6-Sulfatoxymelatonin
ELISA

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

EK-M6S-U 96 tests

Revision date: 20160118

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ENGLISH

INTENDED USE

The BÜHLMANN 6-Sulfatoxymelatonin ELISA Kit provides materials for the direct and quantitative determination of 6-sulfatoxymelatonin (6-SMT) in human urine (1-7).
This product is for research use only. It is not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The BÜHLMANN 6-SMT ELISA is a competitive immunoassay using a capture antibody technique (8). A polyclonal antibody specific for rabbit immunoglobulin has been coated onto the microtiter plate provided in the kit. During the first 3-hours incubation, 6-SMT present in the pre-diluted urine samples, Controls and ready to use Calibrators, respectively, compete with biotinylated 6-SMT for the binding sites of a highly specific rabbit anti-6-SMT antibody, while the formed (biotinylated) 6-SMT-antibody complexes are captured by the second antibody coated on the wells. After washing, the Enzyme Label, streptavidin conjugated to horseradish peroxidase (HRP) is added which binds during a second 30-minutes incubation step to the 6-SMT-biotin-antibody complexes captured on the coated wells. Unbound Enzyme Label is then removed by a second washing step and TMB substrate (tetramethylbenzidin) is added to the wells. In a third 30-minutes incubation step, a colored product is formed in inverse proportion to the amount of 6-SMT originally present in the sample. 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	Quantities	Code	Reconstitution
Microtiter Plate precoated with goat anti-rabbit Ig	8x12 wells	B-M6S-MP	Wash 2x before use
Plate Sealer	3 pcs.		
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 ml	B-M6S-WB	Dilute with 900 ml of deionized water
Incubation Buffer with preservatives	1 bottle 100 ml	B-M6S-IB	Ready to use
Calibrators A to F[1] 6-SMT in a buffer matrix with preservatives	1 vial 2 ml 5 vials 0.5 ml	B-M6S-CASET	Ready to use
Control Low / High[2] Diluted human urine with preservatives	2 vials 0.5 ml	B-M6S-CONSET	Ready to use
Antiserum Rabbit anti-6-SMT in a buffer matrix with preservatives	1 vial 5.5 ml	B-M6S-AS	Ready to use (yellow solution)
Biotin Conjugate 6-SMT conjugated to biotin in a buffer matrix with preservatives	1 vial 5.5 ml	B-M6S-BC	Ready to use (blue solution)
Enzyme Label Streptavidin-HRP in a protein-based buffer with preservatives	1 vial 11 ml	B-M6S-EL	Ready to use (yellow solution)
TMB Substrate citrate buffered with hydrogen peroxide	1 vial 11 ml	B-TMB	Ready to use (colourless)
Stop Solution 0.25 M sulfuric acid	1 vial 11 ml	B-SS	Ready to use **Corrosive agent**

Table 1

[1] The Calibrator A is the Zero Calibrator and does not contain 6-SMT (2 ml/vial). Calibrators B, C, D, E and F effectively contain 4, 10, 25, 62.5 and 200 pg/ml of 6-SMT, respectively (0.5 ml/vial). As the recommended calibrator dilution for urine samples is 1 in 200, the Calibrators B, C, D, E and F are labeled as follows: 0.8, 2, 5, 12.5 and 40 ng/ml, respectively. In this way, the sample dilution is already taken into account for the final calculations.

[2] The Controls contain lot-specific amounts of 6-SMT. Refer to the additional QC Data Sheet for exact concentrations.

