Direct Saliva MELATONIN

ELISA

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

EK-DSM-U 96 tests

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ENGLISH

INTENDED USE

The BÜHLMANN Direct Saliva Melatonin ELISA (EK-DSM) is intended for highly sensitive, quantitative determination of melatonin in human saliva (1-4).

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

PRINCIPLE OF THE ASSAY

The BÜHLMANN Direct Saliva Melatonin ELISA is a competitive immunoassay using a capture antibody (Ab) technique. The polyclonal Kennaway G280 anti-melatonin antibody (5, 6) has been coated onto the microtiter plate, provided in the kit. After the first 16-20 hour overnight incubation, melatonin present in the pre-treated saliva and controls as well as in the calibrators, compete with biotinylated melatonin during a 3 hour incubation for the binding sites of this highly specific antibody. After washing, the enzyme label streptavidin conjugated to horseradish peroxidase (HRP) is added, which binds to the melatonin-biotin-antibody complexes captured on the coated wells during a 60 minute incubation step. Unbound enzyme label is then removed by a washing step and TMB substrate (tetramethylbenzidine) is added to the wells. In a further 30 minute incubation step, a chromophore is formed in inverse proportion to the amount of melatonin present in the sample. 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 G280 anti-melatonin Ab	12x8 wells	B-EKDSM-MP	Wash 2x before use
Plate Sealer	3 pieces	-	Ready to use
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 mL	B-EKDSM-WB	Dilute with 900 mL deionized water
Blanking Reagent[1)] lyophilized	1 vial lyoph	B-EKDSM-BR	Reconstitute in 1mL Incubation Buffer
Incubation Buffer (Zero Calibrator) melatonin-free buffer	1 vial 12 mL	B-EKDSM-IB	Ready to use
Calibrators[2)] lyophilized; do not pretreat	5 vials lyoph	B-EKDSM-CASET	Reconstitute each with 1 mL Incubation Buffer
Control low / high[3)] for pretreatment see page 4	2 vials lyoph	B-EKDSM-CONSET	Reconstitute each with 1 mL Incubation Buffer
Biotin Conjugate	1 vial 5.5 mL	B-EKDSM-BC	Ready to use
Enzyme Label Streptavidin conjugated to HRP	1 vial 11 mL	B-EKDSM-EL	Ready to use
TMB Substrate buffered with citrate	1 vial 11 mL	B-TMB	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 concentration: 0.48, 1.2, 3.2, 8.0 and 20 pg/mL which are corrected for the 20% sample dilution during pretreatment and therefore, labeled with 0.6, 1.5, 4.0, 10, and 25 pg/mL of melatonin, respectively.

[3)] Lot specific amount of melatonin see 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	Return unused strips immediately to the plastic pouch containing the desiccant pack and reseal along the entire edge of zip-seal. Store for up to 2 months at 2-8°C
Pretreatment Reagent	Store at 2-8°C until expiration date printed on the labels.
Neutralizing Solution	
Incubation Buffer	
Wash Buffer diluted	Store at 2-8°C up to 6 months
Blanking Reagent	Stable at 2-8°C up to 4 months.
Calibrators	
Controls	
Biotin Conjugate	Store at 2-8 °C until expiration date printed on the labels.
Enzyme Label	
TMB Substrate	
Stop Solution	

Table 2

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes with disposable tips: 5 µL, 50 µL, 100 µL and 1000 µL pipettes. Repeater or multichannel pipette for 50 µL and 100 µL.
- Disposable polystyrene or polypropylene tubes for the preparation of sample dilutions.
- 1000 mL cylinder for the dilution of the wash buffer.
- Microtiter plate washer or squeeze bottle for wash buffer.
- Blotting paper.
- Refrigerator
- Microtiter plate orbital shaker.
- Microtiter plate reader for measurement of absorbance at 450 nm.
- BÜHLMANN Saliva Collection Devices, B-SLEEP-CHECK16 or B-SVC (50).

