novolytix

MELATONIN ELISA with Extraction Set

MLTN-96/MLTN-EXSET

96 ELISA tests / 80 extractions

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ENGLISH

INTENDED USE

The Melatonin ELISA with Extraction Set (MLTN-96/MLTN-EXSET) is intended for highly sensitive, quantitative determination of melatonin in plasma, serum, urine and other biological fluids after SPE column extraction of the sample.

The MLTN-96/MLTN-EXSET kit is a combination of the former Melatonin ELISA (MLTN-96) and the former Melatonin Extraction Set (MLTN-EXSET) into one single test kit.

PRINCIPLE OF THE ASSAY

Prior to use of the samples in the Melatonin ELISA (MLTN-96) they must be extracted/purified over a Solid-Phase Extraction (SPE) C18 column (MLTN-EXSET). After reconstitution of the extract/eluate it is measured with Melatonin ELISA kit part which is a competitive immunoassay using a capture antibody technique. Melatonin present in extracted serum, plasma or any other biological fluid samples and controls compete with biotinylated melatonin for the binding sites of the highly specific polyclonal Kennaway G280 anti-melatonin antibody during an overnight incubation. During this incubation the formed antibody-melatonin-biotin complexes are captured onto the pre-coated wells of the microplate. After washing away unbound melatonin-biotin conjugate the enzyme label (streptavidin conjugated to horseradish peroxidase) is added, which binds to the free biotin sites captured on the coated wells during a 60-minute incubation step. Unbound enzyme label is then removed by a second washing step and TMB (tetramethylbenzidine) substrate is added to the wells. In a further 30-minute incubation step, a chromophore is formed (turns from colorless to blue) in inverse proportion to the amount of melatonin originally present in the samples, controls and calibrators. The color turns from blue to yellow after the addition of an acidic stop solution and can be measured at 450 nm.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Extraction Columns[1)] C18 reversed phase extraction columns	2 bags 10 pcs. each	B-MEC	Ready to use
Control low / high[2)] human serum base with preservatives	2 vials 1.5 mL	B-MEL-CONSET15	Ready for extraction (see p. 4)
Incubation Buffer with preservatives	1 vial 25 mL	MLTN-IB	Ready to use
Microtiter Plate precoated with antibody capture molecules	12x8 wells	MLTN-MP	Stored under 250 µL of protection buffer; wash 2x before use
Plate Sealer	3 pieces	-	
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 mL	B-WB	Dilute with 900 mL of deionized water
Blanking Reagent[3)] with preservatives	1 vial 1 mL	MLTN-BR	Ready to use
Zero Calibrator with preservatives	1 vial 5 mL	MLTN-0	Ready to use
Calibrators with preservatives	5 vials 1 mL	MLTN-CASET	Ready to use
Biotin Conjugate with preservatives	1 vial 3 mL	MLTN-BC	Ready to use; blue color
Antiserum (G280) with preservatives	1 vial 3 mL	MLTN-AS	Ready to use; yellow color
Enzyme Label Streptavidin conjugated to HRP with preservatives	1 vial 11 mL	MLTN-EL	Ready to use; yellow color
TMB Substrate buffered with citrate	1 vial 11 mL	B-TMBF	Ready to use
Stop Solution 0.25 M sulfuric acid (H_2SO_4)	1 vial 11 mL	B-STS	Ready to use **Irritant**

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) Lot specific amount of melatonin see QC data sheet added to this kit.
3) The Blanking Reagent contains a saturated melatonin solution. Prevent any contamination of other kit reagents.

STORAGE AND SHELF LIFE OF REAGENTS

Sealed / Unopened Reagents	
Store at 2-8°C until expiration date printed on the labels. Extraction Columns may be stored at 0-30°C until expiration date. Do not use past expiration date.	
Opened / Reconstituted Reagents	
Extraction Columns	Used columns can be stored up to 2 months after first use at 18-28°C and protected from dust and light.
Microtiter Plate	Cover unused strips with a plate sealer, return them to the aluminium/plastic pouch and reseal along the entire edge of zip-seal. Store for up to 6 months at 2-8°C.
Wash Buffer diluted	Store at 2-8°C up to 2 months after dilution.
Incubation Buffer	Store at 2-8 °C until expiration date printed on the labels.
Blanking Reagent	
Calibrators	
Controls	
Biotin Conjugate	
Antiserum	
Enzyme Label	
TMB Substrate	
Stop Solution	

Table 2

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes with disposable tips: 25 µL, 100 µL, 200 µL, and 250 µL pipettes. Repeater or multichannel pipette for 25 µL and 100 µL.
- Disposable (borosilicate) glass tubes for the preparation of extracts (*e.g.* disposable glass test tubes from Duran Wheaton Kimble; no. 90106015).
- Extraction vacuum manifold for applying the extraction columns; alternatively, the extraction procedure can also be performed using a centrifuge (see below).
- Methanol (HPLC grade).
- Hexane *(p.a.)*.

