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Instructions for use

Melatonin Research RIA

REF BA R-3900

Σ 100

+2 +8 °C

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

200 kBq

1. Intended use and principle of the test

Radioimmunoassay for the quantitative determination of Melatonin in biological samples. For research use only.
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. Quantification of unknown samples is achieved by comparing their activity with a reference curve prepared with known standards.

2. Precautions, Guidelines and Warnings

- 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.
- Reagents of this kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
- The principles of Good Laboratory Practice (GLP) have to be followed.
- In order to reduce exposure to potentially harmful substances, wear lab coats, disposable protective gloves and protective glasses where necessary.
- 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).
- For the dilution or reconstitution purposes use deionized, distilled, or ultra-pure water.
- Duplicate determination of sample is highly recommended to be able to identify potential pipetting errors.
- 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.
- Incubation times do influence the results. All tubes should be handled in the same order and time intervals.
- To avoid cross-contamination of reagents, use new disposable pipette tips for dispensing each reagent, sample, standard and control.
- A calibrator curve must be established for each run.
- The controls should be included in each run and fall within established confidence limits. The confidence limits are listed in the QC-Report.
- 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.
- Some reagents contain sodium azide (NaN_3) as preservatives. In case of contact with eyes or skin, rinse off immediately with water. NaN_3 may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with a large volume of water to avoid azide build-up.
- 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.
- 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 reagents at 2 - 8 °C until expiration date. Do not use components beyond the expiration date indicated on the kit labels. Do not mix various lots of any kit component within an individual assay. 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 Contents of the kit

REF	Symbol	Reagent	Content	Colour Code	
BA R-0030	PREC-REAG	**Precipitating Reagent**	2 x 55 ml	yellow	ready for use, goat anti-rabbit serum in PEG phosphate buffer *Mix thoroughly before use!*
BA R-3301	STANDARD A	**Standard A**	1 x 4 ml	white	ready for use
BA R-3302	STANDARD B	**Standard B**	1 x 4 ml	light yellow	ready for use
BA R-3303	STANDARD C	**Standard C**	1 x 4 ml	orange	ready for use
BA R-3304	STANDARD D	**Standard D**	1 x 4 ml	dark blue	ready for use
BA R-3305	STANDARD E	**Standard E**	1 x 4 ml	light grey	ready for use
BA R-3306	STANDARD F	**Standard F**	1 x 4 ml	black	ready for use
BA R-3307	STANDARD G	**Standard G**	1 x 4 ml	brown	ready for use
BA R-3310	AS MEL	**Melatonin Antiserum**	1x 5.25 ml	blue	ready for use, from rabbit, blue coloured
BA R-3313	ASSAY-BUFF	**Assay Buffer**	1 x 15 ml	light purple	ready for use
BA R-3315	ENZYME	**Enzyme**	4 x	pink	lyophilized
BA R-3316	ENZYME-BUFF	**Enzyme Buffer**	1 x 15 ml	orange	ready for use
BA R-3320	^{125}I-MEL	**^{125}I – Melatonin**	1 x 3 ml	red	ready for use, activity < 200 kBq, red coloured
BA R-3351	CONTROL 1	**Control 1**	1 x 4 ml	light green	ready for use
BA R-3352	CONTROL 2	**Control 2**	1 x 4 ml	dark red	ready for use
BA R-3961	SYRINGE	**Syringe**	1 x 3 pcs.		ready for use, single-use
BA R-3962	FILTER UNITS	**Filter Units**	1 x 3 pcs.		ready for use, single-use
BA R-3963	PREP TUBES	**Preparation Tubes**	1 x 3 pcs.		ready for use
BA R-3964	CHARCOAL	**Charcoal Suspension**	1 x 25 ml	light purple	ready for use

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.*
The use of conical tubes is highly recommended for the assay.

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 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

	Concentration of the standards:						
Standard	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Melatonin (pg/ml)	0	30	100	300	1 000	3 000	10 000
Melatonin (pmol/l)	0	129	430	1 290	4 300	12 900	43 000
Conversion:	Melatonin (pg/ml) x 4.30 = Melatonin (pmol/l)						

Subtract the mean cpm of the non-specific binding NSB from the mean cpm of standards, controls and samples.

The calibration curve from which the concentrations in the samples can be read off, is obtained by plotting the percentage of (B-NSB)/ (B0-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).

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:**

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

7.1 Quality control

It is recommended to use control samples according national regulations. Use controls at both normal and pathological levels. The kit or other commercial controls should fall within established confidence limits. 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)						
	Sample	**Fresh Sample**	**Freeze and Thaw Stability**	**Mean (pg/ml)**	**Deviation (%)**	**SD (%)**	**CV (%)**
Stability (Freeze and Thaw Stability)	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 updated literature, information about clinical significance or any other information please contact your local supplier.***

Symbols:

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