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Instructions for use

Melatonin ELISA

REF IB89146R

2°C – 8°C

Σ 96

RUO For research use only – Not for use in diagnostic procedures

Melatonin ELISA

1. INTENDED USE

Enzyme immunoassay for the determination of melatonin in human serum and plasma.
For research use only.

2. BACKGROUND

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.
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.

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 streptavidine alkaline phosphatase as marker and p-nitrophenyl phosphate 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.
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. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details. Safety Data Sheets for this product are available upon request directly from the manufacturer.
7. Chemicals and prepared or used reagents have to be treated as hazardous waste according to national biohazard and safety guidelines or regulations.
8. The cleaning staff should be guided by the professionals regarding potential hazards and handling.
9. Avoid contact with Stop solution. It may cause skin irritations and burns.
10. All reagents of this kit containing human serum or plasma have been tested and were found negative for anti-HIV I/II, HBsAg and anti-HCV. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

5. STORAGE AND STABILITY

The kit is shipped at ambient temperature and should be stored at 2 - 8 °C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters.
The microtiter strips are stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2 - 8 °C.
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 may be re-used up to 4 times.

6. SPECIMEN COLLECTION AND STORAGE

Serum, Plasma (EDTA, Heparin)
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.

Storage:	2 - 8 °C	≤ -20 °C (aliquots)	≤ -70 °C (aliquots)	Keep away from heat or direct sun light. Avoid repeated freeze-thaw circle
Stability:	24 h	3 months	12 months	

7. MATERIALS SUPPLIED

⚠ *The reagents provided with this kit are sufficient for single determinations in the sample preparation (extraction) and duplicates in the assay. Additional reagents are available upon request.*

BA E-3331 96 **Microtiter Plate**
Contents: Break apart strips. Coated with anti-rabbit IgG (goat, polyclonal).
Volume: 12 x 8 wells

BA E-3341 BIOTIN **Melatonin Biotin -** lyophilized
Contents: Stabilizers.
Volume: 3 x 2 ml

BA E-3310 MEL-AS **Melatonin Antiserum** - lyophilized
Contents: Antiserum (rabbit, polyclonal), stabilizers.
Volume: 3 x 2 ml

BA E-3340 CONJ CONC 80x **Enzyme Conjugate** - concentrate (80x)
Contents: Streptavidin alkaline phosphatase, Tris buffer, stabilizers.
Volume: 1 x 250 µl

Standards and **Controls** - lyophilized
For exact concentrations see vial labels or QC-Report.

Cat. no.	Component	Concentration (pg/ml)	Volume /Vial
BA E-3301	STANDARD A	0.0	2 ml
BA E-3302	STANDARD B	3.0	2 ml
BA E-3303	STANDARD C	10	2 ml
BA E-3304	STANDARD D	30	2 ml
BA E-3305	STANDARD E	100	2 ml
BA E-3306	STANDARD F	300	2 ml
BA E-3351	CONTROL 1	Concentrations / acceptable ranges see QC-Report	2 ml
BA E-3352	CONTROL 2		2 ml

Contents: Stabilizers.

BA E-3330 WASH-CONC 10x **Wash Buffer** - concentrate (10x)
Contents: Phosphate buffer.
Volume: 1 x 100 ml

BA E-3355 SUBSTRATE **PNPP Substrate Solution** - ready to use
Contents: p-nitrophenyl phosphate (PNPP).
Volume: 2 x 13 ml

BA E-3380 STOP-SOLN **PNPP Stop Solution** - ready to use

Contents: 1 M NaOH, 0.25 M EDTA

Volume: 1 x 15 ml

Hazard identification:

H290 May be corrosive to metals.
H303 May be harmful if swallowed.
H315 Causes skin irritations.
H319 Causes serious eye irritation.

BA E-3385 EXTR COLM 10x **Extraction Columns** - ready to use

Contents: C18 RP, 1 cm^3 / 100 mg.

Amount: 2 x 10

Additional extraction columns can be ordered separately.

3 x Adhesive Foil

8. MATEARIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (Multipette Eppendorf or similar devices, < 3 % CV). Volume: 50; 500 µl
2. Disposable glass test tubes or round-bottom polystyrene test tubes (12 x 75 mm)
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; 100-200 x g
8. alternatively: Vacuum manifold (e.g. Mallinekrodt-Baker or Waters)
9. Methanol (HPLC grade)
10. Evaporator centrifuge (Speed-Vac)
11. alternatively: Sample concentrator by use of nitrogen (e.g. Techne)
12. Microtiter plate reader capable of reading absorbance at 405 nm (reference wavelength 600 - 650 nm)
13. Bidistilled or deionised water
14. Paper towels, pipette tips and timer

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.
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. It is advised to determine samples in duplicate 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. 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.
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).