- Deionized double distilled water (ultrapure; not containing any organic residues).
- Vacuum concentrator or supply for particle free nitrogen.
- Vortex mixer.
- 1000 mL cylinder for the dilution of the Wash Buffer Concentrate.
- Microtiter plate washer or squeeze bottle for diluted Wash Buffer.
- Blotting paper.
- Refrigerator.
- Microtiter plate orbital shaker.
- Microtiter plate reader for measurement of absorbance at 450 nm.

PRECAUTIONS

Safety precautions

- The Stop Solution (B-STS) contains 0.25 M sulfuric acid. It is irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothes. Wear suitable protective clothing, gloves and eye protection. After contact with eyes or skin, wash immediately with plenty of water.
- Unused Stop Solution should be disposed of according to local state and federal regulations.

Clinical / Sampling precautions

- When drawing blood at night, a dim flash light or a yellow light (≤100 lux) should be used in order to avoid a possible light influence on the melatonin profile.
- Meals and posture changes within 30 minutes before next sample collection may stimulate melatonin synthesis and should be avoided.
- Bananas, chocolate, all food and drinks containing caffeine (coffee, black or green tea, iced tea, cola, etc) and alcohol should not be consumed the day before and the during the entire collection period.
- On the sample collection day, if possible, no aspirin and medicines that contain ibuprofen (Brufen®, Algifor® Dismenol®, Dolocyl®, Ecoprofen®) should be taken.
- If a patient or study subject is treated with melatonin, the last melatonin dose must be prescribed not later than two days before the collection period.
- Norepinephrine (noradrenaline) stimulates melatonin synthesis, and such treatments should be stopped at least 36 hours before sample collection.

Technical precautions

Kit components

- Read this instruction for use (IFU) carefully before 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 IFU.
- Residual liquid in the microtiter plate wells result from the production process and stabilizes the coated antibodies. They are removed in the washing step before setting up the assay (assay procedure step 2) and do not affect the results.
- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Every effort should be made to ensure that no cross contamination occurs between reagents, samples or between wells. Be particularly careful by using the Blanking Reagent, as this contains an excess amount of melatonin.
- Microtiter plate wells cannot be re-used.
- Let the reagents adjust to reach room temperature and mix the reagents well before use.

Extraction procedure

- Calibrators must not be extracted; they are ready to use for the ELISA assay procedure.
- Other biological fluids and tissue extracts apart from serum and plasma can be used: Prepare the biological fluid or tissue extract according to your study protocol. Do not use detergents or chaotropic salts for the preparation of samples or tissue extracts as they can interfere with the extraction and/or the ELISA procedure. The procedure calls for 0.25 mL of liquid.
- Extracted (and reconstituted) samples not assayed immediately can be stored at 2-8°C for up to 3 days and for at least 12 months at -20°C. Repeated freeze-thaw cycles should be avoided.
- 0.2 mL of sample are extracted over the SPE C18 column. The dried eluted extract is then reconstituted with 0.25 mL of Incubation Buffer. This 20% dilution is corrected by the concentration of melatonin in the Calibrators (see also footnote 4 in Table 1). No calculation of dilutions is required, the correct concentration of samples and controls can directly be read from the standard curve (see also section RESULTS)
- The Calibrators A, B, C, D and E (MLTN-CASET) are labeled with 0.5, 1.5, 5, 15, and 50 pg/mL of melatonin but contain the following effective concentrations: 0.4, 1.2, 4, 12 and 40 pg/mL, respectively, accounting for the 20% sample dilution during the extraction and reconstitution procedure of samples and controls. Therefore, the effective, final concentration of melatonin in samples and controls can be directly read from the curve without any re-calculations or corrections.

