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## Instructions for use

# Melatonin (Research) RIA

Radioimmunoassay for the determination of Melatonin in biological samples.

For research use only, not for use in diagnostic procedures.

REF

**IB88139**

Σ 100

+2 +8 °C

RUO For Research use only- Not for use in diagnostic procedures

200 kBq

**Melatonin Research RIA**

## 1. Intended use and principle of the test

Radioimmunoassay for the determination of Melatonin in biological samples. For research use only, not for use in diagnostic procedures.

In principle the Melatonin Research RIA kit will work for all kinds of biological samples. As a starting point it is recommended to perform a proof-of-principle (please contact our technical service for details).
As a reference a proof of principle for rat plasma samples was performed using 100 µl of sample volume (please refer to chapter 8.).

Melatonin - the major hormone secreted by the pineal gland - is a key modulator of annual and circadian biorhythms. Its circadian profile in body fluids is an excellent marker for the setting of the endogenous clock. Daytime plasma melatonin levels are low and rise in the evening (onset). Night-time levels peak at around 03.00 hrs. (acrophase) in most healthy humans.
Onset, acrophase and offset have a stable phase relationship even when the phase of the melatonin profile is shifted.

The assay procedure follows the basic principle of radioimmunoassay, involving competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of $^{125}$I-labelled antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the antibody bound radioactivity is precipitated with a second antibody in the presence of polyethylene glycol. The precipitate is counted in a gamma counter. Determination of unknown samples is achieved by comparing their activity with a reference curve prepared with known standards.

## 2. Precautions, Guidelines and Warnings

(1) This kit is intended for professional use only. Users should have a thorough understanding of this protocol for the successful use of this kit. Only the test instruction provided with the kit is valid and has to be used to run the assay. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
(2) The principles of Good Laboratory Practice (GLP) have to be followed.
(3) In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.
(4) The radioactive material ($^{125}$Iodine, half life 60 days, emitting ionizing X-radiation with 28 kev and G-radiation with 35.5 kev) may be received, acquired, possessed and used only by physicians, laboratories or hospitals. In compliance with regulations, a copy of the customer's current radioisotope license must be on file with the supplier. Orders cannot be shipped until the license is received by the supplier (Radiation Protection Act of June 30, 1989).
(5) For the dilution or reconstitution purposes use deionized, distilled, or ultra-pure water.
(6) Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.
(7) Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials and devices are prepared ready at the appropriate time.
(8) Incubation times do influence the results. All tubes should be handled in the same order and time intervals.
(9) To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.
(10) A calibrator curve must be established for each run.
(11) The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.
(12) Do not mix kit components with different lot numbers within a test and do not use reagents beyond expiry date as shown on the kit labels.
(13) For information on hazardous substances included in the kit please refer to Material Safety Data Sheets (MSDS). The Material Safety Data Sheet for this product is available directly on the website of the manufacturer or upon request.
(14) If expected reference values are reported in this test instruction they are only indicative It is recommended that each laboratory establishes its own reference intervals.

## 3. Storage and stability

Store the unopened reagents at 2 - 8 °C until expiration date. Do not use components beyond the expiry date indicated on the kit labels. Once opened the reagents are stable for 1 month when stored at 2 – 8 °C.
Melatonin is sensitive to light-exposure. To avoid photo-oxidative reduction of melatonin, it is necessary to keep it away from direct sunlight and from heat.

## 4. Materials

### 4.1 Content of the kit

**BA R-0030** PREC-REAG **Precipitating Reagent -** Ready to use
Content: Goat anti-rabbit serum in PEG phosphate buffer
Volume: 2 x 55 ml/vial, yellow cap

**BA R-3310** AS MEL **Melatonin Antiserum** - Ready to use
Content: Rabbit anti-melatonin antibody, blue coloured
Volume: 1 x 5.25 ml/vial, blue cap

**BA R-3315** ENZYME **Enzyme** - Lyophilized
Content: Digestive enzyme
Volume: 4 vials, pink cap

**BA R-3313** ASSAY-BUFF **Assay Buffer** - Ready to use
Content: TRIS buffer
Volume: 1 x 15 ml/vial, light purple cap
Hazards identification:

H315 Causes skin irritation.
H319 Causes serious eye irritation.

