Direct Saliva MELATONIN

RIA

For research use only.
Not for use in diagnostic procedures.

RK-DSM2-U 200 tests

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NovoLytiX GmbH
Benkenstrasse 254C
CH - 4108 Witterswil / Switzerland
Tel.: +41 61 721 01 00
Fax: +41 61 530 11 06
info@novolytix.ch

INTENDED USE

The Direct Saliva Melatonin Radio Immunoassay (RIA) test kit is intended for the direct, quantitative determination of melatonin in human saliva (1-4).

PRINCIPLE OF THE ASSAY

The Direct Saliva Melatonin RIA kit measures melatonin by a double-antibody radio immunoassay based on the Kennaway G280 anti-melatonin antibody (5,6). Human saliva samples and reconstituted standards and controls are incubated with the anti-melatonin antibody and ^{125}I-melatonin. ^{125}I-melatonin competes with melatonin present in samples, standards and controls. After 20 hours of incubation, solid-phase second antibody is added to the mixture in order to precipitate the antibody-bound fraction. After aspiration of the unbound fraction, the antibody bound fraction of ^{125}I-melatonin is counted.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Incubation Buffer	2 vials 100 ml	B-DSM-IB	Ready to use
Antiserum anti-melatonin antibody	2 vials 11 ml	B-DSM-AS	Ready to use
Tracer ^{125}I-melatonin	2 vials 11 ml	B-MEL-TR	Ready to use
Calibrator A - E[1)] lyophilized melatonin standards	1 set of 5 vials	B-MEL-CASET	Reconstitute with 5 ml of Incubation Buffer
Controls Low/High[2)] lyophilized melatonin	1 set of 2 vials	B-DSM-CONSET	Reconstitute with 5 ml of Incubation Buffer
2nd Antibody solid phase bound second antibody	2 vials 11 ml	B-AB2	Ready to use

Table 1

[1)] After reconstitution the Calibrators A to E contain 0.5, 1.5, 5, 15 and 50 pg/ml of melatonin, respectively. Reconstitute each vial with 5 ml of Incubation Buffer. **Leave for at least 30 minutes at 2-8°C and vortex.**

[2)] Lot specific amounts of melatonin. Reconstitute each vial with 5 ml of Incubation Buffer. **Leave for at least 30 minutes at 2-8°C and vortex.**

STORAGE AND SHELF LIFE OF REAGENTS

Unopened reagents	
All unopened kit components are stable at 2-8°C until the expiration date.	
Opened / reconstituted reagents	
Incubation Buffer	Stable at 2-8°C until expiration date printed on the labels.
Antiserum	
Tracer	
Calibrators	Stable for at least 4 months after reconstitution at 2-8°C.
Controls	
2nd Antibody	Store refrigerated **(Do not freeze!)** Stable at 2-8°C until expiration date printed on the label.

Table 2

WARNINGS AND PRECAUTIONS

SAFETY PRECAUTIONS

- This kit contains an ionizing gamma-emitter with a half-life of 59.4 days (125-iodinated Melatonin; radionuclid is 125-Iodine, ^{125}I). The activity of the radioactive material in this kit does not exceed 74 kBq (2 µCi) of 125-Iodine.
- Receipt, acquisition, possession, use, and transfer are subject to the local regulations. Unused solutions and radioactive waste should be disposed of according to local State and Federal regulations.

TECHNICAL PRECAUTIONS

- Read carefully the instructions prior to carrying out the test. Test performance will be adversely affected if reagents are incorrectly diluted, modified or stored under conditions other than those as detailed in this instruction for use.
- Incorrect results for standard curve, controls or samples may be obtained, if the 2nd antibody was not mixed sufficiently before pipetting. Do not freeze the 2nd antibody!
- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Reconstitute the lyophilized reagents as indicated. Mix (vortex) well all reagents, particularly the Antiserum, and then let the reagents adjust to reach room temperature before use.
- Counting time should be selected in order to keep statistical counting error small: e.g., at 2000 cpm the counting error is at 5%; at 10000 cpm it is only 1%.
- If the initial concentration of an unknown sample reads above the highest calibrator, the sample should be further diluted with incubation buffer and tested again according to the assay procedure.
- The procedure was tested and validated for human saliva samples. If other saliva specimen have to be used, it is recommended to either validate possible matrix effects with melatonin-free saliva specimen or extract the saliva samples by C18 reversed-phase column extraction (B-MEC).

