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**User's Manual**

# Melatonin direct Saliva (Non-Extraction) ELISA Kit

REF **DEIABL355**

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 direct, quantitative determination of melatonin in human saliva.

## General Description

Melatonin is an intermediate product of tryptophan metabolism and is released into the blood-stream 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. 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 dim light melatonin onset (DLMO). The DLMO allows characterization of the circadian rhythm secretion in multiple matrices (such as saliva). In apparently healthy individuals, DLMO occurs 2 - 3 hours before bedtime.

Disruption to healthy sleep patterns (exposure to bright light at night time 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. Salivary melatonin 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 concentrations in circulation are highly variable. During the day, melatonin levels are very low. At night, melatonin concentrations rise. Illustrative examples for melatonin concentrations can be found in the scientific literature for example in the publications by Ba-Ali et al. and Wiesner et al. 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 pacemaking. Benloucif et al. have published a consensus statement for measuring melatonin in humans in different matrices (such as saliva). Several methods to obtain the DLMO in a consensus statement by Benloucif et al. For example, DLMO can be defined with a threshold calculated at 2 standard deviations above the average baseline of three or more pre-rise samples.

Clinicians should consider the effects of non-modifiable factors (e.g. age) and modifiable factors (lighting, seasonal change, physical activity) on the measurement of melatonin concentration produced in humans that are discussed in detail in peer-reviewed literature. Recommendations for sampling conditions are given in a consensus statement by Benloucif et al. Clinicians should consider the effects of comorbidities (ophthalmic diseases, spinal cord injuries, liver diseases and disorders, kidney diseases and disorders) on the measurement of melatonin concentration produced in humans that are discussed in detail in peerreviewed literature. Clinicians should consider the effects of 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 concentration produced in humans that are discussed in detail in peer-reviewed literature. Laboratories should consider the effect of crossreactivity on the daytime melatonin concentrations that set the baseline for the quantification of melatonin onset.

## Principles of Testing

The assay procedure follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. The amount of biotinylated antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the free biotinylated antigen is removed by a washing step and the antibody bound biotinylated antigen is determined by use of streptavidin-peroxidase as marker and TMB as substrate. Quantification of unknowns is achieved by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards.

## Reagents And Materials Provided

1. MTP Microtiter Plate Break apart strips.

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

2. CAL A and CAL B-F Standard A - F Ready to use. 0.0; 0.5; 1.5; 5.0; 15; 50; pg/mL. Contains: Melatonin and ≤ 0.1 % sodium azide (w/w).
3. CONTROL 1 & CONTROL 2

Control 1+2 Ready to use. Contains: Melatonin (low and high) and ≤ 0.1 % sodium azide (w/w). For exact concentrations see labels or QC certificate.

4. ANTISERUM Melatonin Antiserum Ready to use.

Contains: Antiserum (rabbit, polyclonal), bovine serum albumin and ≤ 0.1% ProClin (w/w).

5. BIOTIN Melatonin Biotin Ready to use.

Contains bovine serum albumin and ≤ 0.1% ProClin (w/w).

6. ENZCONJ Enzyme Conjugate Ready to use.

Contains: streptavidin conjugated to HRP, ≤ 0.02 % methylisothiazolinone (w/w) and ≤ 0.02 % bromonitrodioxane (w/w).

7. WASHBUF CONC Wash Buffer Concentrate (20x)

Phosphate buffer containing ≤ 0.2 % Tween 20 (w/w) and ≤ 0.02% ProClin (w/w).

8. TMB SUBS TMB Substrate Solution Ready to use.

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

9. TMB STOP TMB Stop Solution Ready to use.

Contains 1 M sulfuric acid

10. FOIL Adhesive Foil

## Materials Required But Not Supplied

1. Micropipettes (Multipette Eppendorf or similar devices, < 3 % CV). Volume: 50: 100 µL
2. A suitable sampling device should be used.
3. Orbital shaker (400 - 600 rpm)
4. Vortex mixer

5. 8-Channel Micropipette with reagent reservoirs
6. Wash bottle, automated or semi-automated microtiter plate washing system
7. Centrifuge (preferably refrigerated) 2000 - 3000 x g
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
11. Refrigerator (2 - 8°C)

## 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 are stated in the corresponding chapters.

The unopened reagents are stable until the expiry date indicated. The kit is stable up to 6 months after the first opening (not exceeding the expiry date) when the Microtiter plate is packed in a tightly closed bag, the bottles are closed with their screw caps and the kit is stored at indicated storage temperature.

