Version 29-05/25

User's Manual

Human Melatonin-Sulfate Urine ELISA Kit

REF **DEIA2239**

Σ **96T**

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.

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PRODUCT INFORMATION

Intended Use

Enzyme immunoassay for the quantitative determination of Melatonin-Sulfate (synonyms: 6-hydroxymelatonin sulfate, 6-sulfatoxymelatonin) in human urine.

General Description

Melatonin is an intermediate product of tryptophan metabolism and is released into the bloodstream originating from the pineal gland. Melatonin production is regulated by the circadian timing system. In healthy individuals, it is produced in synchrony to the light/dark cycle, being tightly restricted to the night, provided it is dark. Light stimulus (mainly in the blue range) inhibits melatonin synthesis. The role as a physiological marker has been documented in review articles and textbooks.

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 plasma during the collection period.

Melatonin concentrations play an important role in the regulation of sleep patterns. The start of melatonin production in the human body can we assessed via the timing of the acrophase and/or the dim light melatonin onset (DLMO).

Disruption to healthy sleep patterns (exposure to bright light at nighttime or the participation in night shift work) can alter the DLMO and lead to a phase advance or phase delay of the melatonin concentration in the sample. Urinary melatonin sulfate is a useful biomarker in circadian dysregulation through shift work or exposure to bright light at night. Furthermore, time-resolved quantification of melatonin levels can provide information about diurnal type (morning versus evening).

Melatonin and melatonin sulfate concentrations in circulation are highly variable. During the day, melatonin sulfate levels are very low. At night, melatonin sulfate concentrations rise.

A large variety of methods to assess the onset of melatonin production has led to a formation of an expert group to establish recommendations for the calculation beginning of melatonin secretion. Laboratories should follow consensus guidelines on the generation of data for circadian pace making. Measurements of Melatonin-sulfate in urine show the accumulated amount of 6-sulfatoxymelatonin during an interval of time; overnight Melatonin sulfate levels can be calculated from the first morning void and compared to daytime collected urine fraction.

Clinicians should consider the effects of non-modifiable factors (e.g. age), modifiable factors (lighting, seasonal change, physical activity), comorbidities (ophthalmic diseases, spinal cord injuries, liver diseases and disorders, kidney diseases and disorders) and drugs or nutrition supplements that either increase (melatonin, antidepressants, MAO inhibitors) or decrease (1-adrenergic blockers alpha-2 adrenergic agonist, benzodiazepines) on the measurement of melatonin and its metabolite melatonin sulfate.

Enzyme immunoassay for the quantitative determination of Melatonin Sulfate in human urine. Quantification of melatonin sulfate indicates physiological functions or states of the pineal gland that regulates sleep-wake cycles. Patient population includes healthy adults and adults suspected to be affected by sleep disorders. The assay should not be performed with patient samples that might be affected by interference from exogenous melatonin or samples that might be affected by drugs that interact with melatonin metabolism.

The Melatonin-Sulfate Urine ELISA is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding and measured on an absorbance reader. The assay is semi-automated requiring general purpose laboratory instruments and consumables such as absorbance microplate reader/washer, vortexer and pipettes to execute the test. Test results may be calculated manually from a standard curve and compared to laboratory established reference ranges from healthy adults (i.e., normal ranges). The test kit is intended for professional laboratory use only. The test kit is not for self-testing. The Melatonin-Sulfate Urine ELISA is NOT intended for near-patient testing.

Principles of Testing

Solid phase enzyme-linked immunosorbent assay (ELISA) based on the competition principle. An unknown amount of antigen present in the sample and a fixed amount of enzyme labelled antigen compete for the binding sites of the antibodies coated onto the wells. After incubation, the wells are washed to stop the competition reaction. After the substrate reaction, the intensity of the developed colour is inversely proportional to the amount of the antigen in the sample. Results of samples can be determined directly using the standard curve.

Reagents And Materials Provided

1. Microtiter Plate Break apart strips. 1 × 12 × 8

MTP (12 strips of 8 wells each) coated with goat anti-rabbit antibody in solution containing bovine serum albumin. Vacuum dried.

