BÜHLMANN

MELATONIN
RIA

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

RK-MEL2-U 200 tests

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ENGLISH

INTENDED USE

The BÜHLMANN Melatonin Radio-immunoassay (RIA) is intended for the quantitative determination of melatonin (1-3) in serum, plasma, urine and other biological specimen **upon C18 solid phase extraction.**

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

PRINCIPLE OF THE ASSAY

Melatonin RIA kit measures melatonin by a double-antibody radioimmunoassay based on the Kennaway G280 anti-melatonin antibody (4)*. Reversed-phase column extracted samples and Controls and reconstituted Calibrators are incubated with the anti-melatonin antibody and ^{125}I-melatonin. ^{125}I-melatonin competes with melatonin present in samples, Calibrators 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.

* This publication uses a chloroform ($CHCl_3$) extraction of samples, prior to a ^{125}I-RIA and involves some different ancillary reagents besides the liquid phase second antibodies used in the report. Hence the performance specifications reported therein do not substitute those reported in these instructions.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Extraction Columns[1]	20 Pcs.	B-MEC	C18 reversed phase extraction columns 1 ml
Incubation Buffer	2 vials 100 ml	B-MEL-IB	Ready to use
Antiserum anti-melatonin antibody	2 vials 11 ml	B-MEL-AS	Ready to use
Tracer ^{125}I-melatonin	2 vials 11 ml	B-MEL-TR	Ready to use
Calibrator[2] melatonin Calibrators	5 vials lyoph.	B-MEL-CASET	Reconstitute with 5 ml of Incubation Buffer
Controls Low / High[3] melatonin in a protein buffer matrix	2 vials 5.5 ml	B-MEL-CONSET	Ready for extraction
Second Antibody solid phase bound second antibody	2 vials 11 ml	B-AB2	Ready to use

Table 1

[1] Each extraction column provided with this kit can be utilized up to five times if used according to the extraction procedures described in this instruction for use.

[2] After reconstitution the Calibrators contain 0.5, 1.5, 5, 15 and 50 pg/ml of melatonin. **Reconstitute each calibrator with 5.0 ml of Incubation Buffer, vortex. Leave for at least 30 minutes at 2-8°C and vortex again.**

[3] Lot specific amount of melatonin, see QC Data Sheet added to the kit. Extract the Controls according to the protocol as described on pp. 3ff.

STORAGE AND SHELF LIFE OF REAGENTS

Unopened reagents	
Store at 2-8°C. Do not use past kit expiration date. Columns should be stored at 18-28°C	
Opened / reconstituted reagents	
Extraction Columns	Used columns should be stored at 18-28°C and protected from dust and light.
Incubation Buffer	Stable at 2-8°C until expiration date printed on the label.
Antiserum	
Tracer	
Calibrators	Stable for at least 4 months at 2-8°C after reconstitution.
Controls	After extraction store Controls unreconstituted at -20°C
Second 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-MEL2-U kits contain radioactive material not exceeding 74 kBq (2.0 µCi) of ^{125}I, 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 specimens, we highly recommend to first consult the special local regulations of your country.

Reagents Containing Human Source Material: The Controls (B-MEL-CONSET) of this kit contain components of human origin. Each serum used in the preparation of the kit components was tested by a FDA-approved method and found negative for HBV surface antigen, HCV and HIV1/2 antibodies. Although those methods are highly accurate, there is no guarantee that this material cannot transmit Hepatitis or AIDS. *Therefore, all specimens and kit components should be handled as if capable of transmitting infections.* All products containing human source material should be handled in accordance with good laboratory practices using appropriate precautions.

MATERIALS REQUIRED BUT NOT PROVIDED

- 100, 1000 and 5000 µl precision pipettes (or preferably a 100–1000 µl adjustable multipipette) with disposable tip
- Disposable polystyrene tubes for the RIA (*e.g.* conical tubes from Sarstedt; no. 57.477)
- Disposable borosilicate glass tubes for the preparation of (serum) extracts (*e.g.* disposable CW glass test tubes from Baxter; no. 451296)
- Extraction vacuum manifold for applying the extraction columns (optional)
- Methanol *(HPLC grade)*
- Hexane *(p.a.)*
- Deionized double distilled water (ultrapure; not containing any organic residues)
- Vacuum concentrator or supply for particle free nitrogen
- Refrigerated centrifuge
- Vortex mixer
- Stir bar and magnetic stirrer
- Aspiration device
- Gamma-counter

SPECIMEN COLLECTION AND STORAGE

When drawing blood at night, a dim flash light or a yellow light ($\leq$ 100 lux) should be used in order to avoid a possible light influence on the melatonin profile.