**BA R-3316** ENZYME-BUFF **Enzyme Buffer** - Ready to use
Content: 1 M hydrochloric acid
Volume: 1 x 15 ml/vial, orange cap

**BA R-3320** $^{125}$I-MEL **$^{125}$I – Melatonin** - Ready to use
Content: $^{125}$I labeled Melatonin, red coloured
Volume: 1 x 3 ml/vial, red cap
Hazards identification:

Radioactive, activity < 200 kBq

**Standards** and **Controls** - Ready to use

| Cat. no. | Component | Colour/Cap | Concentration pg/ml | Concentration pmol/l | Volume/ Vial |
|---|---|---|---|---|---|
| **BA R-3301** | STANDARD A | white | 0 | 0 | 4 ml |
| **BA R-3302** | STANDARD B | light yellow | 30 | 129 | 4 ml |
| **BA R-3303** | STANDARD C | orange | 100 | 430 | 4 ml |
| **BA R-3304** | STANDARD D | dark blue | 300 | 1 290 | 4 ml |
| **BA R-3305** | STANDARD E | light grey | 1 000 | 4 300 | 4 ml |
| **BA R-3306** | STANDARD F | black | 3 000 | 12 900 | 4 ml |
| **BA R-3307** | STANDARD G | brown | 10 000 | 43 000 | 4 ml |
| **BA R-3351** | CONTROL 1 | light green | Refer to QC-Report for expected value and acceptable range! | | 4 ml |
| **BA R-3352** | CONTROL 2 | dark red | | | 4 ml |

Conversion: Melatonin (pg/ml) x 4.30 = Melatonin (pmol/l)
Content: TRIS buffer with non-mercury preservatives, spiked with defined quantity of melatonin

**BA R-3961** SYRINGE **Syringe** - Ready to use, single-use
Volume: 1 x 3 pcs.

**BA R-3962** FILTER UNITS **Filter Units** - Ready to use, single-use
Volume: 1 x 3 pcs.

**BA R-3963** PREP TUBES **Preparation Tubes** - Ready to use, single-use
Volume: 1 x 3 pcs.

**BA R-3964** CHARCOAL **Charcoal Suspension** - Ready to use

Content: activated charcoal

Volume: 1 x 25 ml/vials, light purple cap

### 4.2 Additional materials and equipment required but not provided in the kit

- Calibrated precision pipettes to dispense volumes between 20 – 1000 µl; 1,8 ml, 3 ml, 3,6 ml, 7,2 ml
- Conical plastic tubes (polypropylene, polystyrene) and suitable rack
- Centrifuge (preferable refrigerated) capable of at least 3 000 x g
- Suitable device for aspirating or decanting the tubes
- Vortex mixer
- Gamma counter
- Water (deionized, distilled, or ultra-pure)

## 5. Sample storage

The samples have to be stored deep frozen until use.

## 6. Test procedure

Allow all reagents – with the exception of Precipitating Reagent - to reach room temperature and mix thoroughly by gentle inversion before use. Number the assay tubes accordingly. Duplicate determinations are recommended.

⚠ *Pipetted liquids should not adhere to the wall of the RIA tubes. If necessary please centrifuge the tubes for 1 minute at 500 x g to spin down adhering liquids.*

⚠ *For the assay the use of conical tubes is highly recommended.*

### 6.1 Preparation of reagents

**Equalizing Reagent:**

⚠ The preparation of a species and sample specific Equalizing Reagent is mandatory to avoid any matrix effects and false results.
For the preparation of this specific reagent a pool of exact the composition as the sample should be used (e.g. for the measurement of Melatonin in rat plasma, rat plasma has to be used as a starting material for the preparation of the Equalizing Reagent). Through the addition of a charcoal suspension the endogenous melatonin is removed from the specific biological fluid and a Melatonin-free (= "stripped") Equalizing Reagent is produced.

The needed amount of Equalizing Reagent depends on how much sample volume is used for the experiment and this has a strong influence on the limit of detection (please refer to chapter 8.). The following examples demonstrate the relation between number of runs, sample volume, and needed Equalizing Reagent:

| Number of runs (standard curves) | Sample volume (µl) | Tubes (N)* | Volume of Equalizing Reagent needed** |
|---|---|---|---|
| 1 | 100 | 18 | 1.8 ml |
| 2 | 100 | 36 | 3.6 ml |
| 2 | 50 | 36 | 1.8 ml |
| 2 | 200 | 36 | 7.2 ml |

* The number of tubes needed to prepare a standard curve in duplicates including NSB, 7 standards and 2 controls.