EQUIPMENT REQUIRED

- 100 µl, 400 µl, 1000 µl and 5000 µl precision pipettes (or preferably a 100-1000 µl adjustable multipipette) with disposable tips.
- Refrigerated centrifuge.
- Vortex mixer.
- Stir bar and magnetic stirrer.
- Aspiration device.
- Gamma-counter.

MATERIALS RECOMMENDED BUT NOT PROVIDED

- Saliva Collection Devices: B-SLEEPCHECK16-U or B-SVC50-U.
- Disposable polystyrene tubes for the RIA (preferably conical tubes; *e.g.* Sarstedt # 57.477).
- Deionized double distilled water.

SALIVA COLLECTION AND STORAGE

Collection of Saliva

- Collect saliva using a suitable Saliva Collection Device. A suitable cotton swab can absorb up to 3 ml of saliva. The procedure calls for 1 ml of saliva for duplicate determinations. **Do not use cotton swabs containing citric acid**.
- Subjects ideally refrain from eating during the sampling period. If this is not possible, subjects should be permitted to eat immediately after a collection only and rinse their mouths with water 15 minutes before the next collection.
- Avoid beverages containing artificial colorants as well as alcoholic beverages during the sampling period. Coffee is not permitted - it is counter indicated in sleep studies anyway. In view of some recent reports on melatonin in some foods, it is suggested that bananas should not be eaten during the sampling period.
- Subjects should avoid brushing their teeth, with or without toothpaste, during sampling periods. It is likely that subjects with gingivitis will contaminate the saliva with plasma or even whole blood leading to unknown consequences.
- Do not stimulate saliva flow by chewing gums or eating lemons.

- When collecting saliva at night, a dim flashlight or a ≤100 lux yellow light should be used in order to avoid a possible light influence on the melatonin profile.

Storage

Saliva samples absorbed in cotton swabs can be stored in the saliva collection device for up to 7 days at 2-8°C. If not assayed within one week after collection, samples should be frozen and can be stored for at least 6 months at ≤ -20°C. Repeated freeze-thaw cycles should be avoided.
Thaw (if frozen) and centrifuge the saliva samples for 5 minutes at 1000 x g and 2-8°C prior to testing. Transfer the clear supernatant into a new vial.

ASSAY PROCEDURE

1. Label conical polystyrene tubes in duplicates (8x2): A to E for the calibrators, NSB for the blank tubes, MB for the maximum binding tubes and T for the total activity tubes. Label additional tubes in duplicate for controls and saliva samples.

2a. Pipet 500 µl of incubation buffer into the NSB tubes and 400 µl of incubation buffer into the MB tubes.

2b. Pipet 400 µl of the calibrators A to E into the corresponding tubes.

2c. Pipet 400 µl of the controls and saliva samples into each of the correspondingly marked tubes.

3. Add 100 µl of the melatonin antibody to all tubes **except** the NSB and T tubes.
4. Add 100 µl of the ^{125}I-melatonin tracer to all tubes. Vortex. Remove the T tubes, they will need no further processing until counting at step 10.
5. Incubate all tubes for 20± 4 hours at 2-8°C.

6a. Invert the bottle containing the solid phase second antibody several times, add a stir bar and resolve the sediment using a magnetic stirrer.

6b. While stirring the second antibody suspension continuously, add 100 µl of the suspension to all assay tubes (except the T tubes). Vortex.

7. Incubate for 15± 1 minutes at 2-8°C.
8. Add 1 ml of cold, deionized water (2-8°C) to all assay tubes (except the T tubes).
9. Centrifuge for 2 minutes at 2000 x *g* and 2-8°C. Aspirate the supernatants (except the T tubes) and retain the precipitates for counting.
10. Count the tubes for 2 minutes in a gamma-counter.