Serum: The procedure calls for about 2.5 ml of blood or for 1 ml of serum per extraction (if the sample is not diluted after extraction). Collect blood into plain tubes, avoid hemolysis, leave to clot for 45 min at room temperature (18-28°C) protected from light. Centrifuge at 1800 x g for 15 min at room temperature and collect the serum.

Lipemic, hemolytic and icteric samples should not be used in this assay. Lipemic samples can be avoided by asking s to fast for at least 12 hours prior to drawing the.

Plasma: The procedure calls for about 2.5 ml of blood or for 1 ml of plasma per extraction (if the sample is not diluted after extraction). Collect blood into EDTA or Heparin tubes, centrifuge for 15 min at 2-8°C and 1800 x g, collect the plasma. Do not use grossly hemolysed samples.

Specimen Storage: If not extracted immediately, serum or plasma samples should be frozen and can be stored for at least 6 months at -20°C. Repeated freeze-thaw cycles should be avoided.

EXTRACTION OF SAMPLES AND CONTROLS

- Each extraction column provided with this kit can be used up to five times according to the extraction procedures described below. They should be stored at 18-28°C and protected from light and dust.
- Always use HPLC grade methanol and hexane as well as deionized water of ultrapure quality (no organic residues such as oils or detergents) for the extraction procedure.
- In order to avoid clogging of the columns, filter or centrifuge samples containing particles such as fibrin clots prior to the extraction (e.g. heparin plasma that was frozen).
- The extraction method results in recoveries of >90% with either ^{125}I-melatonin or ^{3}H-melatonin using human serum, plasma and urine samples.
- If samples have to be measured containing >50 pg/ml of melatonin, the sample volume may be reduced down to 0.125 ml without a notable change in extraction recoveries. This also indicates that sample volumes as small as 0.125 ml ($\approx$ 0.3 ml of blood) may be used in this method (cf. page 4 for extractive dilution linearity).
- The extraction procedure was tested and validated for human serum, plasma, Saliva and URINE samples. If you intend to measure any other specimen, it is recommended to validate the extraction recovery using ^{125}I-melatonin spiked specimens.

Extraction Procedure using Centrifugation

Column Preparation & Conditioning

- Mark 1 extraction column for each sample to be extracted and place them into polypropylene or glass tubes.
- Add 1 ml of methanol to columns, centrifuge for 1 min at 200 x g.
  Repeat this step once.
- Add 1 ml of H_2O to columns, centrifuge for 1 min at 200 x g.
  Repeat this step once.
- Proceed with sample application without delay.

Sample Application

- Add 1 ml of sample to the correspondingly marked column, centrifuge for 1 min at 200 x g.

Washing

- Add 1 ml 10% methanol in H_2O (v/v) to the columns, centrifuge for 1 min at 500 x g.
  Repeat this step once.
- Add 1 ml of hexane to columns, centrifuge for 1 min at 500 x g.

Elution of Extract

- Place the columns into clean correspondingly marked borosilicate tubes.
- Add 1 ml of methanol to columns, centrifuge for 1 min at 200 x g.
- Use column for extracting the next sample (up to 5 times) or store column at 18-28°C
  Protect from light and dust.

Evaporation & Reconstitution of Extract

- Evaporate the methanol to dryness using a vacuum concentrator with a cold trap. Alternatively, use a 37°C heating block or water bath and evaporate the methanol to dryness with a stream of particle free nitrogen.
- Reconstitute the samples with 1 ml of Incubation Buffer, vortex.
- Equilibrate the extracts for 30 min at 18-28°C, vortex. Store reconstituted extracts capped and frozen if not assayed immediately.

Extraction Procedure using Vacuum Manifold

Note: If not indicated otherwise, always pass the solvent through the column using vacuum and a flow rate of $\leq$ 5 ml/min.