** The charcoal treatment of biological fluids in combination with the ultrafiltration step always leads to a loss of some material. Therefore it is recommended to take more sample volume to prepare the Equalizing Reagent than is needed in the assay (e.g. twice as much).

**Example:** Preparation of Equalizing Reagent for 100 µl sample volume; run in duplicates

| | |
|---|---|
| **1.** | Pipette **7.2 ml** of **Charcoal Suspension** into the **Preparation Tubes.** |
| **2.** | **Spin down** the charcoal. |
| **3.** | Decant the supernatant and remove carefully remaining liquid from the wall of the tubes. |
| **4.** | Pipette **7.2 ml** of the biological fluid (serum, tissue extract etc.) onto the charcoal pellet. |
| **5.** | Close the tube carefully and mix the suspension for **30 min** at **RT** (20 - 25 °C) on a **rotating mixer.** |
| **6.** | **Spin down** the charcoal. |
| **7.** | Remove charcoal fines by filtering the supernatant. One **Filter Unit** is capable of filtering 4 ml of supernatant so that two **Filter Units** (and two **Syringes**) have to be used. |
| **8.** | Mix both filtrates and store deep-frozen. |

**Always check the volumes of the melatonin free Equalizing Reagent prior to its use in the RIA to ensure that the prepared volume is sufficient.**

**Enzyme**
Reconstitute the content of the vial with 3 ml of Enzyme Buffer prior to use. Mix carefully (30 minutes on a rotating mixer). The reconstituted Enzyme cannot be stored and can only be used once. Upon request additional Enzyme vials are provided.

### 6.2 Melatonin RIA

(this exemplary protocol refers to 100 µl sample volume; for other sample volumes please refer to the chart 6.2.1 below)

| | |
|---|---|
| **1.** | Pipette **20 µl** of **Standard A** into the tubes for the **NSB** and into the tubes for the **samples.** |
| **2.** | Pipette **20 µl** of **standards** and **controls** into the respective tubes. |
| **3.** | Add **100 µl** of **Equalizing Reagent** to the tubes with **NSB, standards** and **controls.** |
| **4.** | Pipette **100 µl** of **samples** into the respective tubes. |
| **5.** | Pipette **25 µl** of **Enzyme** in all tubes ***(except totals)*** and vortex. |
| **6.** | Incubate for **1 h** at **RT** (20 – 25 °C). |
| **7.** | Pipette **50 µl** of **Assay Buffer** into all tubes ***(except totals)*** and mix shortly. |
| **8.** | Pipette **25 µl** of the **$^{125}$I Melatonin** into all tubes. |
| **9.** | Pipette **50 µl** of **Melatonin Antiserum** into all tubes ***(except totals and NSB)***; mix thoroughly. |
| **10.** | Cover tubes and incubate for **20 - 24 h at RT** (20 – 25 °C). |
| **11.** | Mix the chilled (2 - 8 °C) **Precipitating Reagent** thoroughly, pipette each **1000 µl** into all tubes ***(except totals)***, and mix on a vortex. |
| **12.** | Incubate for **20 min** at **2 - 8 °C**. |
| **13.** | Centrifuge for **20 min** at **3 000 x g**, if possible in a refrigerated centrifuge. |
| **14.** | **Decant** or aspirate the **supernatant carefully *(except totals)***. Blot the tubes dry and leave them upside down for 2 minutes. |
| **15.** | **Count** all tubes for **1 min** in a gamma counter. |

**6.2.1** For sample volumes differing from 100 µl, the following modifications for the RIA (6.2) in reagent volumes are recommended*:*

| ***Equalizing Reagent*** | ***Sample volume*** | ***Enzyme solution*** | ***Assay Buffer*** | ***Antiserum*** |
|---|---|---|---|---|
| **100 µl** | **100 µl** | **25 µl** | **50 µl** | **50 µl** |
| | | | | |
| *25 µl* | *25 µl* | *25 µl* | *50 µl* | **50 µl** |
| *50 µl* | *50 µl* | *25µl* | *50 µl* | **50 µl** |
| *200 µl* | *200 µl* | *50 µl* | *100 µl* | **50 µl** |

## 7. Calculation of results

Subtract the mean cpm of the non-specific binding NSB from the mean cpm of standards, controls and samples.
The standard curve from which the concentrations in the samples can be read off, is obtained by plotting the percentage of (B-NSB)/ ($B_0$-NSB) measured for the standards (linear, y-axis) against the corresponding standard concentrations (logarithmic, x-axis).
Use a non-linear regression for curve fitting (e.g. spline, 4- parameter, akima).