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, substrate and stop solution: The pretreatment solution (B-EKDSM-PRS) contains sodium hydroxide (NaOH) and the neutralizing solution (B-EKDSM-NS) contains hydrochloric acid (HCl). Substrate and stop solution: The substrate TMB (B-TMB) contains Tetramethylbenzidine. The stop solution (B-STS) contains sulfuric acid (0.25 M). 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 solution should be disposed of according to local state and federal regulations.

Technical precautions

Kit components

- Read the instructions 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 instruction for use.
- Residues in the microtiter plate wells result from the production process. They are removed in the washing step (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.
- Microwells cannot be re-used.
- Let the reagents adjust to reach room temperature. Mix the reagents well (vortex) 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.
- The assay procedure has been optimized for Sleep Check application. Therefore blank reagent and calibrators are assayed in duplicates, whereas controls and samples are measured in single determinations. This approach allows you to test 16 individual profiles (with 5 points each) per microtiter plate. For applications other than Sleep Check duplicate determinations are recommended.

SPECIMEN SHIPMENT AND STORAGE

Shipment: The collected saliva samples must be shipped to the laboratory within two days.

Collected saliva samples must be kept in the fridge at 2-8°C.

Samples should not be sent on Fridays, Saturdays or the day before holiday.

Storage: The saliva samples absorbed in the cotton swab 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. Repeated freeze-thaw cycles should be avoided.

SPECIMEN COLLECTION

Collect saliva using the BÜHLMANN Saliva Collection Devices. The devices can absorb up to 3 mL of saliva. The procedure calls for 0.2 mL of saliva.

- Do not use cotton swabs containing citric acid.
- Do not stimulate saliva flow by chewing gums or eating lemons.
- Individuals should perform the collection on 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.
- Nothing should be eaten during the collection time. The last meal must be taken at least 30 minutes before starting the collection. Bananas and chocolate should not be eaten during the entire day before the collection. Rinse the mouth with water 15 minutes before the collection.
- Drinks containing artificial colorants, caffeine (coffee, black or green tea, iced tea, cola) or alcohol are to be avoided on the evening of 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 plasma or even whole 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 your sleep or sleep-wake rhythm is treated with melatonin, this must be discontinued at least one week before the collection.

SAMPLE PRETREATMENT (LABORATORY)

Sample recovery from saliva collection devices

Centrifuge the collection devices for around 5 min at 3000 rpm (~1500x g). Discard the suspended insert with the swab 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 the pre-treated samples for 5 minutes at 10'000 rpm. Proceed to the ELISA procedure.

ASSAY PROCEDURE

1. Use a plate with enough 8-well strips to test the desired number of blanks, calibrators, controls and samples. Remove excess strips from the holder and re-seal them in the foil pouch together with the two desiccant bags **without delay**. Store refrigerated.
2. Wash the coated 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 incubation buffer (zero calibrator) 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 well H1

Pipet 100 µL of pretreated high control into well H2

3e. Pipet 100 µL of each pretreated sample (single) into the subsequent wells.

4. Cover the plate with a plate sealer and incubate for 16-20 hours at 2-8 °C.
5. Remove and discard plate sealer. Add 50 µL of biotin conjugate (blue solution) to each well. Cover the plate with a plate sealer and place it for 1 min on a plate orbital shaker set at 600 rpm.
6. Incubate for 3 hours (±5 minutes) 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 four 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 minutes (±5 minutes) at 18-28 °C.
10. Remove and discard the plate sealer. Aspirate or invert the plate to empty the solution from each well and wash four times using at least 300 µL of wash buffer per well. Empty the wells and strike the plate firmly onto blotting paper.

Important: *allow the TMB substrate to equilibrate to 18-28°C prior to use.*

11. Add 100 µL of TMB substrate to all wells.
12. Cover the plate, place it on a plate orbital shaker set at 600 rpm, protect the plate from direct light and incubate for 30±5 minutes at 18-28°C.
13. Add 100 µL of stop solution to all wells. Remove air bubbles by pricking them with a pipette tip. 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 instruction for use 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 instruction for use.
Since there are no controls for saliva melatonin commercially available, we recommend using saliva pools containing different levels of melatonin for 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 printed on the additional QC data sheet.
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.