Assay procedure

- The Blanking Reagent contains a saturated melatonin solution. Avoid any contamination of other reagents of this kit. Change disposable tips after each pipetting step.
- It is highly recommended that Blanking Reagent, Calibrators and Controls are assayed in duplicates.

SPECIMEN COLLECTION (SERUM / PLASMA) AND STORAGE

- Serum: The procedure calls for about 0.5 ml of blood or for 0.25 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 3000 x g for 10 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 patients to fast for at least 12 hours prior to blood drawing.

- Plasma: The procedure calls for about 0.5 ml of blood or for 0.25 mL of plasma per extraction (if the sample is not diluted after extraction). Collect blood into EDTA or Heparin tubes, centrifuge for 10 min at 3000 x g for 10 min at room temperature and collect the plasma. Do not use grossly hemolysed samples.
- Other biological fluids and tissue extracts: Prepare the biological fluid or tissue extract according to your study protocol. Do not use detergents or chaotropic salts for the preparation of samples or tissue extracts as they can interfere with the extraction and/or the ELISA procedure. The procedure calls for 0.25 mL of liquid.
- Specimen Storage: If not extracted immediately, serum or plasma samples can be stored at 2-8°C for up to 3 days and for at least 12 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).
- If samples have to be measured containing >50 pg/ml of melatonin, the sample volume may be reduced down to 50 µL without a notable change in extraction recovery. Alternatively, the dried extract may be reconstituted with higher volumes than the recommended volume of 0.25 mL.
- The extraction procedure was tested and validated for human serum, plasma, Saliva and URINE samples. If it is intended to measure another specimen, we recommended to validate the extraction recovery using melatonin-spiked specimens.

Extraction Procedure using Centrifugation

COLUMN PREPARATION & CONDITIONING

- Mark an extraction column for each sample to be extracted and hang it onto 10-mL polypropylene 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 0.2 mL of sample to the correspondingly marked column, centrifuge for 1 min at 200 x g (if sample does not fully run through the column, repeat this step for by centrifuging 1 min at 500 x g; eventually a third time for 1 min at 1000 x g).

WASHING

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

ELUTION OF EXTRACT

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

EVAPORATION & RECONSTITUTION OF EXTRACT

- Evaporate the methanol to dryness using a vacuum concentrator with a cold trap set at 40°C and 1.5 Torr. 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 0.25 mL of Incubation Buffer, vortex.
- Equilibrate the extracts for 30 min at 18-28°C, vortex. If not assayed immediately, cap the glass tubes and store reconstituted extracts for up to 3 days at 2-8°C or at -20°C for up to 12 months.
- Proceed to the ELISA procedure.

Extraction Procedure using Vacuum Manifold

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

COLUMN PREPARATION & CONDITIONING

- Mark an extraction column for each sample to be extracted and fix it onto the Luer connection of the vacuum manifold.
- 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 0.2 mL of sample to the correspondingly marked column, let the solvent pass through slowly (2 mL/min; if sample does not fully run through the column, increase the vacuum).
- Proceed with washing before the column gets dry.

WASHING

- Add 2 x 1 mL of 10% methanol in H_2O (v/v) to the column, let the solvent pass through using vacuum.
- Add 1 mL of hexane to the column, let the solvent pass through using vacuum.

ELUTION OF EXTRACT

- Place a clean correspondingly marked borosilicate tube in the vacuum manifold at the position of the correspondingly marked column. Close vacuum manifold and add the vacuum again.
- Apply vacuum for 1 minute in order to evaporate remaining hexane from the column.
- Add 1 mL of methanol to columns, let the solvent pass through slowly using vacuum and a flow rate of 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 the methanol to dryness using a vacuum concentrator with a cold trap set at 40°C and 1.5 Torr. 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 0.25 mL of Incubation Buffer, vortex.
- Equilibrate the extracts for 30 min at 18-28°C, vortex. If not assayed immediately, cap the glass tubes and store reconstituted extracts for up to 3 days at 2-8°C or at -20°C for up to 12 months.
- Proceed to the ELISA procedure.

ELISA PROCEDURE

1. Use a plate with enough 8-well strips to test the desired number of Calibrators, Controls and samples. Remove excess strips from the holder, cover them again with the attached Plate Sealer and re-seal them in the aluminium/plastic foil pouch. Store refrigerated.
2. Empty the wells and wash the strips twice using at least 300 µL of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.