⚠ *This assay is a competitive assay. This means: the counts are decreasing with increasing concentrations of the analyte. Counts found below the standard curve correspond to high concentrations of the analyte in the sample and have to be reported as being positive.*

The concentrations of the **controls** can be read directly from the respective standard curve.

The concentrations of the **samples** are depending on the sample volume which is used for the assay and concentrations read from the standard curve have to be **multiplied with a volume-factor:**

$$\textbf{Volume factor} = \frac{\textbf{20}}{\textbf{sample volume used for the assay}}$$

**Conversion**
Melatonin (pg/ml) x 4.30 = Melatonin (pmol/l)

### 7.1 Quality control

The confidence limits of the kit controls are indicated on the QC-Report.

## 8. Assay characteristics (for rat plasma, 100 µl sample volume)

| | |
|---|---|
| **Analytical Sensitivity (Limit of Detection)** | 5.9 pg/ml |

| | **Substance** | **Cross Reactivity (%)** |
|---|---|---|
| | | Melatonin |
| **Analytical Specificity (Cross Reactivity)** | Melatonin | 100 |
| | N-Acetylserotonin | 0.98 |
| | 5-Methoxytryptophol | 0.11 |
| | 5-Methoxytryptamine | 0.07 |
| | 6-Methoxytryptamine | < 0.01 |
| | 5-Methoxyindol-3-acetic acid | < 0.01 |
| | Serotonin | < 0.01 |
| | DL-Tryptophan | < 0.01 |
| | DL-5-Methoxytryptophan | < 0.01 |
| | 5-Hydroxy-L-Tryptophan | < 0.01 |

| | | Range | Serial dilution up to | Range (%) |
|---|---|---|---|---|
| **Linearity** | Melatonin Rat | 126 – 2 128 pg/ ml | 1:128 | 83 – 98 |

| | | Mean (%) | Range (%) | % Recovery after spiking |
|---|---|---|---|---|
| **Recovery** | Melatonin Rat | 94 | 82 – 103 | |

| | **Stability (comparison Fresh Sample with Freeze and Thaw Stability)** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Stability (Freeze and Thaw Stability)** | **Sample** | **Fresh Sample** | **Freeze and Thaw Stability** | **Mean (pg/ml)** | **Deviation (%)** | **SD (%)** | **CV (%)** |
| | 1 | 39.5 pg/ml | 40 pg/ml | 39.8 | 1.2 | 0.4 | 0.9 |
| | 2 | 87.1 pg/ml | 89.3 pg/ml | 88.2 | 2.5 | 1.6 | 1.8 |
| | 3 | 248.6 pg/ml | 240.4 pg/ml | 244.5 | 3.3 | 5.8 | 2.4 |
| | **Stability (comparison Fresh Sample with Short-Term Temperature Stability)** | | | | | | |
| | **Sample** | **Fresh Sample** | **Freeze and Thaw Stability** | **Mean (pg/ml)** | **Deviation (%)** | **SD (%)** | **CV (%)** |
| | 1 | 39.5 pg/ml | 46.4 pg/ml | 43 | 17.5 | 4.9 | 11.3 |
| | 2 | 87.1 pg/ml | 85.9 pg/ml | 86.5 | 1.4 | 0.8 | 1.0 |
| | 3 | 248.6 pg/ml | 210.8 pg/ml | 229.7 | 15.2 | 26.7 | 11.6 |

**For orders, please contact:**
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⚠ ***For literature or any other information please contact your local supplier.***

**Symbols:**

| | | | | | |
|---|---|---|---|---|---|
|  | Storage temperature |  | Manufacturer |  | Contains sufficient for <n> tests |
|  | Expiry date | LOT | Batch code | I V D | For in-vitro diagnostic use only! |
|  | Consult instructions for use | CONT | Content | CE | CE labelled |
|  | Caution | REF | Catalogue number | RUO | For research use only! |