STANDARDIZATION

Direct Saliva Melatonin ELISA is calibrated with UV/VIS: ε_{278} = 6300 $M^{-1}cm^{-1}$ in methanol.

RESULTS

Standard Curve

Record the absorbance at 450 nm for each calibrator, incubation buffer 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 incubation buffer (B_0), with the blank-corrected absorbance of the incubation buffer taken as 100 %.

$$B/B_O\ (\%) = \text{percent bound} = \frac{\text{net absorbance}}{\text{net absorbance of incubation buffer}} \times 100$$

Plot the percent bound (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 algorithm.

Samples and controls

- Record the absorbance at 450 nm for each sample, each control and well. 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 incubation buffer (B_0), with the blank-corrected absorbance of incubation buffer taken as 100%.

- Locate the B/B_0 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. *These 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

Intra-Assay Precision: 12.6 %. The intra-assay precision was calculated from the results of four different saliva samples within the standard range, measured 10 times in duplicate in a single run. The results are presented in table 4.

Inter-Assay Precision: 22.9 %. The inter-assay precision was calculated from the results of 17 independent runs with 5 samples within the standard range. The results are presented in table 5.

Detection Limit (LoB): 0.5 pg/mL. 32 wells of incubation buffer (zero calibrator) were assayed in two independent runs. The minimum detectable concentration in 0.1 mL of incubation buffer was calculated by subtracting two standard deviations of averaged refer values from the OD of zero calibrator and intersecting the value with the standard curve obtained in the same run.

Detection Limit (Limit of Quantification - LoQ): 1.6 – 20.5 pg/mL. The limit of quantification of this assay is the melatonin concentration in saliva that can be measured with an inter-assay coefficient of variation (CV) of less than 30 %. The LoQ was determined from 7 different samples from 1.3 **–** 47.3 pg/mL each sample measured 17 times in duplicate in independent runs.

Dilution Linearity/Parallelism: 92.2 %. Three saliva samples with high amount of melatonin were sequentially diluted with incubation buffer and assayed according to the assay procedure. The results are presented in table 6.

Due to the complex matrix of saliva samples dilution with incubation buffer higher than 1:8 will cause a decreased linearity. Therefore sample dilution with incubation buffer higher than 1:4 is not recommended.

Spiking Recovery: 97.9%. Two saliva samples from the same donor, one collected during daytime and one during night time were titrated against each other and assayed according the assay procedure twice, independently. The results are presented in table 7.
Due to the complex and individual nature of the saliva matrix direct spiking of saliva with melatonin can lead to decreased recovery rates.

Specificity: The 50% binding (cross-reactivity) of the melatonin antiserum with different compounds were tested in the Direct Saliva Melatonin Radioimmunoassay (RK-DSM) from BÜHLMANN and are presented in table 8.

METHOD COMPARISON

The comparison was done with 78 saliva samples from 10 different donors collected at different daytimes. The samples were analyzed using the presented EK-DSM assay as well as the Direct Saliva Melatonin Radioimmunoassay (RK-DSM) from BÜHLMANN. The subsequent linear regression analysis resulted in a correlation factor of $R^2 = 0.84$, an intercept of 0.77 pg/mL and a slope of 1.21. The correlation is presented in figure 3.