3a. Pipet 100 µL of Blanking Reagent in duplicate into wells A1+A2.

3b. Pipet 100 µL of Zero Calibrator in duplicate into wells B1+B2.

3c. Pipet 100 µL of Calibrator A in duplicate into wells C1+C2.

Pipet 100 µL of Calibrator B in duplicate into wells D1+D2.

Pipet 100 µL of Calibrator C in duplicate into wells E1+E2.

Pipet 100 µL of Calibrator D in duplicate into wells F1+F2.

Pipet 100 µL of Calibrator E in duplicate into wells G1+G2.

3d. Pipet 100 µL of extracted Low Control into wells H1+H2.

Pipet 100 µL of extracted High Control into well A3+B3.

3e. Pipet 100 µL of each extracted sample into the subsequent wells.

4. Add 25 µL of Biotin Conjugate (blue solution) to each well.
5. Add 25 µL of Antiserum (yellow solution) to each well.
6. Cover the plate with a Plate Sealer, place it for 1 min on a plate orbital shaker set at 600 rpm and then incubate for 16-24 hours at 2-8 °C.
7. Remove and discard the Plate Sealer. Aspirate or invert the plate to empty the solution from each well and wash five times using at least 300 µL of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
8. Add 100 µL of Enzyme Label (yellow solution) to all wells.
9. Cover the plate with a new Plate Sealer, place the plate on a plate orbital shaker set at 600 rpm and incubate for 60 ± 5 minutes at 18-28°C.

Important: *Allow the TMB Substrate to equilibrate to 18-28°C prior to use in step 11.*

10. Remove and discard the Plate Sealer. Aspirate or invert the plate to empty the solution from each well and wash five times using at least 300 µL of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
11. Add 100 µL of TMB Substrate to all wells.
12. Cover the plate to protect it from direct light, place it on a plate orbital shaker set at 600 rpm and incubate for 30 ± 2 minutes at 18-28°C.
13. Add 100 µL of Stop Solution to all wells. Place the plate for 10 seconds on a plate orbital shaker set at 600 rpm and proceed to step 14 within 30 minutes.
14. Read the absorbance at 450 nm in a microtiter plate reader.

QUALITY CONTROL

- A thorough understanding of this IFU is necessary for the successful use of the product. Reliable results will be obtained only by precise laboratory techniques (current GLP guidelines) and accurately following this IFU.
- Since there are no controls for melatonin commercially available, we recommend using internal saliva or plasma/serum pools containing different levels of melatonin as internal quality controls. 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 are printed on the QC data sheet delivered with each test kit.
- If the performance of the assay does not meet the established limits and repetition has excluded errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices; ii) ELISA reader settings; iii) expiration dates of reagents; iv) storage and incubation conditions; v) TMB Substrate solution should be colorless; and vi) purity of water.

STANDARDIZATION

The NovoLytiX Melatonin ELISA with Extraction Set is calibrated with United States Pharmacopeia (USP) Reference Standard material (Merck #1380105), and its correct concentration used to generate the kit Calibrators was confirmed by UV/VIS: Ɛ278 = 6300 M-1cm-1 in ethanol/H_2O solution.

RESULTS

Standard curve

Record the absorbance at 450 nm for each calibrator and blank wells. Average the duplicate values, subtract the average of the blank wells and record averages (=corrected average absorbance). Calculate the binding (B) of each pair of calibrator wells as a percent of Zero Calibrator (B_0), with the blank-corrected absorbance of the Zero Calibrator taken as 100 %.

$$B/B_0\ (\%) = \frac{\text{net absorbance}}{\text{net absorbance of Zero Calibrator}} \times 100$$

Plot the percent bound (B/B_0, vertical axis) versus the concentration of melatonin in pg/mL (horizontal axis) using a lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four-parameter logistic (4-PL) or equivalent algorithm.

Samples and Controls

- Record the absorbance at 450 nm for each control and each sample wells. Subtract the average of the blank wells and record the absorbance (=corrected average absorbance). Calculate, as described above, the binding of each pair of sample wells as a percent of Zero Calibrator (B_0), with the blank-corrected absorbance of the Zero calibrator taken as 100%.
- Locate the B/B_0 value of the samples on the vertical axis, draw a horizontal line intersecting the standard curve and read the melatonin concentration (pg/mL) from the horizontal axis.
- The 20% dilution factor (200 µl sample volume applied to extraction columns; extracts reconstituted in 250 µl of Incubation Buffer) is considered in the concentration specifications of Calibrators A, B, C, D and E of the MLTN-96/MLTN-EXSET ELISA which contain the following melatonin concentration: 0.4, 1.2, 4, 12 and 40 pg/mL, but are labeled with 0.5, 1.5, 5, 15, and 50 pg/mL of melatonin, respectively. Thus, the final results can be directly read / calculated from the standard curve.

