MELATONIN

ELISA

For research use only (RUO)
Not for use in diagnostic procedures

MLTN-96 96 tests

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ENGLISH

INTENDED USE

The Melatonin ELISA (MLTN-96) is intended for highly sensitive, quantitative determination of melatonin in human saliva (1-4) and – upon extraction – plasma, serum and other biological fluids.

For research use only. Not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The Melatonin ELISA is a competitive immunoassay using a capture antibody technique. Melatonin present in pre-treated saliva or extracted serum/plasma samples and controls as well as in the calibrators compete with biotinylated melatonin for the binding sites of the highly specific polyclonal Kennaway G280 anti-melatonin antibody (5, 6) during an overnight incubation. During this incubation the formed antibody-melatonin-biotin complexes are captured onto the pre-coated wells of the microplate. After washing away unbound melatonin-biotin conjugate the enzyme label (streptavidin conjugated to horseradish peroxidase) is added, which binds to the free biotin sites captured on the coated wells during a 60-minute incubation step. Unbound enzyme label is then removed by a second washing step and TMB (tetramethylbenzidine) substrate is added to the wells. In a further 30-minute incubation step, a chromophore is formed (turns from colorless to blue) in inverse proportion to the amount of melatonin originally present in the samples, controls and calibrators. The color turns from blue to yellow after the addition of an acidic stop solution and can be measured at 450 nm.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Pretreatment Solution	1 vial 5 mL	B-EKDSM-PRS	Ready to use **Corrosive agent**
Neutralizing Solution	1 vial 5 mL	B-EKDSM-NS	Ready to use **Irritant**
Microtiter Plate precoated with antibody capture molecules	12x8 wells	MLTN-MP	Stored under 250 µL of protection buffer; wash 2x before use
Plate Sealer	3 pieces	-	
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 mL	MLTN-WB	Dilute with 900 mL of deionized water
Blanking Reagent[1)] with preservatives	1 vial 1 mL	MLTN-BR	Ready to use
Zero Calibrator with preservatives	1 vial 5 mL	MLTN-0	Ready to use
Calibrators[2)] with preservatives	5 vials 1 mL	MLTN-CASET	Ready to use
Control low / high[3)] with preservatives; for pretreatment see p. 4	2 vials 1 mL	MLTN-CONSET	Ready to use
Biotin Conjugate with preservatives	1 vial 3 mL	MLTN-BC	Ready to use; blue color
Antiserum (G280) with preservatives	1 vial 3 mL	MLTN-AS	Ready to use; yellow color
Enzyme Label Streptavidin conjugated to HRP	1 vial 11 mL	MLTN-EL	Ready to use; yellow color
TMB Substrate buffered with citrate	1 vial 11 mL	MLTN-TMBF	Ready to use
Stop Solution 0.25 M sulfuric acid (H_2SO_4)	1 vial 11 mL	B-STS	Ready to use **Corrosive agent**

Table 1

[1)] The Blanking Reagent contains a saturated melatonin solution. Prevent any contamination of other kit reagents.

[2)] The Calibrators A, B, C, D and E contain the following melatonin concentrations: 0.4, 1.2, 4, 12 and 40 pg/mL which are corrected for the 20% sample dilution during pretreatment (saliva) or reconstitution (serum, plasma) and are therefore labeled with 0.5, 1.5, 5, 15, and 50 pg/mL of melatonin, respectively.

[3)] Lot specific amount of melatonin see QC data sheet added to the kit.

STORAGE AND SHELF LIFE OF REAGENTS

Sealed / Unopened Reagents	
Store at 2-8°C until expiration date. Do not use past expiration date.	
Opened / Reconstituted Reagents	
Microtiter Plate	Cover unused strips with a plate sealer, return them to the aluminium/plastic pouch and reseal along the entire edge of zip-seal. Store for up to 6 months at 2-8°C
Pretreatment Reagent	Store at 2-8°C until expiration date printed on the labels.
Neutralizing Solution	
Wash Buffer diluted	Store at 2-8°C up to 6 months.
Blanking Reagent	Store at 2-8 °C until expiration date printed on the labels.
Calibrators	
Controls	
Biotin Conjugate	
Antiserum	
Enzyme Label	
TMB Substrate	
Stop Solution	