## Specimen Collection And Preparation

The donor should not eat, drink, chew gums or brush teeth for 30 minutes before sampling. Rinse mouth thoroughly with cold water 5 minutes prior to sample collection.

Do not collect samples when oral diseases, inflammation or lesions exist (blood contamination).

Reddish colour is indicating blood contamination and leading to wrong results.

A minimum of 0.5 mL liquid should be collected. Saliva flow can be stimulated by chewing on a piece of Parafilm®.

It is recommended to freeze samples at -20°C prior to laboratory testing. After thawing, mix and centrifuge 10 minutes at 2000 – 3000 x g to remove particulate material.

Samples should not be taken from donors that took biotin-containing multivitamins or supplements within last 48 hours.

### Sample Collection Device

Saliva can be collected in a suitable sampling device. Sample collection systems which contain cellulose pads should not be used.

### Specimen storage

Saliva samples can be stored at room temperature for 1 day or at 2 - 8°C for 7 days. Melatonin in saliva is at least stable for 1 month at -20°C. It is recommended that each laboratory conducts and evaluates the further sample stability and establish own specimen storage. Avoid repeated freeze-thaw cycles. Keep away from heat or direct sunlight.

## Reagent Preparation

### Dilution of Samples

Values greater than 50 pg/mL (Standard F) must be diluted with Standard A into the linear range of the standard curve, e.g. by dilution of 1:10 (Example: 50 µL saliva + 450 µL Standard A). Dilution has to be made in glass tubes. Measured results have to be multiplied by dilution factor to obtain corrected results. Values lower than 0 pg/mL should be repeated by an additional measurement.

**Preparation of concentrated components**

Mix 10 mL of WASHBUF CONC with 200 mL of bidistilled water in a 1:20 dilution ratio to create a diluent solution. Ensure vigorous mixing and store the solution at 2 - 8°C. The diluted solution is stable for up to 4 weeks.

## Assay Procedure

**PROCEDURE 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 and 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. It is advised to perform duplicate measurements to be able to identify potential pipetting errors.
5. Use a pipetting scheme to verify an appropriate plate layout.
6. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel micropipette for pipetting of solutions in all wells.
7. 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.
8. 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.
9. 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.

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).

Thimerosal should be avoided in any case.

TEST PROCEDURE

1. Pipette 100 µL of each Standard, Control and sample into the respective wells of the microtiter plate.
2. Pipette 50 µL of Antiserum solution into each well. Cover plate with adhesive foil. Shake plate carefully for

10 seconds.

3. Incubate 16 - 20 hours at 2 - 8°C.
4. 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.
5. Pipette 100 µL of Biotin solution into each well. Cover plate with adhesive foil.
6. Incubate 2 hours at 18 - 25°C (room temperature) on an orbital shaker (500 rpm).
7. 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.
8. Pipette 100 µL of Enzyme Conjugate into each well. Cover plate with adhesive foil.
9. Incubate 1 hour at 18 - 25°C on an orbital shaker (500 rpm).
10. 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.
11. Pipette 100 µL of TMB Substrate Solution into each well.
12. Incubate 15 minutes at 18 - 25°C on an orbital shaker (500 rpm).
13. Stop the substrate reaction by adding 100 µL of TMB Stop Solution into each well. Shake briefly. Color changes from blue to yellow.
14. Measure optical density with a photometer at 450 nm (Reference-wavelength: 600-650 nm) within 15 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.

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.

In case of diluted samples the values have to be multiplied with the corresponding dilution factor.

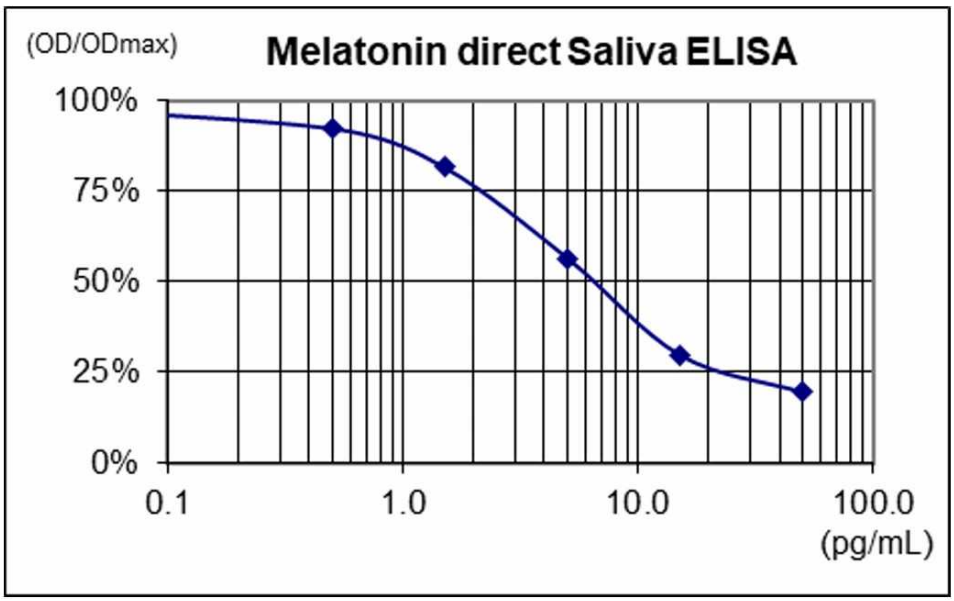
Samples showing concentrations above the highest standard have to be diluted.

