MP Biomedicals
Diagnostics Division
29525 Fountain Parkway
Solon, OH 44139

Direct Salivary Melatonin EIA Kit

For Research Use Only

Catalog Number: 07P634A

Customer Service: (800) 854-0530
Fax: (440) 337-1180
e-mail: custserv@mpbio.com
Website: www.mpbio.com
PT-Revision 3, 12/18
QER NO.: Q19-014
04/19

I. Intended Use and Description

The MP Biomedicals Direct Salivary Melatonin Kit is a rapid, sensitive and specific EIA designed and validated for the direct quantitative measurement of Melatonin in human saliva.

II. Assay Background

Melatonin (N-Acetyl-5-methoxytrptamine) is a biogenic amine that is found in animals and plants. In mammals, melatonin is produced by the pineal gland. Its secretion increases in darkness and decreases during exposure to light. Melatonin is implicated in the regulation of sleep, mood and reproduction. Melatonin is also an effective antioxidant. (1-6)

III. Assay Principle

The MP Biomedicals Direct Salivary Melatonin EIA kit is based on the competition principal and microplate separation. Melatonin in standards (calibrators) and samples compete with a fixed amount of melatonin conjugated to horse radish peroxidase (Melatonin-HRP) for binding sites with a rabbit melatonin monoclonal antiserum bound to GARGG (goat anti-rabbit gamma globulin) coated wells of a microplate. After incubation, unbound components are washed away, enzyme substrate solution is added, and a blue color formed. This reaction is stopped with an acid solution to produce a yellow color. The optical density is then read at 450 nm. The amount of Melatonin-HRP detected is inversely proportional to the amount of melatonin in a sample.

IV. Reagents Provided and Reagent Preparation

Store all other reagents at 2 to 8°C. Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers. Expiration dates and lot numbers are printed on the labels.

1. **GARGG Plate**: One 96 well microplate (12x8 breakable strip wells) coated with goat ani-rabbit gamma globulin placed in a resealable foil bag with desiccant. One (1) 96 well kit is sufficient for 38 duplicate patient measurements.
2. **Concentrated Stock Melatonin (synthetic) solution**: Dilute the 6400 pg/mL stock solution 1:100 (1 part 6400 pg/mL+ 99 parts assay diluent) to obtain the highest working

calibrator (64 pg/mL) then, dilute serially 1:2 (starting with the 64 pg/mL calibrator) to obtain the following concentrations of **working calibrators**: 32 pg/mL, 16 pg/mL, 8 pg/mL, 4 pg/mL, 2 pg/mL and1 pg/mL. "0" calibrator is assay diluent.

3. **Assay diluent**: 1 bottle 20 mL.
4. **Stock Melatonin (synthetic) Control Concentrate 3 ng/mL (3000 pg/mL**): 1 bottle 0.200 mL.

Working Control # 2 Preparation (Example)

Stock Melatonin Control concentrate 3000 pg/mL	Assay Diluent	Dilution	Target	Number of EIA wells per 5 mL volume
0.05 mL (50 µL)	4.950 mL	1:100	30 pg/mL	100

Working Control # 1 Preparation

Melatonin Control #2	Assay Diluent	Dilution	Target	Number of EIA wells per 5 mL volume
0.5 mL	4.5 mL	1:10	3 pg/mL	100

Immediately after use, store the unused portions of the **working calibrators** and the **High** and **Low Controls** at 2-8°C. Discard if not used within **7** days of mixing.

5. **Salivary Melatonin EIA rabbit monoclonal Antibody:** 1 bottle, 3 mL. The solution is blue.
6. **Salivary Melatonin-Horseradish Peroxidase (HRP) concentrate**. 1 amber bottle, 0.7 mL. Melatonin derivative is conjugated to horseradish peroxidase. The solution is yellow and light sensitive.
7. **Melatonin-Horseradish Peroxidase (HRP) conjugate buffer, pH 7.4**: 1 bottle, 6 mL. Use only for the preparation of the **Melatonin-HRP working reagent.**
   Melatonin-HRP working reagent preparation: Determine the amount of **working Melatonin HRP** needed and dilute 1:10 with **conjugate buffer** pH 7.4 (#7). For example, mix 0.5 mL of **Melatonin-HRP concentrate** (#6) plus 4.5 mL with **conjugate buffer,** (#7). This is sufficient for 100 EIA wells.
   The **Melatonin-HRP working reagent** is light sensitive. Immediately after use, wrap the vial with the unused portion of the **Melatonin-HRP working reagent** with aluminum foil or alternatively, prepare the **Melatonin-HRP working reagent** in an amber vial. Store at 2-8°C. Discard if not used within 7 days of mixing.
8. **Wash solution (10X concentrated) EIA #1**: 1 bottle, 50 mL of phosphate buffered saline, pH 7.4. Prior to use dilute 1:10 with deionized water.
9. **Color Development Reagent EIA #1**: 1 amber plastic bottle, 15 mL of Tetramethylbenzidine (TMB) plus hydrogen peroxide. Light sensitive.
10. **Stopping Solution EIA #1**: 1 bottle of a 15 mL mixture of diluted sulfuric and hydrochloric acid solution.