Table 2

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes with disposable tips: 25 µL, 100 µL and 200 µL pipettes. Repeater or multichannel pipette for 25 µL and 100 µL.
- Disposable polypropylene tubes for the pretreatment of saliva samples.
- 1000 mL cylinder for the dilution of the Wash Buffer Concentrate.
- Microtiter plate washer or squeeze bottle for diluted Wash Buffer.
- Blotting paper.
- Refrigerator.
- Microtiter plate orbital shaker.
- Microtiter plate reader for measurement of absorbance at 450 nm.
- Saliva Collection Devices (Salivettes with cotton roll) can be ordered with NovoLytiX (order code: **B-SVC50**).
- Extraction Set (n=80 extractions) for the extraction of serum, plasma, and other biological fluids can be ordered with NovoLytiX (order code: **MLTN-EXSET**).

PRECAUTIONS

Safety precautions

- This test is for research use only, and must be handled by qualified personnel, in accordance with good laboratory practices (GLP).
- Pretreatment/ Neutralizing Solution, TMB Substrate and Stop Solution: The Pretreatment Solution (MLTN-PRS) contains sodium hydroxide (NaOH) and the Neutralizing Solution (MLTN-NS) contains hydrochloric acid (HCl). The Substrate Solution (MLTN-TMBF) contains Tetramethylbenzidine (TMB). The Stop Solution (B-STS) contains 0.25 M sulfuric acid. Each of those reagents is irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothes. Wear suitable protective clothing, gloves and eye protection. After contact with eyes or skin, wash immediately with plenty of water.
- Unused above solutions should be disposed of according to local state and federal regulations.

Technical precautions

Kit components

- Read this instruction for use (IFU) carefully before carrying out the test. Test performance will be adversely affected, if reagents are incorrectly diluted, modified or stored under conditions other than those as detailed in this IFU.
- Residual liquid in the microtiter plate wells result from the production process and stabilizes the coated antibodies. They are removed in the washing step before setting up the assay (assay procedure step 2) and do not affect the results.
- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Every effort should be made to ensure that no cross contamination occurs between reagents, samples or between wells. Be particularly careful by using the Blanking Reagent, as this contains an excess amount of melatonin.
- Microtiter plate wells cannot be re-used.
- Let the reagents adjust to reach room temperature and mix the reagents well before use.

Assay procedure

- The Blanking Reagent contains a saturated melatonin solution. Avoid any contamination of other reagents of this kit. Change disposable tips after each pipetting step.
- It is highly recommended that Blanking Reagent, Calibrators and Controls are assayed in duplicates.

SPECIMEN COLLECTION (SALIVA)

Saliva can be collected by passive drooling, spitting or using saliva collection devices (e.g. Salivettes). The Salivettes can absorb up to 3 mL of saliva. This ELISA procedure calls for 0.2 mL of saliva.

- The use of “neutral” untreated Sarstedt Salivettes with the transparent caps (order code: 51.1534) is recommended. These Salivettes can be ordered with NovoLytiX (order code: **B-SVC50**).
- The use of the Sarstedt Salivettes with the blue caps for cortisol determination (order code: 51.1534.500) is not recommended. This may lead to falsely high values, particularly if saliva volumes of less than 0.5 mL are collected.
- **The use of cotton swabs containing citric acid (i.e. Salivettes with green caps from Sarstedt) leads to wrong and irreproducible results. Do not use them at all.**
- Do not stimulate saliva flow by chewing gums or eating lemons.
- Individuals should perform the saliva collection on a day / an evening without sporting activities and any intense efforts.
- When collecting saliva at night, a dim flash light or a ≤100 lux yellow light should be used in order to avoid a possible light influence on the melatonin profile.
- The last meal and drinks except water must be taken at least 30 minutes before starting a saliva collection. Bananas and chocolate should not be eaten during the entire day before the collection period. Rinse the mouth with water 15 minutes before each collection time point.
- Drinks containing artificial colorants, caffeine (coffee, black or green tea, iced tea, cola) or alcohol are to be avoided during the collection.
- Individuals should avoid brushing their teeth, with or without toothpaste, during sampling periods. It is likely that individuals with gingivitis will contaminate the saliva with blood leading to unknown consequences.
- On the collection day, if possible, no aspirin and medicines that contain ibuprofen (Brufen®, Algifor® Dismenol®, Dolocyl®, Ecoprofen®) should be taken. If a patient or study subject is treated with melatonin, the last melatonin dose must be prescribed not later than two days before the collection period.