**Conversion: Melatonin (pg/mL) x 4.30 = pmol/L**

## Typical Standard Curve

(Example. Do not use for calculation!)

| Standard | Concentration | $OD_{Mean}$ | $OD/OD_{max}$ |
|---|---|---|---|
| A | 0 pg/mL | 2.666 | 100 % |
| B | 0.5 pg/mL | 2.464 | 92 % |
| C | 1.5 pg/mL | 2.176 | 82 % |
| D | 5.0 pg/mL | 1.506 | 56 % |
| E | 15.0 pg/mL | 0.794 | 30 % |
| F | 50.0 pg/mL | 0.519 | 19 % |




Measuring Range: from 0.85 pg/mL (LoQ as functional sensitivity) to 38.4 pg/mL (highest concentration showing linear behavior < highest standard)

## Performance Characteristics

Limit of Blank (LoB)

The LoB study was performed with the zero calibrator (Standard A), measured in 28 replicates in one run. Limit of Blank = 0.4 pg/mL.

Limit of Quantitation (LoQ)

The LoQ study was performed with 10 saliva samples, measured in 10 replicates in one run. Limit of Quantitation = 0.85 pg/mL (CV = 20 %)

Linearity

The linearity study was performed measuring 5 different samples with different concentrations and a serial dilution up to 1:16. The assay showed a linear behavior up to a 1:16 dilution.

## Precision

The intra/inter assay study was conducted with two day samples and three night samples (3.5 - 25 pg/mL) by using 1 kit lot for 20 days with two runs per day and replicate.

The intra-assay precision showed a mean CV of 15.2 % (range: 13.2 - 19.0 %).

The inter-assay precision showed a mean CV of 19.2 % (range: 16.5 - 23.8 %)

To establish between lot precision the following study design assaying five different samples was used: 3 different reagent lots / 5 days / 1 run per day per lot / 5 replicates per run.

The mean between lot variation was 13.4 % (range: 8.8 - 17.7%).

## Specificity

The cross-reactivity of the melatonin antiserum has been measured against various compounds.

The percent of cross-reactivity is expressed as the ratios of melatonin concentration to the concentration of the reacting compound at 50 % binding of the zero standard. The results are shown in the following table.

Substance ------ Cross Reactivity

Serotonin ------ 0.54 %

5- Methoxytryptamine ------ <0.01 %

N-Acetylserotonin ------ <0.01 %

5- Methoxytryptophol ------ <0.01 %

## Recovery

The recovery study was performed measuring four different concentrations in three different saliva samples. Increasing amounts of Melatonin were added to the saliva samples. All samples (spiked and unspiked) were assayed in duplicates. The Melatonin concentrations were measured and the percentage recovery rates were calculated.

The mean recovery of melatonin including all saliva samples was 98 % (range: 74 - 114 %). The relation between expected and measured concentrations of melatonin did not significantly deviate over the concentration range studied.

## Precautions

1. For research use only.
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 us or 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. 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.
12. Avoid contact with Stop solution. It may cause irritations and burns.
13. Some reagents contain ProClin 300 as preservative. In case of contact with eyes or skin, flush immediately with water. When disposing reagents, flush with a large volume of water to avoid built-up.

## Limitations

Samples from donors that took biotin-containing multivitamins or supplements may contain biotin amounts which will cause interference with the assay. Specimen collection and storage have a significant effect on the test results. Thimerosal should be avoided in any case.

The following substances do not have a significant effect (+/- 20 % of expected value) on the test results up to the stated concentrations.

Substance ------ Conc. in saliva

Blood ------ 0.10 %

Sodium azide ------ 0.01 %

Citric acid ------ 0.001 %