2. Melatonin Sulfate Antiserum. Ready to use. 1 × 6 mL

Rabbit anti-melatonin sulfate antibody in Tris buffer containing bovine serum albumin and < 0.1 % Thimerosal(w/w).

3. Enzyme Conjugate,Concentrate (40×). 1 × 0.2 mL

Contains melatonin sulfate conjugated to peroxidase containing Thimerosal < 0.1% (w/w).

4. Standard A-F. Ready to use. 1 × 6 × 0.1 mL

0; 2.0; 6.0; 18.0; 54; 162 ng/mL

0; 6.1;18.3, 54.9; 164.7, 494.1 nmoll/L

Contains melatonin sulfate in Tris buffer, bovine serum albumin and Thimerosal < 0.1 %(w/w).

5. Control 1+2. Ready to use. 1 × 2 × 0.1 mL

Contains melatonin sulfate in Tris buffer, bovine serum albumin and Thimerosal < 0.1%(w/w)

Concentrations / acceptable ranges see QC certificate.

6. Assay Buffer. Ready to use. 1 × 80 mL

Red colored. Contains Tris buffer, bovine serum albumin and Thimerosal < 0.1% (w/w).

7. Wash Buffer, Concentrate (20×). 1 × 50 mL

0. 2 M phosphate buffer containing 0.2 % Tween 20 and Thimerosal < 0.1%(w/w).

8. TMB Substrate Solution. Ready to use. 1 × 15 mL

Contains 3,3',5,5' Tetramethylbenzidine solution

9. TMB Stop Solution. Ready to use. 1 × 15 mL

Contains 1 M sulfuric acid.

10. Adhesive Foil. 3

Materials Required But Not Supplied

1. Micropipettes (Multipette Eppendorf or similar devices, < 3 % CV). Volume: 10; 50; 100; 1000 µL
2. Round-bottom polystyrene test tubes (12 x 75 mm)
3. Rack for test tubes
4. Orbital shaker (500 rpm)
5. Vortex mixer
6. 8-Channel Micropipette with reagent reservoirs
7. Wash bottle, automated or semi-automated microtiter plate washing system
8. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength 600 - 650 nm) 9. Bidistilled or deionised water
10. Paper towels, pipette tips and timer

Storage

The kit is shipped at ambient temperature and should be stored at 2 - 8°C. Keep away from heat or direct sunlight. The storage and stability of specimens and prepared reagents is stated in the corresponding chapters.

The microtiter strips are stable up to the indicated expiry date after the kit is opened. Make sure that the opened bag is tightly closed when stored at 2 - 8°C.

Specimen Collection And Preparation

Urine

It is possible to use spontaneous as well as 24 hours urine. The total volume of urine excreted during a 24 hours period should be collected and mixed in a single bottle. Preservation is not necessary. Determine total volume for calculation of results. Mix and centrifuge samples before use in the assay.

Storage:	2 - 8°C	≤ -20°C (Aliquots)	Keep away from heat or direct sunlight. Avoid repeated freeze-thaw cycles. For more details see: Griefahn et al. (2001)*
Stability:	≤ 7 days	> 7 days	

* Griefahn, B.; Remer, T.; Blaszkewicz, M.; Bröde, P. Long-Term Stability of 6-Hydroxymelatonin Sulfate in 24-h Urine Samples Stored at -20°C. Endocrine, vol. 15, no. 2, 199–202, July 2001

Reagent Preparation

The contents of the kit for 96 determinations can be divided into 3 separate runs. The volumes stated below are for one run with 4 strips (32 determinations).

1. Preparation of concentrated components

Dilute / dissolve	Component		Diluent	Relation	Remarks	Storage	Stability
15 mL	**Wash Buffer**	ad 300 mL	bidist. water	1:20	Resolve crystals at 18 - 25°C.	18 - 25°C	4 weeks
50 µL	**Enzyme Conjugate**	with 2 mL	Assay Buffer	1:41	Prepare freshly and use only once.	18 - 25°C	30 minutes

2. Dilution of Standards, Controls and patient urine samples

1.	Pipette **500 µL** of **Assay Buffer** into each tube.
2.	Pipette **10 µL** of each **Standard, Control and patient urine sample** into polystyrene, polypropylene or glass tubes. Avoid direct sun light. Vortex

Samples containing concentrations higher than the highest standard have to be further diluted with Assay Buffer.