V. Storage and Stability

1. When stored at 2° - 8°C, unopened reagents will retain activity until the expiration date. Do not use reagents beyond this date.
2. Use only reagents supplied with this kit. Do not interchange reagents with different lot numbers.
3. Opened reagents must be stored at 2° - 8°C.
4. Microtiter wells must be stored at 2° - 8°C. Once the foil bag has been opened, care should be taken to reseal tightly.
5. Opened kits retain activity for 28 days if stored as described above.

6. Expiration dates and lot numbers are printed on the labels.

VI. Materials Needed But Not Provided

1. Device to dispense accurately 25 µL and 50 µL.
2. Multichannel pipettors.
3. Microplate or orbital shaker.
4. Vortex Mixer.
5. Microplate washer (not required, plates can be washed manually).
6. Microplate reader capable of reading 450 nm with 4 parameter data reduction or comparable software.
7. Plate Sealers.
8. Suitable saliva sample collection device.

VII. Sample Collection and Processing

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). Saliva can be collected in a suitable sampling device. A minimum of 0.5 mL liquid should be collected.

After collection, refrigerate sample within 30 minutes and freeze at or below -20°C within 4 hours of collection. On day of assay thaw the saliva samples, vortex and centrifuge at 1500x for 15 minutes. Dispense clear sample into appropriate wells.

Sample stability

Storage	Room Temperature 20 – 30 °C	37 °C	2 – 8 °C	≤ -15 °C (freeze / thaw cycles)	≤ - 15 ° C (Long term)
Stability	Up to 7 days	Up to 7 days	Up to 7 days	Up to 7 times	TBD

VIII. Assay Procedure Summary Flow Sheet

Melatonin Calibrators I.D. pg/mL	**Calibrator, Control, Sample (µL)**	**HRP Melatonin Working Solution (µL)**	**Anti-Melatonin (µL)**		**Diluted 10X Wash Solution. (µL)**		**Color Development Reagent (µL)**		**Stopping solution (µL)**	
0	50	50	25	Mix. Incubate for 2 hrs. at Room Temperature, shaking.	300	Wash 3X	125	Mix. Incubate 30 min. at room temperature	125	Mix. Read at 450 nm
1	50	50	25		300		125		125	
2	50	50	25		300		125		125	
4	50	50	25		300		125		125	
8	50	50	25		300		125		125	
16	50	50	25		300		125		125	
32	50	50	25		300		125		125	
64	50	50	25		300		125		125	
Control #1	50	50	25		300		125		125	
Control #2	50	50	25		300		125		125	
Sample	50	50	25		300		125		125	

IX. Assay Procedure

1. It is recommended that the **calibrators**, **controls** and **samples** should be tested in duplicate and the mean value should be used to report the results.
2. To the GARGG microplate dispense **50 µL** of **working Salivary Melatonin EIA calibrators** (0, 1, 2, 4, 8, 16 ,32 and 64 pg/mL), **controls**, and **saliva samples**.
3. Add **50 µL** of **Melatonin-HRP working reagent** to all wells.
4. Add **25 µL** of **Melatonin EIA rabbit monoclonal antibody**.
5. Cover microplate with plastic sealer. Incubate by shaking on a microplate orbital shaker set at 500-900 rpm for **2 hrs.** at room temperature.
6. After incubation, decant the contents of the wells. Wash 3 times with 300 **µL** of **diluted wash solution.** After the 3rd wash, invert GARGG microplate on an absorbent paper and tap dry.
7. Dispense **125 µL of Color Development Reagent EIA #1** into each well. Shake briefly (manual). Cover microplate with plastic sealer. Incubate for **30 minutes at room temperature**.
8. Dispense **125 µL of Stopping Solution EIA #1** into each microtiter well of the GARGG plate. Shake briefly (manual). Color changes from blue to yellow.
9. Read at 450 nm on a microplate reader within 10 minutes.

Note: If samples exceed the upper end of the measuring range of 64 pg/mL, dilute with zero calibrator and make appropriate concentration correction.

X. Typical Results

Typical Calibration Curve (Actual assay)			
Calibrators (pg/mL)	**Mean Absorbance (450 nm)**	**% B/Bo**	**Value (pg/mL)**
0	2.67	100.0	0
1	2.41	90.3	1
2	2.21	82.8	2
4	1.95	73.0	4
8	1.53	57.3	8
16	1.01	37.8	16
32	0.60	22.5	32
64	0.36	13.5	64
Control I	2.11	79.0	2.8
Control II	0.67	25.1	29.7
Sample I	2.32	86.9	1.5
Sample II	1.48	55.4	8.2
Sample III	1.08	40.4	15.0

XI. Determination of Melatonin Concentration

Determine the concentrations of the controls and unknowns by interpolation using Software capable of logistics using a 4-parameter sigmoid minus curve fit.

