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Instructions for use **Melatonin Direct Saliva ELISA**







For research

RUO

use only – Not for use in diagnostic procedures

Melatonin Direct Saliva ELISA

1. INTENDED USE

Enzyme immunoassay for the direct, quantitative determination of melatonin in human saliva. For *research use* only.

2. SUMMARY AND EXPLANATION

Melatonin (N-acetyl-5-methoxy-tryptamine) which is synthesized from the amino acid tryptophan is one of the major hormones of the pineal gland.¹ There is a circadian rhythm associated with Melatonin and the highest levels in plasma are observed during nighttime.² Regulation of the melatonin secretion is influenced by light via a pathway that starts in the retina of the eye. The production of melatonin is stopped abruptly upon exposure to light.¹

Levels of Melatonin in plasma and saliva are correlated throughout the circadian rhythm. The concentration of melatonin in saliva corresponds to about one third of the concentration in serum in average.³

Dim-light melatonin onset (DLMO) can be defined by the rise of melatonin concentration in serum/plasma, saliva and urine, respectively. An absolute threshold of melatonin levels is not necessarily the best method to determine DLMO. For our melatonin Saliva assay, we recommend the following procedure according to Benloucif et. al.⁴ : samples should be taken every 30 to 60 minutes under dim light (< 30 lux) for at least one hour prior and throughout the expected rise in melatonin. The DLMO can then be calculated as the first sample to exceed twice the standard deviation (2 SD) of the first three baseline samples.

Studies of melatonin levels are generally performed in healthy human populations.

Changes in the levels of melatonin and alternation in the pattern of secretion have been reported to coincide with: sleep quality,⁵ insomnia,⁶ daytime sleepiness,⁷ jet lag,⁸ depression,⁹ oxidative stress,¹⁰ pregnancy,¹¹ and blindness.¹²

3. TEST PRINCIPLE

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.

4. WARNINGS AND PRECAUTIONS

- 1. For *research* use only. For professional 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 the manufacturer 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. See MATERIALS SUPPLIED and labels for details. Material Safety Data Sheets for this product are available upon request directly from the manufacturer.
- 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 incident that has 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 donor 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.

5. STORAGE AND STABILITY

The kit is shipped at ambient temperature and should be stored at 2 - 8 $^{\circ}$ 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 after the kit is opened. Make sure that the opened bag is tightly closed when stored at 2 - 8 °C.

6. SPECIMEN COLLECTION AND STORAGE

Specimen collection

Do not eat, drink, chew gums or brush teeth for 30 min before sampling.

Do not collect samples when oral diseases, inflammation or lesions exist (blood contamination). Reddish colour is indicating blood contamination and leading to wrong results.

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

Rinse mouth thoroughly with cold water 5 min prior to sample collection.

Saliva flow can be stimulated by chewing on a piece of Parafilm®.

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

A minimum of 0.5 ml liquid should be collected.

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

Specimen storage

Saliva samples can be stored at room temperature for 1 day or at 2 – 8 °C for 7 days. It is recommended to freeze samples and store at -20 °C for long time storage (< 6 months). Avoid repeated freeze-thaw cycles. Keep away from heat or direct sunlight.

7. MATERIALS SUPPLIED

BA E-3431 III 96 Microtiter Plate

Contents: Break apart strips. Coated with anti-rabbit IgG (goat, polyclonal). Volume: 12 x 8 wells

Standards and Controls - ready to use

For exact concentrations see labels or OC-Report.

Cat. no.	Component	Concentration (pg/ml)	Volume /Vial
BA E-3401	STANDARD A	0	10 ml
BA E-3402	STANDARD B	0.5	1 ml
BA E-3403	STANDARD C	1.5	1 ml
BA E-3404	STANDARD D	5.0	1 ml
BA E-3405	STANDARD E	15.0	1 ml
BA E-3406	STANDARD F	50.0	1 ml
BA E-3451	CONTROL 1	Concentrations /	1 ml
BA E-3452	CONTROL 2	acceptable ranges see QC-Report.	1 ml
Contents:	stabilizers.		
BA E-3410 MEL-AS Melatonin Antiserum - ready to use			
Contents:	Antiserum (rabbit, polyclonal), stabilizers.		
Volume:	1 x 7 ml		
BA E-3441	BIOTIN	Melatonin Biotin - ready to u	ise
Contents:	Stabilizers.		
Volume:	1 x 12 ml		

BA E-3440 Contents: Volume:	CONJUGATE Streptavidin co 1 x 12 ml	Enzyme Conjugate - ready to use onjugated to HRP, stabilizers.
BA E-3430 Contents: Volume:	wash-concleox Phosphate buff 1 x 50 ml	Wash Buffer - concentrate (20x) Fer, Tween, stabilizers.
BA E-3455 Contents: Volume:	SUBSTRATE TMB, Buffer, st 1 x 15 ml	TMB Substrate Solution - ready to use abilizers.
BA E-3480 Contents: Volume: Hazard identification:	STOP-SOLN 1 M H ₂ SO ₄ 1 x 15 ml 4 H290 May be c	TMB Stop Solution - ready to use corrosive to metals.

