy, anorexia nervosa, some forms of cancer, immunological disorders as well as control of sexual maturation during puberty. Most of the circulating melatonin is metabolized in the liver to 6-hydroxymelatonin and subsequently to 6-sulfatoxymelatonin which is excreted into the urine. The concentration of 6-hydroxymelatonin sulfate in urine correlates well with the total level of melatonin in the blood during the collection period.

PRINCIPLE OF THE ASSAY

This assay employs the competitive enzyme immunoassay technique. Endogenous Melatonin of a patient sample competes with a biotinylated-Melatonin for binding to the fixed number of Melatonin antibody binding sites. The amount of bound biotin conjugate is inversely proportional to the

concentration of Melatonin in the sample. After incubation the unbound conjugate is washed off. The antibody-bound biotinylated antigen is determined by the use of streptavidin alkaline phosphatase as marker and p-nitrophenyl phosphate as substrate. Melatonin concentration in the sample is calculated through a calibration curve prepared by using known standards.

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	1 plate (8 X 12 strips)	4°C
Melatonin Biotin	3 X 2 ml (Lyophilized)	4°C
Melatonin Antibody	3 X 2 ml (Lyophilized)	4°C
80 X Streptavidin Alkaline Peroxidase Conjugate	0.25 ml	4°C
Standard 0-5 (0.0, 5.0, 10.0, 32.0, 100.0, 300.0 pg/ml)	6 x 2 ml (Lyophilized)	4°C (Keep aliquots at -20°C)
Control 1 (11.35 pg/ml, accept. Range 6.2 – 16.5 pg/ml)	1 X 2 ml (Lyophilized)	4°C (Keep aliquots at -20°C)
Control 2 (73.15 pg/ml, accept. Range 54.1 – 92.2 pg/ml)	1 X 2 ml (Lyophilized)	4°C (Keep aliquots at -20°C)
10X Wash buffer	100 ml	4°C
Extraction column	2 X 10	4°C
STOP solution	15 ml (Ready to Use)	4°C
PNPP substrate	2 X 13 ml (Ready to Use)	4°C (Protect from light)
Adhesive foil	3	

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 405 nm (reference wavelength 600-650 nm)
- Pipettes and pipette tips
- Deionized or distilled water
- Orbital microplate shaker (500 rpm)
- Methanol (HPLC grade is recommended)
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- 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.
- Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
- If crystals are observed in the 10X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.
- Once the test has been started, all steps should be completed without

interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18 °C- 25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming. Briefly spin down the vials before use.

- Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system.
- Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
- Ensure complete reconstitution and dilution of reagents prior to use.
- Incubation time affects results. All wells should be handled in the same order and time sequences.
- The relative centrifugal force (g) is not equivalent to rounds per minute (rpm) but it has to be calculated depending on the radius of the centrifuge.
- It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.
- Samples contain azide cannot be assayed.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C up to 3 months or at ≤ -70 °C up to 1 year. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C up to 3 months or at ≤ -70 °C up to 1 year. Avoid repeated freeze-thaw cycles.

Note:

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

REAGENT PREPARATION

- 1X Wash buffer: Dilute **10X** Wash buffer into **distilled water** to yield 1X Wash buffer. E.g.: Add 15 ml of 10X Wash buffer into 135 ml of distilled water to a final volume of 150 ml, mix thoroughly. The diluted Wash buffer is stable for 8 weeks at 2-8°C.
- Standards and Controls: Add **2 ml** of **distilled water** into each vial. Incubate for 15 minutes and mix well without foaming. Aliquot and store

at -20°C until expiry date. Avoid repeated freeze-thaw cycles.