Assay Procedure

Assay Notes:

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pre-treatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. 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 - 25°C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each component and specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. Some components contain ≤ 250 µL solution. Take care that the solution is completely on the bottom of the vial before opening.
5. It is advised to perform duplicate measurements to be able to identify potential pipetting errors.
6. Use a pipetting scheme to verify an appropriate plate layout.
7. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.
8. Microtiter plate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microtiter plate 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.
9. 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.

Procedure:

1. Pipette 50 µL of each diluted Standard, diluted Control and diluted patient sample into the respective wells of the Microtiter Plate.
2. Pipette 50 µL of freshly prepared Enzyme Conjugate into each well.

3. Pipette 50 µL of Melatonin Sulfate Antiserum into each well.
4. Cover plate with adhesive foil. Incubate 2 hours at 18 - 25°C (Room temperature) on an orbital shaker (500 rpm).
5. Remove adhesive foil. Discard incubation solution. Wash plate 4 x with 250 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
6. For adding of Substrate and Stop Solution use, if available, an 8-channel Micropipettor. Pipetting should be carried out in the same time intervals for Substrate and Stop Solution. Use positive displacement and avoid formation of air bubbles.
7. Pipette 100 µL of TMB Substrate Solution into each well.
8. Incubate 30 minutes at 18 - 25°C on an orbital shaker (500 rpm).
9. Stop the substrate reaction by adding 100 µL of TMB Stop Solution into each well. Briefly mix contents by gently shaking the plate.
10. Measure optical density with a photometer at 450 nm (Reference-wavelength: 600 - 650 nm) within 60 minutes after pipetting of the Stop Solution.

Quality Control

The test results are only valid if the test has been performed following the instructions. Moreover, the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or comparable standards/laws. User and/or laboratory must have a validated system to get diagnosis according to GLP. All kit controls must be found within the acceptable ranges as stated on the labels and the QC certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

It is recommended to participate at appropriate quality assessment trials.

Calculation

The obtained ODs of the standards (y-axis, linear) are plotted against their concentration (x-axis, logarithmic) either on semi-logarithmic graph paper or using an automated method. A good fit is provided with cubic spline, 4 Parameter Logistics or Logit-Log.

For the calculation of the standard curve, apply each signal of the standards (one obvious outlier of duplicates could be omitted and the more plausible single value used).

The concentration of the samples can be read directly from the standard curve.

The initial dilution has been taken into consideration when reading the results from the graph. Results of samples of higher predilution have to be multiplied with the dilution factor.

Samples showing concentrations above the highest standard have to be diluted as described in Reagent Preparation and re-assayed.

Calculate the 24 hour excretion for each urine sample: µg/24 h = µg/L × L/24 h

Conversion: Melatonin sulfate (ng/mL) × 3.05 = nmol/L

Trueness: Calibration/ Metrological Traceability

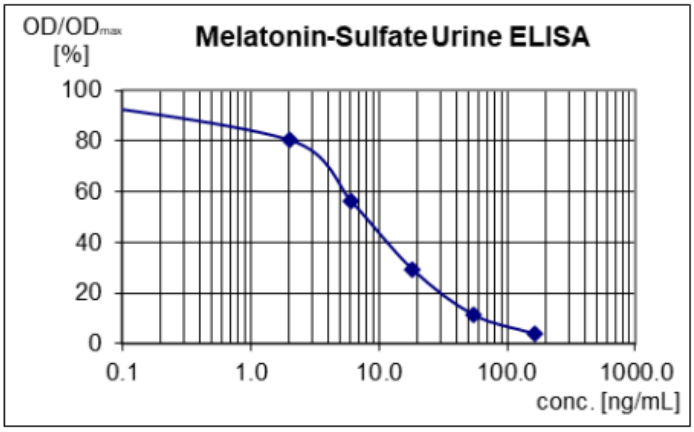
Melatonin Sulfate-Urine ELISA is metrological traceable to the SI unit ng/mL. The concentrations assigned to kit standards and controls are traceable to reference measurement procedure LC-MS through an unbroken chain of calibration according to EN ISO 17511:2021

The combined uncertainty of measurement is calculated as 16.2 % according to the 'Guide to the expression of uncertainty in measurement' (JCGM 100:2008; GUM 1995 with minor corrections).