H314 Causes severe skin burns and eye damage.

3 x Adhesive Foil

8. MATEARIALS REQUIRED BUT NOT SUPPLIED

- 1. Micropipettes (Multipette Eppendorf or similar devices, < 3 % CV). Volume: 50: 100 μ L
- 2. A suitable sampling device should be used.
- 3. Orbital shaker (400 600 rpm)
- 4. Vortex mixer
- 5. 8-Channel Micropipettor 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)

9. 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 pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
- 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. It is advised to determine duplicates 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 Micropipettor 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.

10. PRE-TEST SETUP INSTRUCTIONS

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.

10.1 Preparation of concentrated components

Wash Buffer:

Dilute 10 ml of *Wash Buffer* with bidest. water to a final volume of 200 ml (relation 1:20). Mix vigorously. Storage: 2 - 8 °C Stability: 4 weeks

10.2 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 μ L saliva + 450 μ L 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. Additional Standard A with 100 ml can be ordered separately.

11. TESTPROCEDURE

- **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 h 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 h at RT (18 25 °C) on an orbital shaker (500 rpm).
- 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 h at RT (18 25 °C) on an orbital shaker (500 rpm).
- 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 min at RT (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 min** after pipetting of the Stop Solution.

12. AUTOMATION

Automated protocols can be provided for open ELISA systems, e.g.: DSX®.

For more information please contact the manufacturer.

For the use of products on automated instruments it is absolutely necessary to perform and maintain a validation according to legal requirements. A successful validation of the process is a prerequisite for research use. The responsibility for the implementation and documentation of validation in accordance with the country-specific requirements is assumed by the organization or institution.

13. 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-Report. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls. It is recommended to participate at appropriate quality assessment trials.

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.

14. CALCULATION OF RESULTS

The obtained OD 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 might be omitted and the more plausible single value might be 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 as described in PRE-TEST SETUP INSTRUCTIONS and reassayed.

Conversion:

Melatonin (pg/ml) x 4.30 = pmol/l

Typical Standard Curve

(Example. Do not use for calculation!)

Standard	Melatonin (pg/ml)	OD (Mean)	OD / OD _{max} (%)	
A	0	2.666	100	
В	0.5	2.464	92	
С	1.5	2.176	82	
D	5.0	1.506	56	
E 15.0 F 50.0		0.794	30	
		0.519	19	



Measuring Range: from 0.854 pg/ml (LoQ) to 50 pg/ml (Standard F)

15. 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 (0 - 8 pg/ml) and high levels during night-time (10 - 58 pg/ml) and show a considerable inter-individual variation. The nocturnal melatonin peak among healthy individuals varies significantly. It is recommended that each laboratory establishes its own range of normal values.

16. LIMITATIONS OF THE PROCEDURE

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. See SPECIMEN COLLECTION AND STORAGE for details. For cross-reactivities, see PERFORMANCE.

Thimerosal should be avoided in any case.

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

Substance	Conc. in saliva		
Blood	0.125 %		
BSA	0.125 %		
NaN ₃	0.125 %		
Citric acid	0.01 %		

17. PERFORMANCE

17.1 Analytical Specificity (Cross Reactivity)

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		

17.2 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.

17.3 Limit of Quantitation (LoQ)

The LoQ study was performed with 10 saliva samples, measured in 10 replicates in one run. Limit of Quantitation = 0.854 pg/ml (CV = 20 %)

17.4 Metrological traceability

Traceability was proved by comparing the results of measurements between Melatonin Direct Saliva ELISA, with LC-MS/MS measurements, performed by a certified independent laboratory (ZRT Laboratory, USA).

The results obtained with the Melatonin Direct Saliva ELISA are metrological traceable to the SI-Unit pg/ml by mass spectrometry using 10 saliva QC samples.

The calculated maximum uncertainty of Melatonin Direct Saliva ELISA is 14.7 %.

$y = Melatonin Direct Saliva ELISA \qquad y = 0.9388 LC-MS/MS + 0.088 \qquad 1 - 0.991$

17.5 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.

17.6 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.

17.7 Method Comparison

A method comparison with a commercial ELISA was performed. 71 saliva samples were measured with the following result: r = 0.9899.

A method comparison was performed with the Melatonin RIA. 82 saliva samples were measured with the following result: r = 0.9539.

17.8 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 for day samples (< 8 pg/ml) of 17.0 % (15.0 - 19.0 %) and for night samples (> 10 pg/ml) of 13.9 %, Range 13.2 - 14.4 %.

The Inter assay precision showed a mean CV for day samples (< 8 pg/ml) of 20.5 % (17.1 - 23.8 %) and for night samples (> 10 pg/ml) of 18.4 %, Range 16.5 - 19.7 %.

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 %).

18. PRODUCT LITERATURE REFERENCES

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▲ For updated literature or any other information please contact your local supplier. Symbols:

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	+2 +2	Storage temperature	***	Manufacturer	Σ	Contains sufficient for <n> tests</n>
	\sum	Expiry date	LOT	Batch code		
	i	Consult instructions for use	CONT	Content		
	Â	Caution	REF	Catalogue number	RUO	For research use only!