RESULTS & STANDARDIZATION

1. Record the cpm for all tubes (T, NSB, MB, Calibrators A, B, C, D, E, samples and controls) and calculate the mean cpm for each pair of tubes.
2. Subtract the mean assay blank (NSB tubes) from the respective mean of each pair of tubes:

   $\text{Net cpm} = \text{cpm}_{\text{Average}} - \text{cpm}_{\text{Average NSB}}$

3. Calculate the binding of each pair of tubes as a percent of maximum binding (MB tubes), with the NSB-corrected cpm of the MB tubes taken as 100%:

$$\text{percent bound} = \frac{\text{net cpm}}{\text{net MB cpm}} \times 100$$

4. Prepare a lin/log graph paper and plot the percent bound on the vertical axis against the melatonin concentration (pg/ml) on the horizontal axis for each of the calibrators. Draw the best fitting curve or calculate the standard curve using a four-parameter logistic (4-PL), a spline smoothed or an equivalent algorithm.
5. Determine the melatonin concentrations for the patient controls and samples from this standard curve. Alternative data reduction methods are equally acceptable.

See Table 3 and Figure 1 for examples of a standard curve. This standard curve is for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.

Standardization: Direct Saliva MELATONIN RIA is calibrated with UV/VIS: ε_{278} = 6300 $M^{-1}cm^{-1}$ in ethanol.

QUALITY CONTROL

A thorough understanding of this instruction for use (IFU) is necessary for the successful use of the product. Reliable results will be obtained only by using precise laboratory techniques (current GLP guidelines) and accurately following this instruction for use.
The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the Controls are lot-specific and printed on the QC Data Sheet added to the kit.
If the precision of the assay does not correlate with the established limits and repetition excludes errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices ii) expiration dates of reagents iii) storage and incubation conditions iv) purity of water.

LIMITATIONS

Melatonin results should be interpreted in conjunction with information available from clinical assessment of the patient and other diagnostic procedures.

PERFORMANCE CHARACTERISTICS

The assay performance characteristics have been validated in duplicates.

Intra-Assay Precision (Within-Run): 2.6-20.1%. The intra-assay precision was calculated from results of 20 pairs of values from each saliva sample from different daytime in a single run. The values are presented in Table 12.
Inter-Assay Precision (Run-to-Run): 6.6-16.7%. The inter-assay precision was calculated from results of 20 pairs of values from each saliva sample from different daytime in 20 different runs. The values are presented in Table 5.
Detection Limit (LoB): 0.2 pg/ml (0.9 pmol/l). Twenty zero Calibrator (MB) replicates were assayed in a single run. The minimum detectable concentration of melatonin in 400 µl incubation buffer was calculated by subtracting two standard deviations of averaged zero Calibrator duplicates from the counts at maximum binding and intersecting this value with the standard curve obtained in the same run.
Detection Limit (LoQ): 0.9 pg/ml (4.0 pmol/l). The functional least detectable dose (FLDD) of the assay is the minimal melatonin concentration in saliva that can be measured with an intra-assay coefficient of variation (CV) of less than 10%. The limit of quantification was determined from 13 different saliva samples each measured at least 10 times in a single run.
Dilution Linearity/Parallelism: 93.1%. Two human saliva sample spiked with 40 pg/ml of melatonin were diluted with Incubation Buffer and subsequently assayed according to the assay procedure. The values are presented in Table 6.
Spiking Recovery: 106%. Three saliva samples were spiked with increasing amounts of melatonin and analyzed according to the assay procedure. The values are presented in Table 7.
Specificity: In Table 8 the following cross-reactivities of the melatonin antiserum were found at 50 % binding.

Table 3 **Example of Results**

	cpm	B/T [%]	B/B_0 [%]	Conc. [pg/ml]	cpm_{CV} [%]
Total	13650	100.0			
Total	13496	100.0			
Total Avg	13573	100.0			0.80
NSB	352	2.6			
NSB	357	2.6			
NSB Avg	355	2.6			0.96
MB	5401	37.2	100.0		
MB	5271	36.2	100.0		
MB Avg	5336	36.7	100.0		1.73
A Std	4934	33.7	91.9		
A Std	4879	33.3	90.8		
A Std Avg	4906	33.5	91.4	0.5	0.79
B Std	4263	28.8	78.5		
B Std	4388	29.7	81.0		
B Std Avg	4326	29.3	79.7	1.5	2.04
C Std	3264	21.4	58.4		
C Std	3145	20.6	56.0		
C Std Avg	3205	21.0	57.2	5.0	2.63
D Std	1781	10.5	28.6		
D Std	1764	10.4	28.3		
D Std Avg	1773	10.5	28.5	15.0	0.68
E Std	919	4.1	11.3		
E Std	940	4.3	11.7		
E Std Avg	930	4.2	11.5	50.0	1.60