Column Preparation & Conditioning

- Mark 1 extraction column for each sample to be extracted and place them into polypropylene or glass tubes.
- Add 2 x 1 ml of methanol to columns, let the solvent pass through using vacuum.
- Add 2 x 1 ml of H_2O to columns, let the solvent pass through using vacuum.
- Proceed with sample application before the column gets dry.

Sample Application

- Add 1 ml of sample to the correspondingly marked column, let the solvent pass through slowly ($\leq$ 2 ml/min).
- Proceed with washing before the column gets dry.

Washing

- Add 2 x 1 ml of 10% methanol in H_2O (v/v) to columns, let the solvent pass through using vacuum.
- Add 1 ml of hexane to the columns, let the solvent pass through using vacuum.
- Apply vacuum for 1 more min. in order to evaporate remaining hexane in the column.

Elution of Extract

- Place the columns into clean correspondingly marked borosilicate tubes.
- Add 1 ml of methanol to columns, let the solvent pass through slowly using vacuum and a flow rate of $\leq$ 2 ml/min.
- Use column for extracting the next sample (columns can be used up to 5 times) or store column at 18-28°C and protected from light and dust.

Evaporation & Reconstitution of the Extract

- Evaporate methanol to dryness using a vacuum concentrator with a cold trap. Alternatively, use a 37°C heating block or water bath and evaporate the methanol to dryness with a stream of particle free nitrogen.
- Reconstitute the samples with 1 ml of Incubation Buffer, vortex.
- Equilibrate the extracts for 30 min at 18-28°C, vortex. Store reconstituted extracts capped and frozen if not assayed immediately.

ASSAY PROCEDURE

1. Label 8 conical polystyrene tubes in duplicate: A to E for the Calibrator tubes, NSB (non-specific binding) for the blank tubes, MB for the maximum binding tubes and T for the total activity tubes. Label additional tubes in duplicate for samples and Controls.
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 extracted samples and Controls into each of the correspondingly marked tubes.
3. Add 100 µl of the Antiserum to all tubes except the NSB and T tubes, vortex.
4. Add 100 µl of ^{125}I-melatonin tracer to all tubes. Vortex. Remove T tubes, they will need no further processing until counting at step 10.
5. Incubate all tubes for 20 ($\pm$ 4) hours at 2-8°C.
6a. Invert the bottle containing the solid phase Second Antibody several times, add a stir bar and place the bottle on a magnetic stirrer.
6b. While stirring the Second Antibody suspension continuously, add 100 µl of the suspension to all assay tubes (except T tubes), vortex.

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

RESULTS

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

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

- 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%.

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

- 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 spline smoothed fitting algorithm.
- Determine melatonin concentrations for the samples and Controls 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 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 (Run-to-Run) of RIA: 7.9%. The intra-assay precision was calculated from results of 20 pairs of values from each sample in a single run. The results are presented in Table 4.

Inter-Assay Precision (Run-to-Run) of RIA: 11.7%. The inter-assay precision was calculated from results of 20 pairs of values in 20 different runs. The results are presented in Table 5.

Intra-Assay Precision (Run-to-Run) of Column Extraction and RIA combined: 8.2%. A daytime human serum was extracted 12 times in parallel using 12 separate extraction columns. Subsequently, all extracts were analyzed in a single run according to the assay procedure. The results are presented in Table 6.

Dilution/Linearity-Parallelism: 103.7%. Two human serum samples containing an elevated concentration of elevated concentration of melatonin was extracted both, diluted and undiluted with Incubation Buffer and subsequently assayed according to the assay procedure. The results are presented in Table 7.

Extractive Dilution Linearity: 108.8 %. Varying amounts of a human serum sample containing an elevated concentration of melatonin were applied onto extraction columns, extracted according to the protocol and subsequently assayed according to the assay procedure. The results are presented in Table 8.

Extraction Recovery: 99.9 %. Two serum samples were spiked with increasing amounts of melatonin, extracted and analyzed according to the assay procedure. The results are presented in Table 9.

Functional Sensitivity: 0.9 pg/ml. The functional least detectable dose (FLDD) was calculated to be 0.9 pg/ml (cut-off of intra-assay CV = 10%).