STORAGE AND SHELF LIFE OF REAGENTS

Unopened Reagents	
All unopened kit components are stable at 2-8°C until the expiration date printed on the labels.	
Opened / Reconstituted Reagents	
Microtiter Plate	Return unused strips immediately to the aluminum pouch containing the desiccant packs and reseal along the entire edge of zip-seal. Store for up to 2 months at 2-8°C.
Wash Buffer	Store at 2-8°C until expiration date printed on the labels.
Incubation Buffer	
Calibrators	
Controls	
Antiserum	
Biotin Conjugate	
Enzyme Label	
Substrate Solution	
Stop Solution	Store at 18-28°C until expiration date printed on the label

Table 2

PRECAUTIONS

SAFETY PRECAUTIONS

- The Calibrators (B-M6S-CASET) and the controls (B-M6S-CONSET) of this kit contain components of human origin. Although tested and found negative for HBV surface antigen, HCV and HIV1/2 antibodies, the reagents should be handled as if capable of transmitting infections and should be handled in accordance with good laboratory practices using appropriate precautions.
- **Substrate and Stop Solution:** The Substrate Solution (B-TMB) contains Tetramethylbenzidine (TMB), hydrogen peroxide and dimethylformamide. The Stop Solution (B-STS) contains sulfuric acid. Each of those reagents is irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. After contact with eyes or skin, wash immediately with plenty of water.
- Unused solution should be disposed of according to local State and Federal regulations.

TECHNICAL PRECAUTIONS

Kit components

- 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.
- Concentrated wash buffer **may contain salt crystals**. Make sure these crystals have completely dissolved after dilution of the concentrate by stirring the diluted buffer at RT. Stir the solution at RT before usage in the assay.
- **Residues in the microtiter plate** wells result from the production process. They are removed in the washing step (Assay procedure step 3) and do not affect the results.
- **Steps 3- 9:** Use cold (2-8°C) reagents and keep them cold during pipetting.
- If an **automated washer is used,** “plate mode” should be chosen so that dispensing is performed sequentially on all strips before aspirating.
- 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.
- Microwells cannot be re-used.
-

- The enzyme used as the label is inactivated by oxygen and is highly sensitive to sodium azide, thimerosal, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Therefore, use only deionized high quality water.
- If the initial concentration of a sample is exceeds the highest calibrator, the urine sample should be further diluted with Incubation Buffer and assayed again according to the assay procedure. The additional dilution must be considered when calculating the actual concentration of 6-SMT present in the sample.
- If the initial concentration of a sample is lower than the lowest calibrator, the urine sample should be less diluted with Incubation Buffer (e.g. by a factor of 20 instead of 200) and assayed again according to the assay procedure. The lower dilution factor must be considered when calculating the actual concentration of 6-SMT present in the sample.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes with disposable tips: 5 µl, 50 µl, 100 µl and 1000 µl pipettes.
- Disposable polystyrene or polypropylene tubes for the preparation of sample dilutions.
- 1000 ml cylinder for the dilution of the Wash Buffer Concentrate.
- Microtiter plate washer or squeeze bottle for Wash Buffer.
- Blotting paper.
- Refrigerator.
- Microtiter plate rotator.
- Microtiter plate reader for measurement of absorbance at 450nm.

SPECIMEN COLLECTION AND STORAGE

The procedure calls for <10 µl of urine. Collect urine, centrifuge for 1 minute at 12,000 x g or 5 minutes at 2000 x g and transfer aliquots to fresh micro-tubes. Urinary 6-SMT is stable for several weeks even at ambient temperatures. However, due to potential growth of microorganisms it is recommended to store the urine samples at ≤-20°C. Samples are stable for >1 year if stored at ≤-20°C. Avoid repeated freeze-thaw cycles. Frozen samples should be thawed and mixed thoroughly by vortexing prior to use.

ASSAY PROCEDURE

1. Dilute all urinary samples 1:200 with Incubation Buffer (e.g. 5 µl of urine + 1 ml of Incubation Buffer).
2. Prepare a plate with sufficient strips to test the desired number of Calibrators, Controls and samples. Remove excess strips from the holder and reseal them in the foil pouch together with the desiccant packs **without delay**. Store refrigerated.
   Important: Use refrigerated reagent solutions in steps 3. to 9. only.
3. Wash the coated wells twice using at least 300 µl of refrigerated Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.

4a. Pipet 100 µl of Calibrator A in duplicate into wells A1+A2 (Blank wells).