SPECIMEN SHIPMENT AND STORAGE

Storage: The saliva samples absorbed in the cotton swabs may be stored in the saliva collection device for up to 7 days at 2-8°C. If not assayed within one week after collection, samples should be frozen and may be stored for at least 6 months at ≤ -20°C. Do not add biocides to the saliva samples as this may lead to false results.

Shipment: Home- or outpatient-collected saliva samples can be shipped at ambient temperatures with a duration for up to three days. Before shipment such collected saliva samples can be kept in the refrigerator at 2-8°C for several days. There is no deterioration of melatonin, however bacterial and fungal growth may happen.

SERUM, PLASMA, AND OTHER BIOLOGICAL FLUIDS

Serum, plasma and other biological fluids can be measured with the NovoLytiX MLTN-96 ELISA upon SPE C18 column extraction. The instructions can be found in the IFU of the

Melatonin Extraction Set which allows for the extraction of 80 samples (order code: **MLTN-EXSET**).

SALIVA SAMPLE PRETREATMENT

Sample recovery from saliva collection devices

Centrifuge the collection devices for 5 min at 3000 rpm (~1500x g). Discard the suspended insert with the oral swab (cotton roll) and store the tube at 2-8°C or -20°C.

Pre-treatment of saliva samples and controls

- Pipet 200 µL of controls and saliva samples, respectively, into correspondingly marked polypropylene tubes.
- Add 25 µL of Pretreatment Solution to each tube using a multichannel or repeater pipette.
- Vortex for 5 seconds and leave the tubes for 10 minutes at 18-28 °C.
- Add 25 µL of Neutralizing Solution to each tube using a multichannel or repeater pipette. Vortex for 5 seconds.
- Centrifuge for 5 min at 5000 rpm and 18-28°C.
- Proceed with the supernatant to the ELISA procedure.

ASSAY PROCEDURE

1. Use a plate with enough 8-well strips to test the desired number of Calibrators, Controls and samples. Remove excess strips from the holder, cover them again with the attached Plate Sealer and re-seal them in the aluminium/plastic foil pouch. Store refrigerated.
2. Empty the wells and wash the strips twice using at least 300 µL of wash buffer per well. Empty the wells and strike the plate firmly onto blotting paper.

3a. Pipet 100 µL of Blanking Reagent (blank) in duplicate into wells A1+A2.

3b. Pipet 100 µL of Zero Calibrator (Cal 0) in duplicate into wells B1+B2.

3c. Pipet 100 µL of Calibrator A in duplicate into wells C1+C2.

Pipet 100 µL of Calibrator B in duplicate into wells D1+D2.

Pipet 100 µL of Calibrator C in duplicate into wells E1+E2.

Pipet 100 µL of Calibrator D in duplicate into wells F1+F2.

Pipet 100 µL of Calibrator E in duplicate into wells G1+G2.

3d. Pipet 100 µL of pretreated Low Control into wells H1+H2.

Pipet 100 µL of pretreated High Control into well A3+B3.

3e. Pipet 100 µL of each pretreated or extracted sample into the subsequent wells.

4. Add 25 µL of Biotin Conjugate (blue solution) to each well.
5. Add 25 µL of Antiserum (yellow solution) to each well.
6. Cover the plate with a Plate Sealer, place it for 1 min on a plate orbital shaker set at 600 rpm and then incubate for 16-24 hours at 2-8 °C.
7. Remove and discard the Plate Sealer. Aspirate or invert the plate to empty the solution from each well and wash five times using at least 300 µL of wash buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
8. Add 100 µL of Enzyme Label (yellow solution) to all wells.
9. Cover the plate with a new Plate Sealer, place the plate on a plate orbital shaker set at 600 rpm and incubate for 60 ± 5 minutes at 18-28 °C.