Typical Standard Curve

(Example. Do not use for calculation!)

Standard	Melatonin sulfate	OD_{Mean}	OD/OD_{max}
A	0.0 ng/mL	2.090	100 %
B	2.0 ng/mL	1.679	80 %
C	6.0 ng/mL	1.174	56 %
D	18.0 ng/mL	0.613	29 %
E	54.0 ng/mL	0.237	11 %
F	162 ng/mL	0.080	4 %



The measuring range for the Melatonin-Sulfate Urine ELISA is between 2.18 ng/mL (LoQ as functional sensitivity) and 162 ng/mL (Standard F).

Reference Values

Published literature identified that the concentration of melatonin sulfate in urine reflects the circadian melatonin rhythm, with a concentration peak during nighttime and very low levels during daytime.

A high the intra-subject night–day ratio are reported, as the melatonin production and therefore the melatonin sulfate excretion is effected by non-modifiable factors (e.g. age), modifiable factors (lighting, seasonal change, physical activity), comorbidities (ophthalmic diseases, spinal cord injuries, liver diseases and disorders, kidney diseases and disorders) and drugs or nutrition supplements that either increase (melatonin, antidepressants, MAO inhibitors) or decrease (1-adrenergic blockers alpha-2 adrenergic agonist, benzodiazepines).

Van Faassen et al. (2021)* published the expected values of melatonin sulfate (in nmol/day) for 24 hours collected urine in individuals without sleeping disorders, measured with LC-MS and categorized per age. The decline in the excretion of melatonin sulfate in urine with age was confirmed with the measurement of 125 samples with the Melatonin Sulfate Urine ELISA.

Age	LC-MS as indicated in published literature van Faassen et al. 2021			Melatonin-Sulfate Urine DEIA2239		
	Range (interquartile)	Median	n	Range (min - max)	Average	n
20 - 29 years	30 - 71 nmol/24h	47.7 nmol/24h	40	19 - 229 nmol/24h	85 nmol/24h	56
30 - 39 years	28 - 62 nmol/24h	48.7 nmol/24h	40	39 - 237 nmol/24h	105 nmol/24h	14
40 - 49 years	21 - 48 nmol/24h	32.8 nmol/24h	40	16 - 167 nmol/24h	70 nmol/24h	32
50 - 59 years	16 - 36 nmol/24h	23 nmol/24h	40	21 - 285 nmol/24h	81 nmol/24h	17
60 - 69 years	12 - 30 nmol/24h	21 nmol/24h	40	13 - 92 nmol/24h	38 nmol/24h	4
70 - 79 years	9.2 - 30 nmol/24h	19 nmol/24h	40	28 - 29 nmol/24h	29 nmol/24h	2

Expected values for affected population present a reduction in the intra-individually melatonin sulfate excretion rates, which reflect the circadian melatonin rhythm. Therefore, the melatonin sulfate urine does not show the expected concentration peak during night-time nor very low levels during daytime. It is recommended the comparison of day and night samples, to assess the sleep-wake cycle which is linked to the circadian melatonin rhythm.

* van Faassen M. et al. Mass spectrometric quantification of urinary 6-sulfatoxymelatonin: agedependent excretion and biological variation. Clin Chem Lab Med 2021; 59(1): 187–195. https://doi.org/10.1515/cclm-2020-0455

Precision

The intra-assay and inter-assay study was conducted for 20 days using one reagent lot. Two runs were performed per day with high and low kit controls and with a panel of 5 urine samples. Each sample was run in duplicate.

Intra-Assay			
Sample	Mean conc.	SD	CV
1	12.1 ng/mL	0.9 ng/mL	7.8 %
2	6.3 ng/mL	0.5 ng/mL	8.4 %
3	37.2 ng/mL	2.2 ng/mL	5.8 %
4	62.5 ng/mL	4.5 ng/mL	7.2 %
5	2.7 ng/mL	0.4 ng/mL	15.7 %

The intra-assay precision calculated from five human urine samples showed a mean CV of 9.0 % and range of 5.8 % - 15.7 %.