APPENDIX I

TABLES AND FIGURES

Examples of results

	Conc. (pg/mL)	Absorbance (OD)	B/B0 (%)	CV Conc. (%)	Calc. Conc. (pg/mL)
Blank Blank **Avg.**		0.075 0.067 **0.071**		**5.6**	
Zero Calibrator Zero Calibrator **Avg.**	**0.0**	1.715 1.766 **1.741**	98.5 101.5 **100.0**	**2.1**	
Cal A Cal A **Avg.**	**0.6**	1.484 1.514 **1.499**	85.3 87.0 **86.1**	**1.4**	
Cal B Cal B **Avg.**	**1.5**	1.292 1.274 **1.283**	74.2 73.2 **73.7**	**1.0**	
Cal C Cal C **Avg.**	**4.0**	0.755 0.769 **0.762**	43.4 44.2 **43.8**	**1.3**	
Cal D Cal D **Avg.**	**10**	0.364 0.359 **0.362**	20.9 20.6 **20.8**	**1.0**	
Cal E Cal E **Avg.**	**25**	0.179 0.182 **0.181**	10.3 10.5 **10.4**	**1.2**	
Ctrl. high Ctrl. high **Avg.**		0.560 0.553 **0.557**	32.2 31.8 **32.0**	**0.9**	6.2 6.3 **6.2**
Ctrl. low Ctrl. low **Avg.**		0.929 0.874 **0.902**	53.4 50.2 **51.8**	**4.3**	2.9 3.3 3.1
Sample 01 Sample 01 **Avg.**		0.414 0.404 **0.409**	23.8 23.2 **23.5**	**1.7**	8.9 9.2 **9.0**
Sample 02 Sample 02 **Avg.**		0.970 0.908 **0.939**	55.7 52.2 **54.0**	**4.7**	2.7 3.0 **2.9**
Sample 03 Sample 03 **Avg.**		1.215 1.162 **1.189**	69.8 66.8 **68.3**	**3.2**	1.6 1.8 **1.7**

Table 3

ED20 = 10.9 pg/mL ED50 = 3.3 pg/mL ED80 = 1.0 pg/mL

Example of Standard Curve (OD_{450})

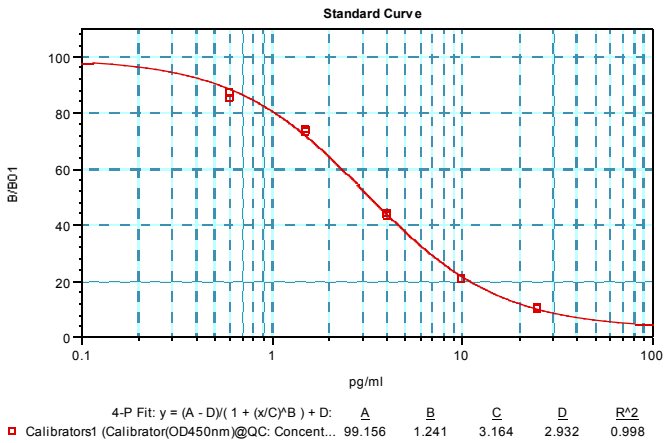

Figure 1

Pipetting Scheme

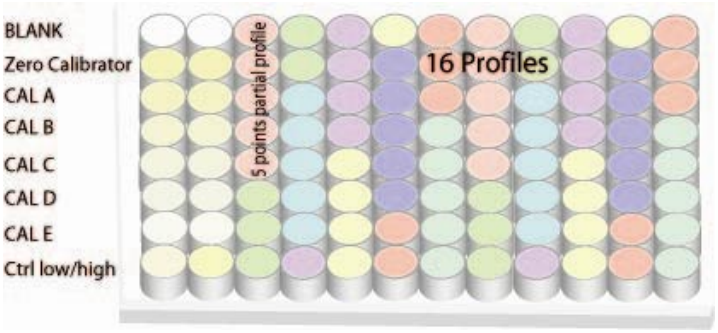

Figure 2

Intra-Assay Precision

Sample	Mean [pg/mL]	SD [pg/mL]	CV [%]
S01	1.7	0.19	11.2
S04	5.2	1.20	22.9
S03	13.7	1.48	10.8
S05	15.9	0.84	5.3
Mean			12.6

Table 4

Inter-Assay Precision

Sample	Mean [pg/mL]	SD [pg/mL]	CV [%]
Ctrl low	2.6	0.59	23.8
S11	2.4	0.41	17.2
S12	4.6	1.32	28.8
Ctrl high	5.2	1.20	23.2
S13	13.7	3.05	22.3
Mean			22.9

