



Direct Saliva MELATONIN

RIA

This product is for research use only
It is not intended for use in diagnostic procedures

RK-DSM2-U

200 tests

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ENGLISH

INTENDED USE

The BÜHLMANN Direct Saliva Melatonin Radio Immunoassay (RIA) test kit is intended for the direct, quantitative determination of melatonin in human saliva (1-4). This product is for research use only. It is not intended for use in diagnostic procedures.

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 ¹²⁵I-melatonin. ¹²⁵I-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 ¹²⁵I-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 - E¹⁾ lyophilized melatonin standards	5 vials	B-MEL-CASET	Reconstitute with 5 ml of Incubation Buffer
Controls low/high²⁾ lyophilized melatonin	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	
2 nd Antibody	Store refrigerated (Do not freeze!) Stable at 2-8°C until expiration date printed on the label.

Table 2

WARNINGS AND PRECAUTIONS

Radioactive Material: The RK-DSM2 kits contain radioactive material not exceeding 74 kBq (2.0 µCi) of ¹²⁵I, respectively.

The receipt, acquisition, possession, use and transfer are subject to the local regulations.

Concerning the proper precautions for the handling and disposal of kit reagents, radioactive material, radioactive waste and samples, we highly recommend to first consulting the special local regulations of your country.

Reagents Containing Human Source Material: No reagents of this kit contain components of human origin. Samples should be handled as if capable of transmitting infections and should be handled in accordance with good laboratory practices (GLP) using appropriate precautions.

MATERIALS REQUIRED BUT NOT PROVIDED

- BÜHLMANN Saliva Collection Device (Order Code: B-SLEEPCHECK16 or B-SVC/5)
- 100 µl, 400 µl, 1000 µl and 5000 µl precision pipettes (or preferably a 100-1000 µl adjustable multipipette) with disposable tips
- Disposable polystyrene tubes for the RIA (preferably conical tubes; e.g. Sarstedt # 57.477)
- Deionized double distilled water
- Refrigerated centrifuge
- Vortex mixer
- Stir bar and magnetic stirrer
- Aspiration device
- Gamma-counter

SALIVA COLLECTION AND STORAGE

Precautions for Collection of Saliva

- 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 flash light or a ≤100 lux yellow light should be used in order to avoid a possible light influence on the melatonin profile.

Collection and Storage: Collect saliva using the BÜHLMANN Saliva Collection Device (Order Code: B-SLEEPCHECK16 or B-SVC/5). One of these cotton swabs 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.**

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.

PROCEDURAL NOTES

- Saliva samples containing more than 50 pg/ml of melatonin must be further diluted with Incubation Buffer.
- The procedure was tested and validated for human saliva samples. If other saliva specimens 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).

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 ¹²⁵I-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

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 algorithm or equivalent.
5. Determine the melatonin concentrations for 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.

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.

PERFORMANCE CHARACTERISTICS

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 4.

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.

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.

Functional Sensitivity: 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 FLDD was determined from 13 different saliva samples each measured at least 10 times in a single run.

Analytical Sensitivity: 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.

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

ED₂₀: 23.7 pg/ml D₅₀: 6.6 pg/ml ED₈₀: 1.47 pg/ml

Figure 1 Example of Standard curve

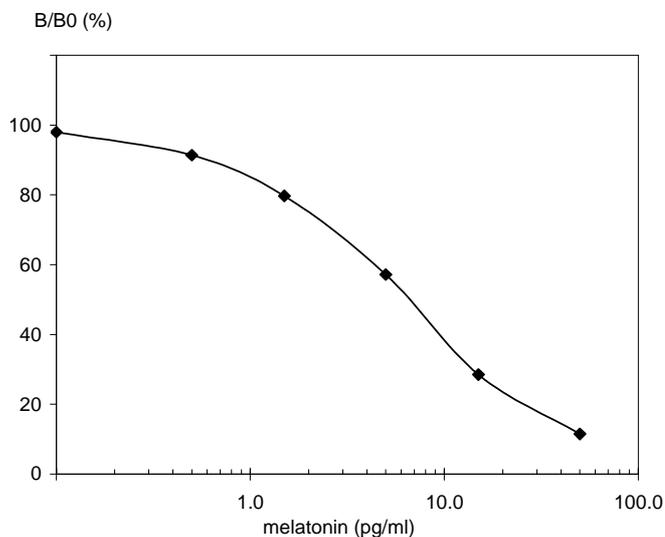


Table 4 Intra-Assay Precision

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
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
Methoxy psoralen	<0.001

**APPENDIX II
REFERENCES**

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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	
Control HIGH	--	400	100	100	100	aspirate supernatant (except T tubes) and count for 2 minutes	
Samples	--	400	100	100	100		

Table 4

Symbol	Explanation
	Use By
REF	Catalogue number
LOT	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Temperature limitation
	Radioactive Material

Symbol	Explanation
BUF INC	Incubation Buffer
Ab	Antiserum
TR	Tracer
CALA - CALE	Calibrator A - E
CONTROL L	Control Low
CONTROL H	Control High
Ab2	2 nd Antibody