10.1 Preparation of lyophilized or concentrated components

Wash Buffer:
Dilute 15 ml of *Wash Buffer Concentrate* with bidist. water (relation 1:10) to a final volume of 150 ml.
Warm up at 37 °C to dissolve crystals, if necessary. Mix vigorously.
Storage: 2 - 8 °C
Stability: 8 weeks

Standards and Controls:
Dissolve *Standards and Controls* with 2.0 ml bidist. water. Let stand for 15 min. mix without foaming.
Storage: ≤ -20 °C (aliquots)
Stability: until Exp. date

Biotin:
Dissolve *Biotin* with 2.0 ml *diluted Wash Buffer*. Let stand for 15 min. mix without foaming.
Prepare freshly and use only once.

Antiserum:
Dissolve *Antiserum* with 2.0 ml bidist. water. Let stand for 15 min. Mix without foaming.
Prepare freshly and use only once.

Enzymconjugate:
Dilute 70 µl *Enzymconjugate* with 5.6 ml diluted *Wash Buffer* (relation 1:81).
Prepare freshly and use only once.

Methanol (diluted)
Dilute 10 ml *Methanol* (undiluted) with bidest. water to a final volume of 100 ml [relation 10 % (v/v)].

If a larger volume is needed, vials can be pooled. Avoid repeated freeze-thaw cycles.

10.2 Dilution of Samples

Samples suspected to contain concentrations higher than the highest standard have to be diluted with *diluted Wash Buffer* prior to extraction step.

10.3 Extraction of Samples, Standards and Controls (Extraction Column)

The yield of extraction with this procedure is approx. 90 - 100 %.
Filter or centrifuge the samples prior to extraction in order to avoid clogging of the columns.

⚠ *Each sample, Standard and Control has to be extracted. Extraction may be performed in advance.*
The dried extracts (after evaporation of methanol) may be stored at 2 - 8 °C or ≤ -20 °C for up to 24 h.
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 may be re-used up to 4 times. In case of re-use, start again with A.1 (Column Conditioning).

A. Standard version: Procedure for Centrifuge and Evaporator Centrifuge

1. Column Conditioning:

1.	Place the Extraction Columns into polystyrene or glass tubes (12 x 75 mm).
2.	Add **1 x 1 ml** of **methanol (undiluted)** to the columns. Let the solvent pass through the column by centrifugation for 1 min at 120 x g. Discard eluate.
3.	Add **1 x 1 ml** of **bidist. water** to the columns. Let the solvent pass through the column by centrifugation for 1 min at 120 x g. Discard eluate.
4.	Proceed with sample application without delay in order to avoid the columns getting dry.

2. Sample Application:

5.	Place the Extraction Columns into correspondingly marked polystyrene or glass tubes (12 x 75 mm).
6.	Add **0.5 ml** of **Standards, Controls and samples** to the columns.
7.	Add **0.5 ml** of **bidist. water** to the columns. Let pass through the column by centrifugation for 5 min at 120 x g. Discard eluate.

3. Washing:

8.	Add **2 x 1 ml** of **10 % methanol in bidist. water (v/v)** to the columns. Let the solvent pass through the column by centrifugation for 5 min at 120 x g. Discard eluate.

4. Elution of Extract:

9.	Place the Extraction Columns into new, correspondingly marked polystyrene or glass tubes (12 x 75 mm).
10.	Add **1 ml of methanol (undiluted)** to the columns. Let the solvent pass through the column by centrifugation for 5 min at 120 x g.
11.	Remove columns from the tubes. Avoid drops to be left at the columns. Use columns for extraction of the next samples or store at 2 - 8 °C protected from dust. Extraction Columns may be re-used up to 4 times.

5. Evaporation and Reconstitution of Extract:

12.	**Evaporate** the methanol **to dryness** by use of evaporator centrifuge.
13.	**Reconstitute** samples with **0.15 ml** of **bidist. water.**
14.	Vortex at least 1 min and assay immediately.

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

For the extraction scheme follow points A 1-5 accordingly. The volumes remain unchanged.
Let the **solvent** pass through the column using vacuum and a flow rate of **≤ 5 ml/min.**
For the samples and extracts use a flow rate of **≤ 2 ml/min**.
The evaporation of the solvent may be performed by using an evaporator centrifuge or by nitrogen.

11. TESTPROCEDURE

1.	Pipette **50 µl** of each extracted **Standard**, extracted **Control** and extracted **sample** into the respective wells of the Microtiter Plate.
2.	Pipette **50 µl of Melatonin Biotin** into each well.
3.	Pipette **50 µl of Melatonin Antiserum** into each well.
4.	Cover plate with adhesive foil. Shake plate carefully. **Incubate 14 - 20 h at 2 - 8 °C.**
5.	Remove adhesive foil. Discard incubation solution. Wash plate **3 x with 250 µl** of **diluted Wash Buffer**. Remove excess solution by tapping the inverted plate on a paper towel.
6.	Pipette **150 µl** of freshly prepared **Enzyme Conjugate** into each well.
7.	Cover plate with new adhesive foil. Incubate **120 min at RT (18 - 25 °C)** on an orbital shaker (500 rpm).
8.	Remove adhesive foil. Discard incubation solution. Wash plate **3 x with 250 µl** of diluted **Wash Buffer.** Remove excess solution by tapping the inverted plate on a paper towel.
9.	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.
10.	Pipette **200 µl of PNPP Substrate Solution** into each well.
11.	**Incubate 40 min** at **RT (18 - 25 °C)** on an orbital shaker (500 rpm).
12.	Stop the substrate reaction by adding **50 µl of PNPP Stop Solution** into each well. Briefly mix contents by gently shaking the plate.
13.	**Measure** optical density with a photometer at **405 nm** (Reference-wavelength: 600-650 nm) within **60 min** after pipetting of the Stop Solution.