See Table 3 and Figure 1 for examples of results and standard curves. *Results and standard curves are for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.*

PERFORMANCE CHARACTERISTICS

Precision (including Column Extraction): 8.2%.
Three human plasma samples were extracted 12 times in parallel using 12 separate extraction columns. Subsequently, all extracts were analyzed in a single assay run according to the assay procedure of the NovoLytiX Melatonin ELISA with Extraction Set. The results are presented in Table 4.

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 NovoLytiX Melatonin ELISA with Extraction Set. The results are presented in Table 5.

Spiking Recovery (including Extraction): 99.9 %.
Two plasma samples were spiked with increasing amounts of melatonin, extracted and analyzed according to the assay procedure of the NovoLytiX Melatonin ELISA with Extraction Set. The results are presented in Table 6.

Specificity.

The 50% binding (cross-reactivity) of the melatonin antiserum with different compounds was tested in the NovoLytiX Melatonin Radioimmunoassay (RK-MEL2) and are presented in table 7. The data can be transferred to the NovoLytiX Melatonin ELISA with Extraction Set as the same antibody (G280) in very similar assay buffer systems as compared to the RK-DSM2 assay are used.

Re-usability of Extraction Columns.

One serum sample was 5-times extracted over 2 separate extraction columns (B-MEC). The extraction columns were re-conditioned after each extraction run and re-used up to 4-times according to the extraction protocols described on pages 4 and 5. The reconstituted extracts were assayed with the NovoLytiX Melatonin ELISA with Extraction Set in one single. The results are presented in Table 8.

Method Comparison.
The comparison was done with 21 plasma samples from 2 different donors collected at different daytimes. The samples were analyzed using the NovoLytiX Melatonin ELISA with Extraction Set (MLTN-96/MLTN-EXSET) as well as the NovoLytiX Melatonin Radioimmunoassay (RK-MEL2). The subsequent Passing-Bablok regression analysis resulted in a correlation factor of $R^2 = 0.980$, an intercept of -0.47 pg/mL and a slope of 1.19. The correlation graph is presented in Figure 2 (*MLTN-PL means MLTN-96/MLTN-EXSET*).

APPENDIX I

TABLES AND FIGURES

Examples of Results

	Conc. (pg/mL)	Absor-bance (OD)	B/B0 (%)	CV Conc. (%)	Calc. Conc. (pg/mL)
Blank Blank **Avg.**		0.140 0.146 **0.143**		**3.0**	
Zero Calibrator Zero Calibrator **Avg.**	**0**	2.106 2.197 **2.151**	97.7 102.1 **100.0**	**3.0**	
Cal A Cal A **Avg.**	**0.5**	1.850 1.877 **1.868**	86.0 87.3 **86.7**	**0.9**	
Cal B Cal B **Avg.**	**1.5**	1.655 1.620 **1.638**	76.9 75.7 **76.3**	**1.1**	
Cal C Cal C **Avg.**	**5**	1.115 1.121 **1.118**	51.8 52.1 **52.0**	**0.2**	
Cal D Cal D **Avg.**	**15**	0.562 0.530 **0.550**	26.1 25.0 **25.6**	**0.7**	
Cal E Cal E **Avg.**	**50**	0.204 0.255 **0.230**	10.3 10.5 **10.4**	**1.7**	
Ctrl. High Ctrl. High **Avg.**		0.477 0.538 **0.557**	22.2 25.0 **23.6**	**11.7**	19.4 16.4 **17.9**
Ctrl. Low Ctrl. Low **Avg.**		1.435 1.379 **1.407**	66.7 64.1 **65.4**	**8.6**	2.5 2.8 **2.7**
Sample 01 Sample 01 **Avg.**		0.576 0.549 **0.563**	26.8 25.5 **26.2**	**4.8**	14.9 16.0 **15.4**
Sample 02 Sample 02 **Avg.**		1.313 1.261 **1.287**	61.0 58.6 **54.0**	**4.7**	3.2 3.6 **3.4**
Sample 03 Sample 03 **Avg.**		1.888 1.806 **1.847**	87.8 84.0 **85.9**	**23.5**	0.6 0.9 **0.8**