- Melatonin Biotin: Add 2 ml of 1X Wash buffer into vial. Incubate for 15 minutes and mix well without foaming. Prepare freshly and use only once.
- Melatonin Antibody: The antiserum is a rabbit polyclonal antibody with stabilizers. Add 2 ml of distilled water into vial. Incubate for 15 minutes and mix well without foaming. Prepare freshly and use only once.
- Streptavidin Alkaline Peroxidase Conjugate: Dilute 1:81, or add 70 µl of Streptavidin Alkaline Peroxidase Conjugate into 5.6 ml of 1X Wash buffer. Prepare freshly and use only once.
- 10% Methanol: Add 10 ml of undiluted methanol into distilled water to a final volume of 100 ml.
- Samples: Samples suspected to contain concentrations higher than the highest standard have to be diluted with 1X Wash buffer prior to extraction step.

EXTRACTION OF SAMPLES, STANDARDS, and CONTROLS

NOTE:

- The yield of extraction of this procedure is 90-100%.
- Filter or centrifuge the samples prior to extraction in order to avoid clogging of columns.
- Each sample, standard and control has to be extracted. Extraction can be performed in advance. The dried extracts (after evaporation of methanol) may be stored at 2-8°C or ≤-20°C for up to 24h.
- After elution with methanol, the Extraction Columns may be used for extraction of the next samples or stored at 2-8°C protected from dust.

Extraction Columns can be re-used up to 4 times. In case of re-use, start with Step A.1 (Column conditioning).

PROCEDURE:

A. Standard version: Procedure for Centrifuge and Evaporator Centrifuge

1. Column conditioning:
   (a) Place the Extraction column into polystyrene or glass tubes (12 X 75 mm).
   (b) Add **1 ml** of **methanol (undiluted)** into each column. Let the solvent pass through the column by centrifugation for 1 min at 120xg. Discard eluate.
   (c) Add **1 ml** of **distilled water** into each column. Let the solvent pass through the column by centrifugation for 1 min at 120xg. Discard eluate.
   (d) Proceed to sample application without delay to avoid columns getting dry.
2. Sample application:
   (a) Place the Extracted Column into corresponding labeled polystyrene or glass tubes (12 X 75mm)
   (b) Add **0.5 ml** of **Standards, Samples and Controls** into columns.
   (e) Add **0.5 ml** of **distilled water** to columns. Let pass through the column by centrifugation for 5 min at 120xg. Discard eluate.
3. Washing:
   (a) Add **2 x 1 ml** of **10% methanol** to the columns. Let the solvent pass through the column by centrifugation for 5 min at 120xg. Discard eluate.

(b) Repeat washing once as according to step (a).

4. Elution of Extract:

(a) Place the Extraction Columns into new corresponding labeled polystyrene or glass tubes (12 X 75 mm)

(b) Add **1 ml** of **methanol (undiluted)** into each column. Let the solvent pass through the column by centrifugation for 5 min at 120xg. **DO NOT DISCARD ELUATE!**

(c) Remove columns from the tubes. Avoid drops to be left at the columns.

(d) The Extraction Columns may be used for extraction of the next samples or stored at 2-8°C protected from dust. Extraction Columns can be re-used up to 4 times.

5. Evaporation and Reconstitution of Extract:

(a) Evaporate the methanol (from 4-(b)) to dryness by use of evaporator centrifuge.

(b) Reconstitute Standards, Controls, and Samples with **0.15 ml** of **distilled water.**

(c) Vortex at least 1 min and assay immediately.

B. Alternative version: Procedure for Vacuum Manifold instead of a Centrifuge

1. The volumes remain unchanged for the extraction scheme follow points A. 1 to A. 5.
2. Let the solvent pass through the column using vacuum and a flow rate of **≤ 5 mL/min**.
3. For the samples and extracts use a flow rate of **≤ 2 mL/min**.
4. The evaporation of the solvent may be performed by using an **evaporator**

centrifuge or by nitrogen.