12. 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-Report. If the criteria are not met, the run is not valid and should be repeated. 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.

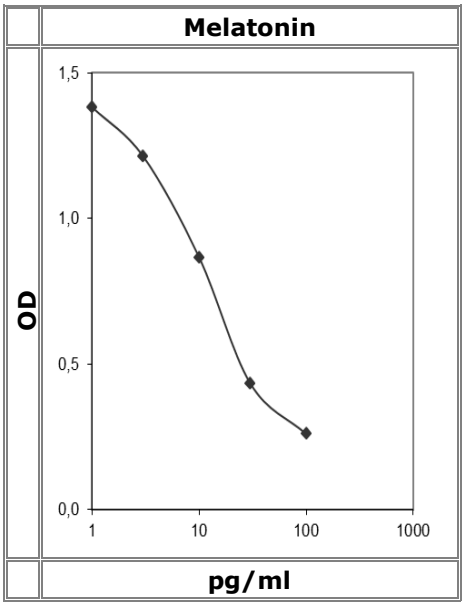
13. CALCULATION OF RESULTS

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	0.0	1.517	100.0
B	3.0	1.383	91.1
C	10	1.214	80.1
D	30	0.867	57.1
E	100	0.434	28.6
F	300	0.260	17.1



14. EXPECTED VALUES

A study with apparently healthy subjects has shown that the melatonin levels in humans have a marked circadian rhythmicity characterised by very low levels during day-time and high levels 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).
In a group of six healthy volunteers the circadian rhythm of melatonin was studied. The mean value reaches a minimum of about 4.6 pg/ml during daytime at 4 p.m. and a maximum of about 77.5 pg/ml during nighttime at 4 a.m. The nocturnal melatonin peak among healthy individuals varies significantly.

Melatonin in Serum
Apparently healthy subjects show the following values:

Time	n	Mean Melatonin in Serum	90 % percentile
03:00 A.M.	129	78.2 pg/ml	18.5 - 180 pg/ml
08:00 A.M.	128	28.5 pg/ml	3.8 - 80.4 pg/ml

Reference: Terzieva et al. Clin Lab (2009)

It is recommended that each laboratory establishes its own range of normal values.

15. LIMITATIONS OF THE PROCEDURE

Specimen collection has a significant effect on the test results. See SPECIMEN COLLECTION AND STORAGE for details.
For cross-reactivities, see PERFORMANCE.
The following blood components do not have a significant effect (+/- 20% of expected) on the test results up to the below stated concentrations:

Hemoglobin	8.0 mg/ml
Bilirubin	0.36 mg/ml

16. PERFORMANCE

	Substance	Cross Reactivity (%)
		Melatonin
Analytical Specificity (Cross Reaction)	5-Methoxy-Tryptophole	1.2
	N-Acetyl-Serotonin	1.2
	5-Methoxy-Tryptamine	2.5
	Cross-reactivity of other substances tested < 0.01 %	

	Melatonin
Analytical Sensitivity (Limit of Detection)	1.6 pg/ml
	Mean Signal (Zero Standard) - 2 SD

Precision					
Intra-Assay			**Inter-Assay**		
	Range (pg/ml)	CV (%)		Range (pg/ml)	CV (%)
Melatonin	8.8 - 151.7	3.0 - 11.4	Melatonin	5.6 - 134.3	6.4 - 19.3

Linearity		Range (pg/ml)	Serial dilution up to	Range (%)
	Melatonin	80.7 - 191.4	1:16	73 - 135

Recovery		Mean (%)	Range (%)	% Recovery after spiking
	Melatonin	102.4	83 - 125	

Method Comparison versus commercial RIA	Melatonin	ELISA = 1.01 x RIA + 4.6	r = 0.98; n = 50

Method Comparison versus other commercial RIA	Melatonin	ELISA = 0.86 x other RIA + 5.33	r = 0.96; n = 46

17. PRODUCT LITERATURE REFERENCES

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10. Czeisler CA et al. Suppression of melatonin secretion in some blind patients by exposure to bright light.N. Engl. J. Med., 332: 6-11 (1995)

⚠ ***For updated literature or any other information please contact your local supplier.***

Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Expiry date	LOT	Batch code		
	Consult instructions for use	CONT	Content		
	Caution	REF	Catalogue number	RUO	For research use only!