4b. Pipet 50 µl of Calibrator A (Zero Standard) in duplicate into wells B1+B2.
Pipet 50 µl of Calibrator B in duplicate into wells C1+C2.
Pipet 50 µl of Calibrator C in duplicate into wells D1+D2.
Pipet 50 µl of Calibrator D in duplicate into wells E1+E2.
Pipet 50 µl of Calibrator E in duplicate into wells F1+F2.
Pipet 50 µl of Calibrator F in duplicate into wells G1+G2.
Pipet 50 µl of Low Control in duplicate into wells H1+H2.
Pipet 50 µl of High Control in duplicate into wells A3+A4.

4c. Pipet 50 µl of each diluted sample in duplicate into the subsequent wells.

5. Add 50 µl of M6S-Biotin Conjugate (blue solution) to all wells.
6. Add 50 µl of Antiserum (yellow solution) to all wells, **except Blank wells** (wells A1+A2). Cover the plate with a plate sealer and place it for 60 seconds on a plate mixer set at 800-1000 rpm.
7. Incubate for 3 hours (± 5 min) at 2-8°C.
8. Remove and discard the Plate Sealer. Empty the wells and wash four times using at least 300 µl of refrigerated Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
9. Add 100 µl of Enzyme Label (yellow solution) to all wells.
10. Cover the plate with a Plate Sealer and incubate for 30 minutes (± 5 min) at 2-8°C.
    Important: Allow the TMB substrate solution to reach 18-28°C.
11. Remove and discard the Plate Sealer. Empty the wells and wash four times using at least 300 µl of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
12. Add 100 µl of the TMB Substrate Solution to each well.
13. Cover the plate with a Plate Sealer, place the plate on a plate mixer set at 800-1000 rpm, protect the plate from direct light and incubate for 15 minutes (± 2 min) at 18-28°C.
14. Add 100 µl of Stop Solution to all wells. Remove air bubbles with a pipette tip. Proceed to step 15 within 30 minutes.
15. Read the absorbance at 450 nm in a microtiter plate reader. If wavelength correction is available, set instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 600 to 620 nm.

RESULTS & STANDARDIZATION

Standard Curve: Record the absorbance at 450 nm for each calibrator and blank (NSB) well. Average the duplicate values, subtract the average of the blank wells (NSB) 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 NSB-corrected absorbance of the Zero Calibrator taken as 100 %.

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

Plot the percent bound (vertical axis) versus the concentration of M6S in ng/ml (horizontal axis) using a lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four parameter algorithm.

Samples and Controls: Record the absorbance at 450 nm for each sample well. Average the duplicate values, subtract the average of the blank wells and record the averages (=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 NSB-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 M6S concentration (ng/ml) from the horizontal axis.

NOTE: If the Microtiter Plate reader is not capable of reading absorbance greater than 2 or greater than the absorbance of the Zero Calibrator, a second reading at a wavelength of 490 or 492 nm is recommended (reference filter at 600 or 620 nm if available). In this case, proceed to construct a second standard curve with the absorbance readings of all calibrators at 490 or 492 nm. The concentration of the off-scale samples at 450 nm are then read from the new standard curve as described above. The readings at 490 or 492 nm should not replace the on-scale readings at 450 nm.

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

Standardization: MELATONIN 6 SULFATE ELISA is calibrated against UV/VIS: ε_{222} = 39'727 $M^{-1}cm^{-1}$ in H_2O.

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.
Since there are no controls for urinary 6-SMT commercially available, we recommend to use urine pools containing different levels of 6-SMT for 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 printed on the additional QC data sheet.
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) ELISA reader settings iii) expiration dates of reagents iv) storage and incubation conditions v) TMB Substrate Solution should be colorless vi) purity of water.

PERFORMANCE CHARACTERISTICS

Intra-Assay Precision (Within-Run): 7.1%. The intra-assay precision was calculated from the results of 24 pairs of values obtained in a single run from three urine samples with different amounts of 6-SMT. The results are presented in Table 4.