ED_{20}: 23.7 pg/ml D_{50}: 6.6 pg/ml ED_{80}: 1.47 pg/ml

Figure 1 **Example of Standard curve**

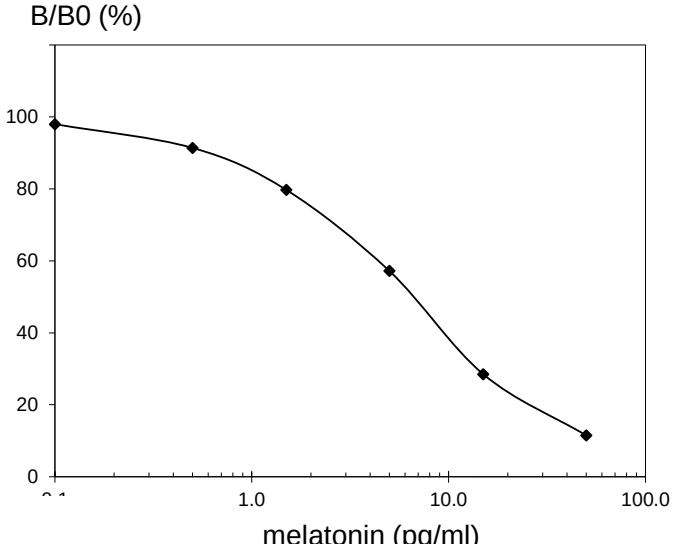

Table 4 **Intra-Assay Precision**

Sample	Mean [pg/ml]	SD [pg/ml]	CV (%)
daytime	0.60	0.121	20.1
evening	3.56	0.145	4.1
nighttime	24.42	1.169	4.8
early morning	7.24	0.188	2.6
Mean			7.9

Table 5 **Inter-Assay Precision**

Sample	Mean [pg/ml]	SD [pg/ml]	CV (%)
daytime	0.82	0.137	16.7
evening	8.99	0.593	6.6
nighttime	25.58	2.148	8.4
early morning	3.39	0.256	7.5
Mean			9.8

Table 6 **Dilution Linearity/Parallelism**

Sample	Dilution	Observed [pg/ml]	Expected [pg/ml]	O/E [%]
spiked Saliva 1	spiked: 40 pg/ml	44.4	---	---
	1 in 2 (50.0%)	25.1	22.2	112.9
	1 in 4 (25.0%)	11.3	11.1	101.6
	1 in 8 (12.5%)	4.92	5.55	88.6
	1 in 16 (6.3%)	2.60	2.78	93.5
	1 in 32 (3.1%)	1.51	1.39	108.6
	1 in 64 (1.6%)	0.60	0.69	87.0
spiked Saliva 2	spiked: 40 pg/ml	47.2	---	---
	1 in 2 (50.0%)	22.6	23.6	95.6
	1 in 4 (25.0%)	10.8	11.8	91.2
	1 in 8 (12.5%)	4.97	5.9	84.2
	1 in 16 (6.3%)	2.65	2.95	89.9
	1 in 32 (3.1%)	1.23	1.48	83.5
	1 in 64 (1.6%)	0.60	0.74	81.1
Mean				93.1

Table 7 **Spiking Recovery**

Basic Value	Added [pg/ml]	Expected [pg/ml]	Observed [pg/ml]	Recovery [%]
0.69	1	1.69	1.68	99
	2	2.69	2.50	93
	5	5.69	5.82	102
	10	10.69	11.94	112
	20	20.69	25.03	121
	40	40.69	42.95	106
1.35	1	2.35	2.51	107
	2	3.35	3.45	103
	5	6.35	7.39	116
	10	11.35	12.12	107
	20	21.35	25.11	118
	40	41.35	45.11	109
0.74	2.5	3.24	3.31	102
	5	2.74	7.02	122
	10	10.74	10.79	100
	20	20.74	21.20	102
	40	40.74	36.88	91
	80	80.74	82.60	102
Mean				106

Table 8 **Specificity**

Compound	Crossreactivity [%]
Melatonin	100
6-Sulfatoxymelatonin	0.002
Serotonin	<0.001
5-Hydroxy-Indole Acetic Acid	<0.001
N-Acetylserotonin	0.027
5-Methoxytryptamine	0.003
5-Methoxytryptophane	0.001
5-Methoxytryptophol	0.001
Methoxypsoralen	<0.001

Table description: cf. “Results” and “Performance Characteristics” (page 3).