Important: *Allow the TMB Substrate to equilibrate to 18-28°C prior to use in step 11.*

10. Remove and discard the Plate Sealer. Aspirate or invert the plate to empty the solution from each well and wash five times using at least 300 µL of wash buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
11. Add 100 µL of TMB Substrate to all wells.
12. Cover the plate to protect it from direct light, place it on a plate orbital shaker set at 600 rpm and incubate for 30 ± 2 minutes at 18-28°C.
13. Add 100 µL of Stop Solution to all wells. Place the plate for 10 seconds on a plate orbital shaker set at 600 rpm and proceed to step 14 within 30 minutes.
14. Read the absorbance at 450 nm in a microtiter plate reader.

QUALITY CONTROL

A thorough understanding of this IFU is necessary for the successful use of the product. Reliable results will be obtained only by precise laboratory techniques (current GLP guidelines) and accurately following this IFU.
Since there are no controls for melatonin commercially available, we recommend using internal saliva or plasma/serum pools containing different levels of melatonin as internal quality controls. The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the Controls are lot-specific and are printed on the QC data sheet delivered with each test kit.
If the performance of the assay does not meet the established limits and repetition has excluded errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices; ii) ELISA reader settings; iii) expiration dates of reagents; iv) storage and incubation conditions; v) TMB Substrate solution should be colorless; and vi) purity of water.
Each new batch of Calibrators is tested against the United States Pharmacopeia (USP) Melatonin reference material.

STANDARDIZATION

The Calibrators of the Melatonin ELISA are standardized with UV/VIS: $\varepsilon_{278} = 6300\ M^{-1}cm^{-1}$ in ethanol.
Each new batch of Calibrators is tested against the United States Pharmacopeia (USP) Melatonin reference material.

RESULTS

Standard Curve

Record the absorbance at 450 nm for each calibrator and blank well. Average the duplicate values, subtract the average of the blank wells and record averages (=corrected average absorbance). Calculate the binding (B) of each pair of calibrator wells as a percent of Zero Calibrator (B_0 or Total, T), with the blank-corrected absorbance of the Zero Calibrator taken as 100 %.

$$B/B_0\ (\%) = \quad B/T\ (\%) = \frac{\text{net absorbance}}{\text{net absorbance of Zero Calibrator}} \times 100$$

Plot the percent bound (B/B_0 or B/T, vertical axis) versus the concentration of melatonin in pg/mL (horizontal axis) using a lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four-parameter logistic (4-PL) algorithm.

Samples and controls

- Record the absorbance at 450 nm for each control and each sample wells. Subtract the average of the blank wells and record the absorbance (=corrected average absorbance). Calculate, as described above, the binding of each pair of sample wells as a percent of Zero Calibrator (B_0 or T), with the blank-corrected absorbance of the Zero calibrator taken as 100%.
- Locate the B/B_0 (B/T) value of the samples on the vertical axis, draw a horizontal line intersecting the standard curve and read the melatonin concentration (pg/mL) from the horizontal axis.

See Table 3 and Figure 1 for examples of results and standard curves. ***Results and standard curves are for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.***

PERFORMANCE CHARACTERISTICS

Specificity: The 50% binding (cross-reactivity) of the melatonin antiserum with different compounds were tested in the Direct Saliva Melatonin Radioimmunoassay (RK-DSM2) from NovoLytiX, the former "BÜHLMANN RIA" and are presented in Table 4.

METHOD COMPARISON

The comparison was done with 87 saliva samples from 14 different donors collected at different daytimes. The samples were analyzed using this Melatonin ELISA (MLTN-96) as well as the Direct Saliva Melatonin Radioimmunoassay (RK-DSM2) from NovoLytix, the former "BÜHLMANN RIA". The subsequent linear regression analysis resulted in a correlation factor of R^2 = 0.931, an intercept of -0.12 pg/mL and a slope of 1.07. The correlation is presented in Figure 2.