Inter-Assay			
Sample	Mean conc.	SD	CV
1	12.1 ng/mL	1.2 ng/mL	9.6 %
2	6.3 ng/mL	1.0 ng/mL	15.3 %
3	37.2 ng/mL	3.6 ng/mL	9.7 %
4	62.5 ng/mL	7.3 ng/mL	11.7 %

The inter-assay precision calculated from four human urine samples showed a mean CV of 11.6 % and of range 9.6 % - 15.3 %.

The inter lot precision study was conducted during 5 days of testing. Each run was performed with high and low kit controls and with a panel of 5 urine samples. Each sample was tested five times per run with 3 different reagent lots.

Inter-Lot			
Sample	**Mean conc.**	**SD**	**CV**
1	27.8 ng/mL	3.8 ng/mL	13.5 %
2	11.9 ng/mL	1.3 ng/mL	10.9 %
3	44.5 ng/mL	7.8 ng/mL	17.8 %
4	5.2 ng/mL	0.5 ng/mL	10.4 %
5	2.6 ng/mL	0.3 ng/mL	11.3 %

The inter lot precision showed a mean CV of 12.8 % and a range of 10.4 % - 17.8 %.

Detection Limit

Limit of Blank (LoB)

The LoB study was performed with five blank samples (Standard A), measured in 4 replicates per sample over three days with two kit lots.

Limit of Blank: 1.27 ng/mL

Limit of Detection (LoD)

The LoD study was performed using five different low concentrated samples, measured in 4 replicates per sample over three days with two kit lots.

Limit of Detection: 2.18 ng/mL

Limit of Quantitation (LoQ as functional sensitivity)

The LoQ (functional sensitivity) study was performed using five low and three higher concentrated samples, measured in 4 replicates over three days with two kit lots.

Limit of Quantitation (Functional Sensitivity): 2.18 ng/mL

Specificity

Substance	Cross Reactivity
Melatonin	0.002 %
6-Hydroxymelatonin	0.001 %
N-Acetyl-5-hydroxytryptamine	0.0005 %
N-Acetyl-L-tryptophan	< 0.0001 %
5-Methoxytryptamine	< 0.0001 %
Tryptamine	< 0.0001 %
5-Methoxytryptophol	< 0.0001 %
5-Methoxy-3-indoleacetic acid	< 0.0001 %
DL-5-Methoxytryptophan	< 0.0001 %
5-Hydroxyindoleacetic acid	< 0.0001 %
5-Hydroxy-L-tryptophan	< 0.0001 %
6-Methoxytryptamine hydrochloride	< 0.0001 %
DL-Tryptophane	< 0.0001 %

Linearity

The linearity study shows acceptable linearity melatonin sulfate throughout the concentration range of kit standards. The tested concentration range (3.98 ng/mL up to Standard F 162 ng/mL) supports claimed measured ranges from LoQ (functional sensitivity, LoQ = 2.18 ng/mL) to Standard F, which covers the expected concentration range of melatonin sulfate for apparently healthy and affected population.

Precautions

1. For professional use only by GLP trained professionals.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
3. In case of severe damage of the kit package please contact your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
4. Broken glass may cause injury. Handle glass vessels with caution.
5. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
6. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
7. Reagents of this kit containing hazardous material may cause eye and skin irritations.
8. Chemicals and prepared, used, unused or expired reagents have to be treated as hazardous waste according to national biohazard and safety guidelines or regulations.
9. The cleaning staff should be guided by the professionals regarding potential hazards and handling.
10. All serious incidents that have occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.
11. Avoid contact with Stop solution. It may cause skin irritations and burns.
12. The device contains material of animal origin, which is certified apparently free of infectious or contagious diseases and injurious parasites. Still the material should be handled with extreme caution.

Limitations

Specimen collection and storage have a significant effect on the test results. See Specimen Collection And Preparation for details. For cross-reactivities, see Specificity.

	Component	Concentration
The following substances do not have a significant effect (+/- 20 % of expected value) on the test results up to the stated concentrations.	HSA	50 mg/mL
	Creatinine	5 mg/mL
	Whole blood	1% (v/v)