1. Koorengevel, KM*, et al. A forced desynchrony study of circadian pacemaker chrarcteristics in seasonal affective disorder.* J Biol Rhytms **17**, 463-75 (2002).
2. Wirz-Justice, A*, et al. No evidence for a phase delay in human circadian rhythms after a single morning melatonin administration*. J Pineal Res **32**, 1-5 (2002).
3. Graw, P*, et al. Early morning melatonin administration impairs psychomotor vigilance.* Behav Brain Res **121**, 167-72 (2001).
4. Danilenko, KV*, et al. Is sleep per se a Zeitgeber in humans.* J Biol Rhytms **18**, 170-8 (2003).
5. Vaughan G M: *New sensitive serum melatonin radioimmunoassay employing the Kennaway G280 antibody: Syrian hamster morning adrenergic response.* J Pineal Res **15**, 88-103 (1993).
6. Voultsios, A*, et al. Salivary melatonin as a circadian phase marker: validation and comparison to plasma melatonin*. J Biol Rhythms 12, 457-466 (1997)

RADIOIMMUNOASSAY PROCEDURE

Polystyrene tubes in duplicate	Incubation Buffer (µl)	Standard, Control, Sample (µl)	Antiserum (µl)	Tracer (µl)		Second Antibody (µl)	
Total	--	--	--	100		--	**Vortex and incubate for 15±2 minutes at 2-8°C**
NSB	500	--	--	100		100	
MB	400	--	100	100		100	
Std A 0.5 pg/ml	--	400	100	100	**Vortex and incubate at 2-8°C for 20±4 hours**	100	**add 1 ml of deionized water (except T tubes) and centrifuge for 2 minutes at 2-8°C and 2000 x g**
Std B 1.5 pg/ml	--	400	100	100		100	
Std C 5.0 pg/ml	--	400	100	100		100	
Std D 15.0 pg/ml	--	400	100	100		100	
Std E 50.0 pg/ml	--	400	100	100		100	
Control LOW	--	400	100	100		100	**aspirate supernatant (except T tubes) and count for 2 minutes**
Control HIGH	--	400	100	100		100	
Samples	--	400	100	100		100	

Table 9

APPENDIX III

SYMBOLS

Symbol	Explanation
	Use by Verwendbar bis Utiliser jusqu'au Utilizzare entro Fecha de caducidad
REF	Catalogue number Bestellnummer Référence du catalogue Numero di catalogo Número de catálogo
LOT	Batch code Chargenbezeichnung Code du lot Codice del lotto Codigo de lote
IVD	*In Vitro* Diagnostic Medical Device *In Vitro* Diagnostikum Dispositif médical de diagnostic *in vitro* Dispositivo medico-diagnostico *in vitro* Producto sanitario para diagnóstico *in vitro*
	Consult Instructions for Use- Gebrauchsanweisung beachten Consulter le mode d'emploi Consultare le istruzioni per l'uso Consulte las instrucciones de uso
	Contains sufficient for <n> tests Ausreichend für „n" Ansätze Contenu suffisant pour „n" tests Contenuto sufficiente per „n" saggi Contenido suficiente para <n> ensayos
	Temperature limitation Zulässiger Temperaturbereich Limites de température Limiti di temperatura Limite de temperatura
	Radioactive Material Radioaktives Material Matériel radioactif Materiale radioattivo Material radiactivo

Symbol	Explanation
BUF INC	Incubation Buffer Inkubations-Puffer Tampon d'incubation Tampone d'incubazione Tampón de incubación
Ab	Antiserum Antiserum Antisérum Antisiero Antisuero
TR	Tracer Tracer Traceur Elemento tracciante Trazador
CAL A - CAL E	Calibrator A - E Kalibrator A - E Calibrateur A - E Calibratore A - E Calibrador A - E
CONTROL L	Control Low Kontrolle tief Contrôle bas Controllo basso Control bajo
CONTROL H	Control High Kontrolle hoch Contrôle élevé Controllo alto Control alto
Ab2	2nd Antibody 2. Antikörper 2ème Anticorps Secondo anticorpo Segundo anticuerpo

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