Analytical Sensitivity: 0.3 pg/ml. Twenty Zero Calibrator (MB) replicates were assayed in a single run. The minimum detectable concentration of melatonin in 400 ml of unextracted Incubation Buffer was calculated to be 0.3 pg/ml (1.3 pmol/l) by subtracting 2 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 10 the crossreactivities of the melatonin antiserum were found at 50 % binding.

Table 3 — **Expamples of results**

	cpm	B/T [%]	B/B$_0$ [%]	Conc. [pg/ml]	cpm$_{CV}$ [%]
Total	17145.9	100.0			
Total	16942.3	100.0			
Total Avg	**17044.1**	**100.0**			**0.8**
NSB	479.1	2.81			
NSB	434.6	2.55			
NSB Avg	**456.9**	**2.68**			**6.9**
MB	5501.7	32.28	100.0		
MB	5478.6	32.14	100.0		
MB Avg	**5490.2**	**32.21**	**100.0**		**0.8**
Cal A	5029.2	29.51	91.0		
Cal A	5020.7	29.46	90.9		
Cal A Avg	**5025.0**	**29.48**	**90.9**	**0.5**	**0.1**
Cal B	4218.6	24.75	74.9		
Cal B	4285.2	25.14	76.2		
Cal B Avg	**4251.9**	**24.95**	**75.5**	**1.5**	**1.1**
Cal C	3020.9	17.72	51.0		
Cal C	2949.6	17.31	49.6		
Cal C Avg	**2985.3**	**17.51**	**50.3**	**5.0**	**1.7**
Cal D	1921.1	11.27	29.1		
Cal D	1866.2	10.95	28.1		
Cal D Avg	**1893.6**	**11.11**	**28.6**	**15.0**	**2.1**
Cal E	950.6	5.58	9.8		
Cal E	1006.9	5.91	10.9.		
Cal E Avg	**978.8**	**5.74**	**10.4**	**50.0**	**4.1**
Con L	3666.3		63.8	2.69	
Con L	3669.6		63.8	2.68	
Con L Avg	**3668.0**		**63.8**	**2.69**	**0.2**
Con H	1607.7		22.9	21.20	
Con H	1580.4		22.3	21.96	
Con H Avg	**1594.1**		**22.6**	**21.58**	**2.5**

ED$_{20}$ = 25.55 pg/ml   ED$_{50}$ = 5.04 pg/ml   ED$_{80}$ = 1.14 pg/ml

Figure 1 — **Example of Standard Curve**

B/B0 (%)

Melatonin (pg/ml)
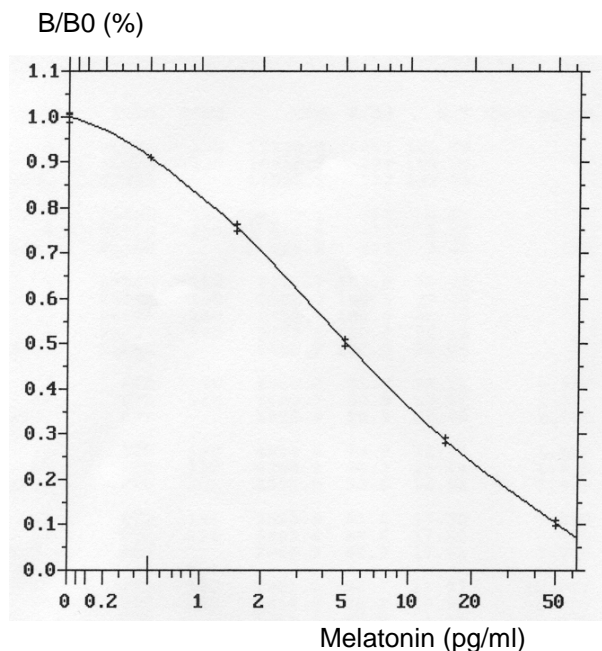
Table 4 — **Intra Assay Precision**

Sample	Mean [pg/ml]	SD [pg/ml]	CV [%]
Low Control	4.6	0.31	6.7
High Control	14.3	1.08	7.5
Low Serum	1.9	0.19	10.0
High Serum	21.9	1.58	7.2
Mean			7.9

Table 5 — **Inter-Assay Precision**

Sample	Mean [pg/ml]	SD [pg/ml]	CV [%]
Low Control	2.3	0.38	16.2
High Control	18.7	1.35	7.2
Mean			11.7