APPENDIX I

TABLES AND FIGURES

Examples of Results

	Conc. (pg/mL)	Absorbance (OD)	B/B0 (B/T) (%)	CV Conc. (%)	Calc. Conc. (pg/mL)
Blank Blank **Avg.**		0.140 0.146 **0.143**		**3.0**	
Zero Calibrator Zero Calibrator **Avg.**	**0**	2.106 2.197 **2.151**	97.7 102.1 **100.0**	**3.0**	
Cal A Cal A **Avg.**	**0.5**	1.850 1.877 **1.868**	86.0 87.3 **86.7**	**0.9**	
Cal B Cal B **Avg.**	**1.5**	1.655 1.620 **1.638**	76.9 75.7 **76.3**	**1.1**	
Cal C Cal C **Avg.**	**5**	1.115 1.121 **1.118**	51.8 52.1 **52.0**	**0.2**	
Cal D Cal D **Avg.**	**15**	0.562 0.530 **0.550**	26.1 25.0 **25.6**	**0.7**	
Cal E Cal E **Avg.**	**50**	0.204 0.255 **0.230**	10.3 10.5 **10.4**	**1.7**	
Ctrl. High Ctrl. High **Avg.**		0.477 0.538 **0.557**	22.2 25.0 **10.7**	**11.7**	19.4 16.4 **17.9**
Ctrl. Low Ctrl. Low **Avg.**		1.435 1.379 **1.407**	66.7 64.1 **65.4**	**8.6**	2.5 2.8 **2.7**
Sample 01 Sample 01 **Avg.**		0.576 0.549 **0.563**	26.8 25.5 **26.2**	**4.8**	14.9 16.0 **15.4**
Sample 02 Sample 02 **Avg.**		1.313 1.261 **1.287**	61.0 58.6 **54.0**	**4.7**	3.2 3.6 **3.4**
Sample 03 Sample 03 **Avg.**		1.888 1.806 **1.847**	87.8 84.0 **85.9**	**23.5**	0.6 0.9 **0.8**

Table 3

ED80 = 1.2 pg/mL ED50 = 5.4 pg/mL ED20 = 20.4 pg/mL

Example of Standard Curve (OD_{450})

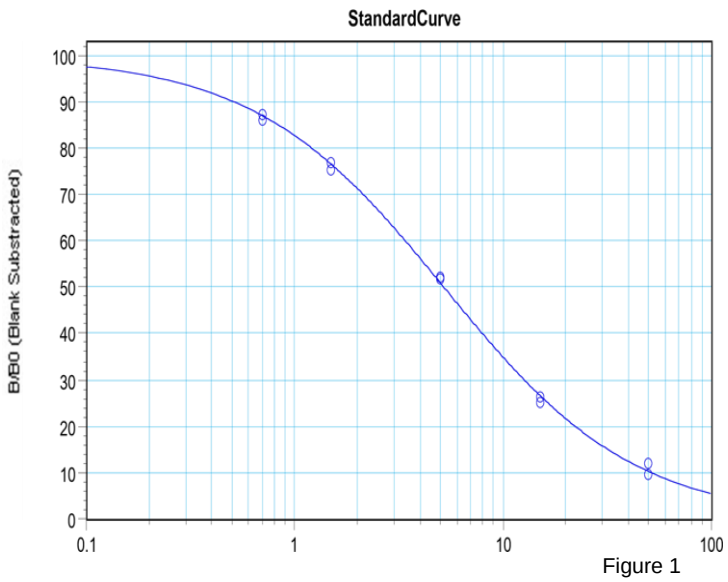

Figure 1

Specificity

Compound	Crossreactivity [%]
melatonin	100
serotonin	< 0.001
6-sulfatoxymelatonin	< 0.001
N-acetylserotonin	0.045
5-hydroxy-indole acetic acid	< 0.001
5-methoxytryptamine	0.007
5-methoxytryptophane	< 0.001
2-methyl-5-hydroxytryptamine	< 0.001
5-methoxypsoralen	< 0.001
5-methoxytryptophol	0.002
chloromelatonin	1.3
L-arginine	< 0.001
Arg-vasopressin	<0.001
noradrenalin (norepinephrin)	<0.001
caffeine	<0.001
caffeine acid	<0.001
soluble coffee	<0.001
soluble coffee decaffeinated	<0.001