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 18-25°C) before use. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **50 µl** of **extracted standards**, **extracted controls** and **extracted samples** in duplicate into the respective wells of the Microtiter Plate.
3. Add **50 µl** of **melatonin biotin** into each well.
4. Add **50 µl** of **melatonin antibody** into each well. Cover with adhesive foil. **Incubate for 14-20 hour at 2-8°C**.
5. Remove adhesive foil. Aspirate each well and wash, repeating the process 2 times for a **total 3 washes**. Wash by filling each well with **1× Wash Buffer (250 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.
6. Add **150 µl** of freshly prepared **Streptavidin Alkaline Peroxidase Conjugate** into each well. Cover plate with new adhesive foil. Incubate **120 min at RT** on an orbital microplate shaker (500 rpm).
7. **Wash** as according to **step 5.**
8. Add **200 µl** of **PNPP substrate** to each well. Incubate for **40 minutes** with shaking (500rpm) on a microplate shaker **at room temperature in dark.**
9. Add **50 µl** of **Stop Solution** to each well. Briefly mix contents by gently shaking the plate.

10. **Read** the OD with a microplate reader at **405 nm** (Optional: Read reference-wavelength at 600-650 nm) immediately or within 60 min after pipetting of the Stop Solution.

CALCULATION OF RESULTS

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

2. Using linear, semi-log or log-log 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. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. 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.

5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for detail. (https://www.arigobio.com/elisa-analysis)

6. Conversion: Melatonin (pg/ml) X 4.30 = pmol/L.

7. Test Validity: A test run is valid if:

(a) The mean absorbance of standard 0 (0.0 pg/ml) is > 1.00

(b) The mean absorbance of standard 5 (300 pg/ml) is < 0.50

(c) The mean of control 1 is determined between 6.2 – 16.5 pg/ml

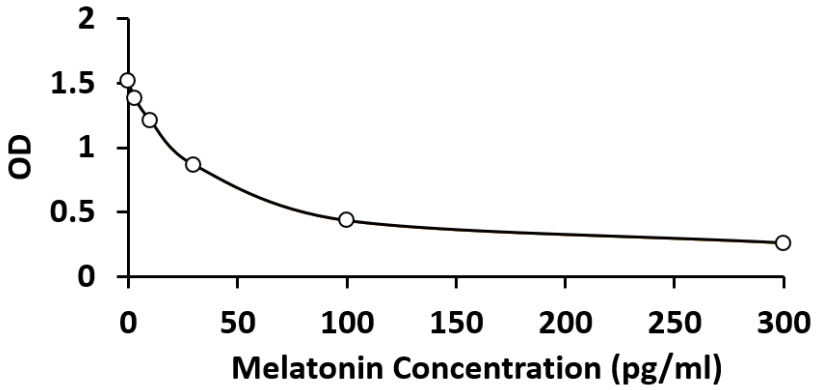
(d) The mean of control 2 is determined between 54.1 – 92.2 pg/ml

If the above mentioned quality criteria are not met, repeat the test and make

sure that the test procedure is correct (Extraction steps and incubation times and temperatures, sample and wash buffer dilution, washing steps etc.).

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.



EXPECTED VALUES

A study with apparently healthy subjects has shown that the melatonin levels in humans have a marked circadian rhythmicity characterized by very low levels during day time and high level during night time, and show a considerable inter-individual variation. Furthermore, the melatonin concentration is age-dependent. The highest concentrations were found in samples of infants (up to 3 years).

Melatonin in Serum:

Apparently healthy subjects show the following values:

Time	n	Mean Value	90% Percentile
03:00 AM	129	78.2 pg/ml	18.5-180 pg/ml
08:00 AM	128	28.5 pg/ml	3.8-80.4 pg/ml

QUALITY ASSURANCE

Specificity

Substance	Cross reactivity
5-Methoxy Tryptophole	1.2 %
N-acetyl Serotonin	1.2 %
5-Methoxy Tryptamine	2.5 %

Cross-reactivity of other substances tested < 0.01%

Sensitivity

The minimum detectable dose (MDD) of Melatonin was found to be 1.6 pg/ml.

Intra-assay and Inter-assay precision

The CV value of intra-assay precision was 3-11.4% and inter-assay precision was 6.4-19.3%.

Recovery

83-125%

Linearity

73 – 135

Interfering Substances

Any improper handling of samples or modification of this test might influence

the results. Haemoglobin (up to 8 mg/mL) and Bilirubin (up to 0.36 mg/mL) have no influence on the assay results.