Table 6 — **Intra-Assay Precision Column Extraction and RIA**

Sample	Mean [pg/ml]	SD [pg/ml]	CV [%]
Low Serum1	1.6	0.22	14.0
Low Serum2	2.8	0.17	6.1
High Serum	19.5	0.88	4.5
Mean			8.2

Table 7 — **Dilution/Liniearity-Parallelism**

Sample	Dilution	Obs. (O) [pg/ml]	Exp. (E) [pg/ml]	% O/E
High Control	1:1	18.9	-	-
	1:2	9.6	9.5	101.4
	1:4	5.2	4.7	109.0
	1:8	2.7	2.4	114.3
	1:16	1.4	1.2	119.7
High Serum	1:1	36.3	-	-
	1:2	16.8	18.2	92.7
	1:4	8.3	9.1	91.6
	1:8	4.3	4.5	95.4
	1:16	2.3	2.3	100.7
	1:32	1.2	1.1	108.3
Mean				103.7

Table 8 — **Extractive Dilution-Linearity**

Sample	Volume applied [ml]	Obs. (O) [pg/ml]	Exp. (E) [pg/ml]	% O/E
High Serum	1	29.1	-	-
	0.5	15.1	14.6	103.8
	0.25	7.8	7.28	107.2
	0.125	4.2	3.64	115.4
Mean				108.8

Table 9 — **Extraction Recovery**

Sample	Basic Value	Added [pg/ml]	Observed [pg/ml]	Expected [pg/ml]	Recovery [%]
A	0.46	1	1.2	1.5	83
		2	2.2	2.5	88
		5	5.2	5.5	95
		10	10.5	10.5	100
		20	21.7	20.5	106
		40	41.0	40.5	101
B	0.61	1	1.9	1.6	120
		2	2.3	2.6	87
		5	5.5	5.6	98
		10	11.8	10.6	111
		20	22.1	20.6	107
		40	41.3	40.6	102
Mean					99.9

Table 10   **Specificity**

Compound	Crossreactivity [%]
melatonin	100
6-sulfatoxymelatonin	0.002
serotonin	< 0.001
5-hydroxy-indoleacetic acid	< 0.001
N-acetylserotonin	0.027
5-methoxytryptamine	0.003
5-methoxytryptophan	0.001
5-methoxytryptophol	0.001

APPENDIX II
REFERENCES

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2. Luboshitzky R. et al. *Daily and seasonal variations in the concentration of melatonin in the human pineal gland.* Brain Res Bull 47, 271-6 (1998)

3. Danilenko K. et al. *Phase advance after one or three simulated dawns in humans.* Chronobiol Int 17, 659-68 (2000)

4. 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).

APPENDIX III
SHORT PROTOCOL

EXTRACTION PROCEDURE

C18 column
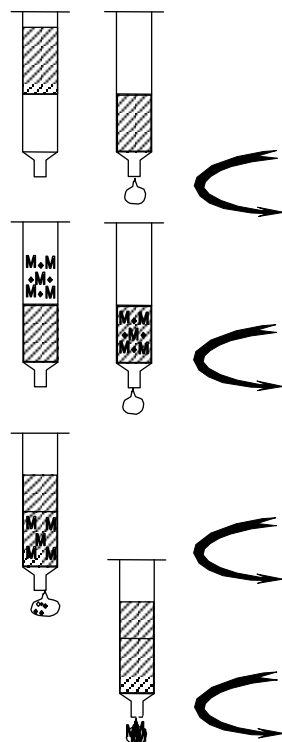
Conditioning

2 x 1 ml methanol
2 x 1 ml water

aspirate or centrifuge

Load column

1 ml of sample

aspirate or centrifuge

Wash column

2 x 1 ml 10% (v/v) methanol
1 ml hexane

aspirate or centrifuge

Elute Melatonin

1 ml methanol

aspirate or centrifuge

Evaporate to dryness
and reconstitue in 1 ml of
Incubation Buffer

Figure 2

Table 11

RADIOIMMUNOASSAY PROCEDURE

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

Symbol	Explanation
⧗	Use By
REF	Catalogue number
LOT	Batch code
🌡	Temperature limitation
📖	Consult Instructions for Use
∑	Contains sufficient for <n> tests
RADIOACTIVE ☢ RADIOAKTIV	Radioactive Material

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