Table 3

ED80 = 1.2 pg/mL ED50 = 5.4 pg/mL ED20 = 20.4 pg/mL

Example of Standard Curve (OD_{450})

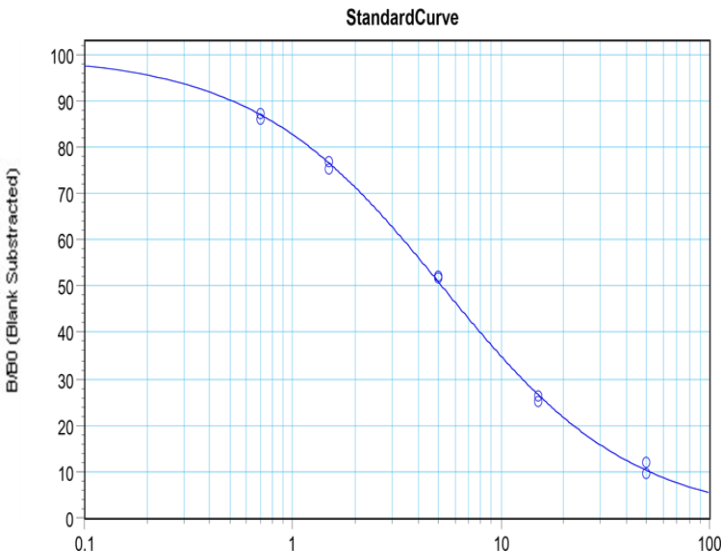


Figure 1

Precision (including Extraction)

Sample	Mean [pg/mL]	SD [pg/mL]	CV [%]
Low Plasma 1	1.6	0.22	14.0
Low Plasma 2	2.8	0.17	6.1
High Plasma 3	19.5	0.88	4.5
Mean			**8.2**

Table 4

Extractive Dilution Linearity

Sample	Volume applied [mL]	Obs. (O) [pg/mL]	Exp. (E) [pg/mL]	% O/E
High Serum 4	1	29.1	-	-
	0.5	15.1	14.6	103.8
	0.25	7.8	7.3	107.2
	0.125	4.2	3.6	115.4
Mean				**108.8**

Table 5

Spiking Recovery (including Extraction)

Sample	Basic Value [pg/mL]	Added [pg/mL]	Obs. (O) [pg/mL]	Exp. (E) [pg/mL]	Recovery (O/E) [%]
Plasma 5	0.5	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
Plasma 6	0.6	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 6

Specificity

Compound	Crossreactivity [%]
melatonin	100
serotonin	< 0.001
6-sulfatoxymelatonin	< 0.001
N-acetylserotonin	0.045
5-hydroxy-indole acetic acid	< 0.001
5-methoxytryptamine	0.007
5-methoxytryptophane	< 0.001
2-methyl-5-hydroxytryptamine	< 0.001
5-methoxypsoralen	< 0.001
5-methoxytryptophol	0.002
chloromelatonin	1.3
L-arginine	< 0.001
Arg-vasopressin	<0.001
noradrenalin (norepinephrin)	<0.001
caffeine	<0.001
caffeine acid	<0.001
soluble coffee	<0.001
soluble coffee decaffeinated	<0.001

Table 7

Re-usability of Extraction Columns

Extrac-tion Column	Extrac-tion run	Melatonin recovered [pg/ mL]	Mean [pg/mL]	SD [pg/mL]	CV [%]
1	1	11.7			
	2	11.7			
	3	12.6			
	4	12.8			
	5	12.4	12.2	0.51	4.2
2	1	10.8			
	2	13.1			
	3	11.9			
	4	12.5			
	5	11.9	12.0	0.85	7.1
Mean			**12.1**		**6.0**