Table 4

Comparison

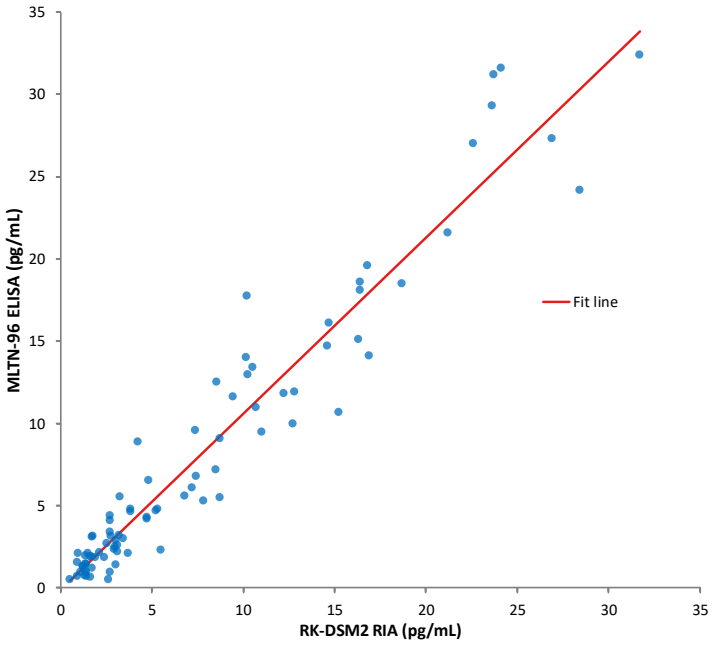

Figure 2

APPENDIX II

PIPETTING PROTOCOLS

Sample Pretreatment (Saliva & Controls)

Clean polypropylene tube

200 µL Saliva Sample or Control

add 25 µL Pretreatment Solution

↓ ↻ *Vortex 5 sec*

Incubate 10 min, 18-28°C

add 25 µL Neutralization Solution

↓ ↻ *Vortex 5 sec*

Centrifuge 5 min, 5000 rpm, 18-28°C

Proceed to ELISA procedure

ELISA Procedure

Precoated Microtiter Plate

↓ ↻ *Wash 2x with ≥300 µL Wash Buffer*

100 µL Calibrators, Pretreated Controls or Samples

↓

add 25 µL Melatonin-Biotin-Conjugate

↓

add 25 µL Antiserum

↓ ↻ *1 minute on a plate orbital shaker*

↻ *16-22 hours at 2-8°C*

↻ *Wash 5x with ≥300 µL Wash Buffer*

add 100 µL Enzyme Label

↓ ↻ *60 minutes at 18-28°C on a plate orbital shaker*

↻ *Wash 5x with ≥300 µL Wash Buffer*

add 100 µL TMB Substrate

↓ ↻ *Incubate 30 minutes at 18-28°C on a plate orbital shaker*

add 100 µL Stop Solution

➡ Read absorbance at 450 nm (within 30 minutes)

APPENDIX IV

SYMBOLS

Symbol	Explanation
	Use By
REF	Order Code
LOT	Batch Code
	Contains sufficient for <n> tests
	Consult Instructions for Use
	Temperature Limitation
REAG PRE	Pretreatment Solution
SOLN NEUT	Neutralizing Solution
MP	Microtiter Plate
AS	Antiserum

Symbol	Explanation
BUF WASH 10X	Wash Buffer Concentrate (10x)
REAG BLANK	Blanking Reagent
CAL 0	Zero Calibrator
CAL A - CAL E	Calibrator A - E
CONTROL L	Control Low
CONTROL H	Control High
BC	Biotin Conjugate
EL	Enzyme Label
SUBS TMB	TMB Substrate
SOLN STOP	Stop Solution

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