Analytical measuring range (AMR)	1 – 64 pg/mL

XII. Quality Control

The expected values for the controls are stated on the certificate of analysis which are included in the kit. The results can only be accepted if the expected values are met. Follow federal, state and local guidelines for testing quality control materials.

XIII. Expected Melatonin Normal Ranges

Saliva samples from apparently healthy subjects collected in the PM (bedtime), AM (arise), and noon show the following results below:

Time	Subjects (Number)	Median (pg/mL)	Range (pg/mL)
PM	27	6.4	1.4 – 24.2
AM	27	6.3	1.6 – 22.6
NOON	27	2.7	0.2 – 10.2

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

XIV. Performance Characteristics

A. Specificity of the Antiserum

Compounds	% Cross-reactivity
N-Acetylserotonin	0.38
5-MethoxyTryptophol	<0.001
5-Methoxy-DL-Tryptophan	<0.001
Serotonin Hydrochloride	<0.001
5-Methoxytryptamine	0.15
6-Hydroxymelatonin	<0.001

B. Sensitivity

Analytical Sensitivity

The lower limit of sensitivity was determined by interpolating the mean optical density minus 2 SDs of 20 values at the 0 pg/mL level. The minimal concentration of Melatonin that can be distinguished from 0 is 0.62 pg/mL.

C. Precision and Reproducibility:

Intra-assay

The intra-assay precision was determined from the mean of 20 replicates of low, medium and high pools.

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	20	5.5	0.275	5.0
Medium	20	9.9	0.507	5.1
High	20	27.6	1.330	4.8

Inter-assay

The inter-assay precision was determined from the mean average of the duplicates for 12 separated assays with low, medium and high pools.

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	12	5.6	0.620	11.1
Medium	12	9.9	0.971	9.8
High	12	26.6	2.194	8.3

Inter-lot Variation

The inter-lot precision was determined by duplicate measurements of three (3) saliva pools and three (3) individual saliva samples, using three (3) different reagent lots.

Saliva Samples ID	Lot # 001 mean (pg/mL)	Lot # 002 mean (pg/mL)	Lot # 003 mean (pg/mL)	Inter-lot mean (pg/mL)	Inter-lot Std. Dev. (pg/mL)	Inter-lot CV (%)
Sample 1	23.3	25.7	25.8	24.9	1.415	5.7
Sample 2	11.1	12.9	13.5	12.5	1.249	10.0
Sample 3	4.3	5.1	4.6	4.7	0.404	8.7
Pool 1	2.5	2.7	2.7	2.6	0.115	4.4
Pool 2	18.2	20.9	20.1	19.7	1.387	7.0
Pool 3	34.4	35.8	34.2	34.8	0.872	2.5

D. Dilution Study:

Sample I.D.	Dilution factor	Expected pg/mL	Observed pg/mL	Recovery (%)
1			27.0	
	1:2	13.500	13.800	102.2
	1:4	6.750	7.200	106.7
	1:8	3.375	3.300	97.8
	1:16	1.688	1.700	100.7
2			17.1	
	1:2	8.550	9.500	111.1
	1:4	4.275	4.500	105.3
	1:8	2.138	2.200	102.9
	1:16	1.069	1.100	102.9
3			23.3	
	1:2	11.650	10.900	93.6
	1:4	5.825	5.700	97.9
	1:8	2.913	2.900	99.6
	1:16	1.456	1.600	109.9
4			39.9	
	1:2	19.950	21.400	107.3
	1:4	9.975	9.900	99.2
	1:8	4.988	5.600	112.3
	1:16	2.494	2.500	100.3

E. Recovery

Four saliva samples with different levels of endogenous Melatonin were spiked with known quantities of Melatonin.

Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	11.0	20.0	31.0	33.4	107.7
2	8.0	10.0	18.0	17.0	94.4
3	2.1	20.0	22.1	22.9	103.6
4	17.2	10.0	27.2	29.1	107.0

XV. Limitations of the Procedure

1. The MP Biomedicals Direct Salivary Melatonin EIA Kit reagents are optimized to measure melatonin in human saliva.
2. Avoid the use of samples containing blood contamination.
3. Samples containing Azide or thimerosal are unsuitable for this assay.
4. Avoid repeated freezing and thawing of saliva samples after the initial freeze/thaw.

XVI. Precautions

1. Only physicians, clinical labs, research labs and hospital labs may acquire, possess and use the kit.
2. Compare contents and packing list, if there is breakage or shortage, notify MP Biomedicals immediately.
3. Do not pipet reagents by mouth.
4. Do not smoke, eat or drink while performing assay.
5. Wear disposable rubber gloves.
6. Treat all saliva samples as potentially infectious.
7. Do not mix reagent lot numbers or alter in any way the reagents in this kit. If this is done, MP Biomedicals will not be responsible for the performance of the assay.
8. Avoid contact with Color Development Reagent (TMB). It contains solvents that can irritate skin and mucus membranes. If contact is made, wash thoroughly with water.
9. Avoid contact with stopping solution. It contains acid. If contact is made, rinse thoroughly with water.

XVII. Selected and Cited References

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