



Pantex

Division of Bio-Analysis, Inc.

Providing Diagnostic Technology for a Better Tomorrow

DIRECT MELATONIN SERUM/PLASMA Enzyme Immunoassay Kit For Research Use Only (RUO)

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

Catalog Number: 534
RUO

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1



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| TABLE OF CONTENTS | | |
|--------------------------|---|----------------|
| | | Page(s) |
| I | Intended Use and Description | 3 |
| II | Assay Background | 3 |
| III | Assay Principle | 3 |
| IV | Reagents Provided and Reagent Preparation | 3-4 |
| V | Storage and Stability | 4 |
| VI | Materials Needed but not Supplied | 5 |
| VII | Sample Collection, Processing and Storage | 5 |
| VIII | Assay Procedure Summary Flow Sheet | 6 |
| IX | Assay Procedure | 6-7 |
| X | Typical Results | 7 |
| XI | Calculation | 7 |
| XII | Quality Control | 8 |
| XIII | Expected Values | 8 |
| XIV | Performance Characteristics | 8 |
| | A. Specificity of the Antiserum | 8 |
| | B. Sensitivity | 8 |
| | C. Precision and Reproducibility | 9 |
| | D. Dilution Study | 10 |
| | E. Recovery | 10 |
| XV | Limitations | 11 |
| XVI | Precautions | 11 |
| XVII | References | 12 |



I. Intended Use and Description

The Pantex Direct Melatonin Serum/Plasma Kit, Cat #534 is a rapid, sensitive and specific EIA designed and validated for the Direct quantitative measurement of Melatonin in human serum and plasma.

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 Pantex Direct Melatonin Serum/Plasma Kit, Cat #534 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

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



VI. Materials Needed but not Supplied

- Device to dispense accurately 25 μ L and 50 μ L.
- Multichannel pipettors.
- Microplate or orbital shaker
- Vortex Mixer
- Microplate washer (not required, plates can be washed manually).
- Microplate reader capable of reading 450 nm with 4 parameter data reduction or comparable software.
- Plate Sealers
- 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 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°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 Reagent (µL) | Anti-Melatonin (µL) | Mix. Incubate for 2 hrs. at Room Temperature, shaking. | Diluted 10X Wash Solution. (µL) | Wash 3X | Color Development Reagent (µL) | Mix. Incubate 30 min. at room temperature | Stopping Solution (µL) | Mix. Read at 450 nm |
|----------------------------------|----------------------------------|------------------------------------|---------------------|--|---------------------------------|---------|--------------------------------|---|------------------------|---------------------|
| 0 | 25 | 50 | 50 | | 125 | | 125 | | | |
| 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 Working Reagent** to all wells.
4. Add **50 µL** of **Anti-Melatonin EIA rabbit monoclonal antibody**.
5. Cover microplate with plastic sealer. Incubate by shaking on a microplate orbital shaker set a 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 μ L 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 μ L 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. Calculation

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 |
|----------------------------------|-----------------|



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 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) 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-Thyptophan | <0.001 |
| Serotonin Hydrochloride | <0.001 |
| 5-Methoxytryptamine | 0.15 |
| 6-Hydroxy melatonin | <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/mL) | 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

- The Pantex Direct Melatonin Serum/ Plasma EIA Kit reagents are optimized to measure melatonin in human serum or plasma.
- Avoid the use of samples containing blood contamination.,
- Samples containing Azide or thimerosal are unsuitable for this assay.
- Avoid repeated freezing and thawing of serum or plasma samples after the initial freeze/thaw.

XVI. Precautions

- Only physician, clinical labs, research labs and hospital labs may acquire, possess and use the kit.
- Compare contents and packing list, if there is breakage or shortage, notify Pantex immediately.
- Do not pipet reagents by mouth.
- Do not smoke, eat or drink while performing assay.
- Wear disposable rubber gloves.
- Treat all serum or plasma samples as potentially infectious.
- Do not mix reagent lot numbers or alter in any way the reagents in this kit. If this is done, Pantex will not be responsible for the performance of the assay.
- 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.
- Avoid contact with the stopping solution. It contains acid. If contact is made, rinse thoroughly with water.



XVII. References

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