Table 8

Method Comparison

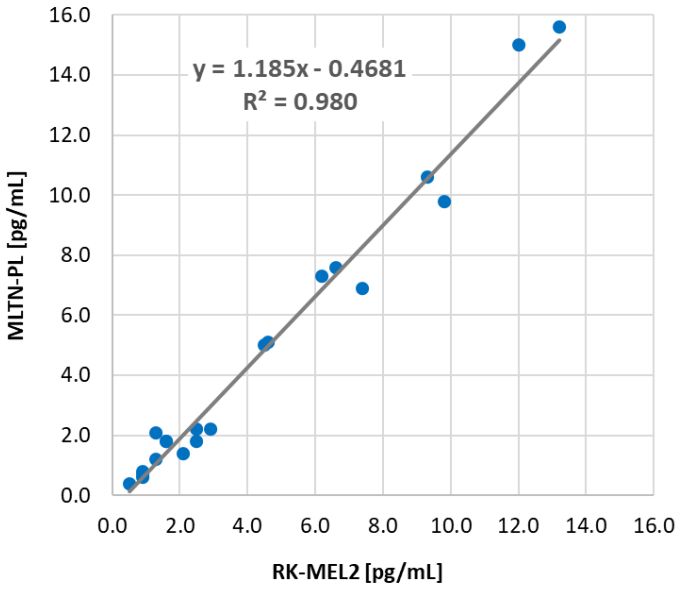
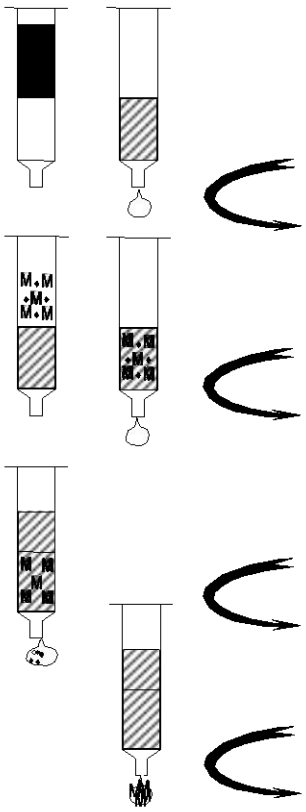


Figure 2

EXTRACTION PROCEDURE

SPE C18 column



Conditioning

2 x 1 mL methanol
2 x 1 mL water

aspirate or centrifuge

Load column

0.2 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 0.25 mL
of Incubation Buffer

ELISA Procedure

Precoated Microtiter Plate

↓ *Wash 2x with ≥300 µL Wash Buffer*

100 µL Calibrators, Pretreated Controls or Samples

↓

add 25 µL Melatonin-Biotin-Conjugate

↓

add 25 µL Antiserum

↓ *1 minute on a plate orbital shaker*

16-22 hours at 2-8°C

Wash 5x with ≥300 µL Wash Buffer

add 100 µL Enzyme Label

↓ *60 minutes at 18-28°C on a plate orbital shaker*

Wash 5x with ≥300 µL Wash Buffer

add 100 µL TMB Substrate

↓ *Incubate 30 minutes at 18-28°C on a plate orbital shaker*

add 100 µL Stop Solution

➡ Read absorbance at 450 nm (within 30 minutes)

APPENDIX III

CHANGE LOG

Date	Version	Reason for change
2022-01-11 / 2022-02-02	01	- Release of MLTN-96 and MLTN-EXSET as two single kits after its declaration of conformity, respectively
2024-10-10	02	- First combined version without any change in reagent compositions and technical and clinical performance, respectively, between MLTN-96/MLTN-EXSET and MLTN-96 combined with MLTN-EXSET; - More assay performance data added, *e.g.* comparison to a “gold-standard” radioimmunoassay (RIA)
2026-04-06	03	- MLTN-96 and MLTN-EXSET combined into one kit box and named MLTN-96/MLTN-EXSET - Single, unified Instructions for Use (IFU)

APPENDIX IV

SYMBOLS

Symbol	Explanation
	Use By
REF	Order Code
LOT	Batch Code
Σ	Contains sufficient for <n> tests
	Consult Instructions for Use
	Temperature Limitation
MEC	Extraction Column
BUF INC	Incubation Buffer
MP	Microtiter Plate
AS	Antiserum

Symbol	Explanation
BUF WASH 10X	Wash Buffer Concentrate (10x)
REAG BLANK	Blanking Reagent
CAL 0	Zero Calibrator
CAL A - CAL E	Calibrator A - E
CONTROL L	Control Low
CONTROL H	Control High
BC	Biotin Conjugate
EL	Enzyme Label
SUBS TMB	TMB Substrate
SOLN STOP	Stop Solution

CE

IVD