Table 5

Dilution Linearity/ Parallelism

Sample	Dilution Factor	Observed [pg/mL]	Expected [pg/mL]	Recovery O/E [%]
S06	1:1 1:2 1:4 [1:8]	14.9 7.8 3.0 [1.1]	-- 7.5 3.7 [1.9]	-- 104.7 80.5 [59.1]
S07	1:1 1:2 1:4 [1:8]	22.7 11.6 5.0 [1.8]	-- 11.4 5.7 [2.8]	-- 102.2 88.1 [63.4]
S08	1:1 1:2 1:4 [1:8]	20.4 13.1 6.4 [2.7]	-- 13.6 6.8 [3.4]	-- 96.3 94.1 [79.4]
Mean				92.2

Table 6

APPENDIX I

TABLES AND FIGURES

Spiking Recovery

Sample	Titration Ratio S5/S8	Expected [pg/mL]	Observed [pg/mL]	Recovery O/E [%]
S5/S8	5/0	1.2	1.2	--
	4/1	4.3	4.4	102.8
	3/2	7.4	5.3	72.0
	2/3	10.4	9.8	93.9
	1/4	13.5	13.9	102.8
	0/5	16.6	16.6	--
S5/S8	5/0	0.9	0.9	--
	4/1	3.7	3.6	96.8
	3/2	6.5	6.4	97.9
	2/3	9.4	10.5	112.2
	1/4	12.2	12.8	105.1
	0/5	15.0	15.0	--
Mean				97.9

Table 7

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
chloramelatonin	1.3
caffeine	< 0.001
caffeine acid	<0.001
soluble coffee	<0.001
soluble coffee decaffeinated	<0.001

Table 8

Correlation EK-DSM/ RK-DSM

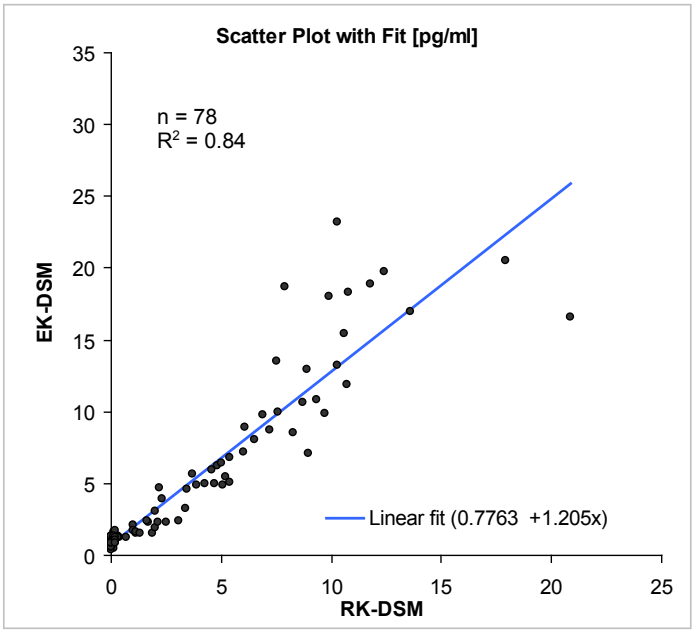

Figure 3

APPENDIX II

PIPETTING PROTOCOL

Direct Saliva Melatonin
Sample Pretreatment (Saliva& Controls)

Clean polypropylene tube

200 µL Saliva Sample or Control
25 µL Pretreatment Solution

Vortex, 5 sec

Incubate, 10 min, 18-28°C

25 µL Neutralization Solution

Vortex, 5 sec

Centrifuge at 10'000 rpm for 5 min

Proceed to ELISA procedure

Direct Saliva Melatonin
ELISA Procedure

Precoated Microtiter Plate

Wash 2x with ≥300µL wash buffer

100 µL Calibrators, Pretreated Controls or Samples

Incubate 16-20 hours at 2-8°C

Add 50 µL Melatonin-Biotin-Conjugate

1 minute on a plate orbital shaker

3 hours at 2-8°C

Wash 4x with ≥300µL wash buffer

add 100 µL Enzyme Label

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

Wash 4x

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 III

NOTES

APPENDIX III

NOTES

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 Reagent
SOLN NEUT	Neutralizing Solution
MP	Microtiter Plate

Symbol	Explanation
BUF WASH 10X	Wash Buffer Concentrate (10x)
REAG BLANK	Blanking Reagent
BUF INC	Incubation Buffer
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