



MP Biomedicals
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Direct Melatonin Serum/Plasma EIA Kit

**A non-extraction immunoassay for the measurement of
Melatonin in human serum and plasma**

For Research Use Only

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07P534A**

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I. Intended Use and Description

The MP Biomedicals Direct Melatonin Serum/Plasma Kit is a rapid, sensitive and specific EIA designed and validated for the direct quantitative measurement of Melatonin in human serum and plasma. This test is for research use only.

II. Assay Background

Melatonin (N-Acetyl-5-methoxytryptamine) 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 Melatonin Serum/Plasma Kit is based on the competition principal and microplate separation. Melatonin in 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 anti-rabbit gamma globulin placed in a resealable foil bag with desiccant. One (1) 96 well kit is sufficient for 40 duplicate patient measurements.
2. **Melatonin Calibrators ready to use:** 1 bottle each, 0.5 mL. 0, 3, 10, 30, 100 and 300 pg/mL.
3. **Melatonin Control #1:** 1 bottle, 0.5 mL. The concentration is stated on the label.
4. **Melatonin Control #2:** 1 bottle, 0.5 mL. The concentration is stated on the label.
5. **Melatonin EIA rabbit monoclonal Antibody:** 1 bottle, 6 mL. The solution is blue.
6. **Melatonin-Horseradish Peroxidase (HRP) conjugate.** 1 amber bottle, 6 mL. Melatonin derivative is conjugated to horseradish peroxidase. The solution is yellow and light sensitive.
7. **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.
8. **Color Development Reagent EIA #1:** 1 amber plastic bottle, 15 mL of Tetramethylbenzidine (TMB) plus hydrogen peroxide. Light sensitive.
9. **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 serum or plasma sample collection device.

VII. Sample Collection, Processing 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.

Sample Stability

Storage:	2°C - 8°C	≤ -20°C (Aliquots)	≤ -40°C (Aliquots)	Keep away from heat or direct sun light. Avoid repeated freeze-thaw cycles.
Stability	24 hrs.	3 months	1 year	

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	25	50	50	Mix. Incubate for 2 hrs. at Room Temperature, shaking.	125	Wash 3X	125	Mix. Incubate 30 min. at room temperature	125	Mix. Read at 450 nm
3	25	50	50		125		125			
10	25	50	50		125		125			
30	25	50	50		125		125			
100	25	50	50		125		125			
300	25	50	50		125		125			
Control 1	25	50	50		125		125			
Control 2	25	50	50		125		125			
Sample	25	50	50		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 **25 µL** of ready to use **serum Melatonin EIA Calibrators** (0, 3, 10, 30, 100 and 300 pg/mL), **controls**, and **samples**.
3. Add **50 µL** of **Melatonin-HRP Conjugate** to all wells.
4. Add **50 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 hours** 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 the GARGG microplate on 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 300 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	2.81	100	0
3	2.51	89.3	3
10	1.97	70.1	10
30	1.29	46.0	30
100	0.55	19.6	100
300	0.23	8.2	300
Control I	2.24	79.7	6.1
Control II	0.63	22.4	86.9
Sample I	2.39	85.1	4.3
Sample II	1.35	48.0	26.8
Sample III	0.61	21.7	91.2

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)	3.0 – 300 pg/mL
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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

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) 55(9-10), 359-61

Studies of Melatonin Levels in humans show a considerable inter-individual variation. Therefore, it is recommended that each laboratory establish 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 2.5 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 samples.

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	20	23.1	2.200	9.5
Medium	20	141.6	8.614	6.1
High	20	270.6	16.987	6.3

Inter-assay

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

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	%CV
Low	12	21.8	2.147	9.8
Medium	12	138.5	7.254	5.2
High	12	270.1	18.024	6.7

Inter-lot Variation

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

Samples ID	Lot # 001 mean (pg/mL)	Lot # 002 mean (pg/L)	Lot # 003 mean (pg/mL)	Inter-lot mean (pg/mL)	Inter-lot Std. Dev. (pg/mL)	Inter-lot CV (%)
Sample 1	9.3	10.6	10.2	10.0	0.666	6.6
Sample 2	29.6	28.6	29.7	29.3	0608	2.1
Sample 3	92.0	91.2	89.4	90.9	1.332	1.5
Pool 1	24.3	24.8	24.5	24.5	0.252	1.0
Pool 2	161.2	147.3	149.5	152.7	7.472	4.9
Pool 3	273.6	267.8	253.9	265.1	10.124	3.8

D. Dilution Study:

Sample I.D.	Dilution factor	Expected pg/mL	Observed pg/mL	Recovery (%)
1			30.8	
	1:2	15.400	14.5	94.2
	1:4	7.700	8.4	109.1
	1:8	3.850	3.9	101.3
	1:16	1.925	2.2	114.3
2			78.0	
	1:2	39.000	39.9	102.3
	1:4	19.500	18.0	92.3
	1:8	9.750	10.8	110.8
	1:16	4.875	5.0	102.6
3			279.2	
	1:2	139.600	152.5	109.2
	1:4	69.800	79.1	113.3
	1:8	34.900	32.8	94.0
	1:16	17.450	16.3	93.4

E. Recovery

Three serum 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	5.4	50.0	55.4	58.5	105.6
2	6.9	100.0	106.9	121.4	113.6
3	4.3	200.00	204.3	208.6	102.1

XV. Limitations of the Procedure

1. The MP Biomedicals Direct Melatonin Serum/ Plasma EIA Kit reagents are optimized to measure melatonin in human serum or plasma.
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 serum or plasma 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 serum or plasma 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 the 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 the stopping solution. It contains acid. If contact is made, rinse thoroughly with water.

XVII. Selected and Cited References

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