Inter-Assay Precision (Run-to-Run): 11.9%. From 6 urine samples with different amounts of 6-SMT the inter-assay precision was calculated from the results of 10 pairs of values obtained in 10 different runs. The results are presented in Table 5.

Dilution Linearity/Parallelism: 97.8%. Three urine samples with high amount of 6-SMT were sequentially diluted with Incubation Buffer and assayed according to the assay procedure. The Results are presented in Table 6.

Spiking Recovery: 119%. Three human urine samples were spiked with increasing amounts of 6-SMT and assayed according the assay procedure. The Results are presented in Table 7.

Analytical Sensitivity: 0.14 ng/ml. 23 duplicates of Incubation Buffer (Standard A) were assayed in a single run. Mean and standard deviation were calculated for the absorbance values. The minimum detectable dose of 6-SMT was calculated to be 0.14 ng/ml by substracting two standard deviations to the mean absorbance of the Incubation Buffer and intersecting this value with the standard curve obtained in the same run.

Functional Sensitivity: 1.5 ng/ml. The Functional Least Detectable Dose **(**FLDD) of this assay is the minimum 6-SMT concentration in urine that can be measured with an inter-assay coefficient of variation (C.V.) of less than 15%. The FLDD was determined from 7 different urinary samples each measured in one duplicate pair of tubes over 10 assays. The FLDD was calculated to be 1.5 ng/ml (at a sample dilution of 1:200).

Specificity: The following cross-reactions of the Rabbit anti-6-SMT antibody have been determined at 50 % binding. The results are presented in Table 8.

Method Comparison: 42 urine samples were analyzed using the BÜHLMANN 6-Sulfatoxymelatonin ELISA and a other commercially available reagent set for measuring 6-SMT by means of an ^{125}I-radioimmunoassay, which is regarded as the gold standard assay in the scientific literature (9-11).
The linear regression analysis (Figure 2) of the data yielded the following statistics:

Bühlmann ELISA = 0.75 x comm. RIA + 1.70 ng/ml
r = 0.964 (n = 42)

APPENDIX I
TABLES

Table 3: **Example of Results**

	Conc. (ng/ml)	Absorbance (OD)	B/B_0 (%)	Calc. Conc. (ng/ml)	CV Conc. (%)
Blank		0.127			
Blank		0.119			
Blank Avg.		**0.123**			**4.6**
Std. A	0.0	2.213	100.0		
Std. A	0.0	2.176	100.0		
Std. A Avg.	**0.0**	**2.194**	**100.0**		**1.2**
Std. B	0.8	1.960	88.7	0.8	
Std. B	0.8	1.966	89.0	0.8	
Std. B Avg.	**0.8**	**1.963**	**88.8**	**0.8**	**2.1**
Std. C	2.0	1.699	76.1	2.0	
Std. C	2.0	1.705	76.4	2.0	
Std. C Avg.	**2.0**	**1.702**	**76.2**	**2.0**	**1.0**
Std. D	5.0	1.205	52.2	4.9	
Std. D	5.0	1.192	51.6	5.1	
Std. D Avg.	**5.0**	**1.199**	**51.9**	**5.0**	**1.5**
Std. E	12.5	0.719	28.8	12.2	
Std. E	12.5	0.697	27.7	12.8	
Std. E Avg.	**12.5**	**0.708**	**28.2**	**12.5**	**3.7**
Std. F	40.0	0.365	11.7	40.0	
Std. F	40.0	0.365	11.7	40.0	
Std. F Avg.	**40.0**	**0.365**	**11.7**	**40.0**	**0.0**
Ctrl. LOW		1.485		3.1	
Ctrl. LOW		1.444		3.3	
Ctrl. L. Avg.		**1.464**		**3.2**	**5.1**
Ctrl. HIGH		0.593		17.0	
Ctrl. HIGH		0.579		17.7	
Ctrl. H. Avg.		**0.586**		**17.3**	**3.0**
Sample 1		0.808		10.1	
Sample 1		0.794		10.3	
Sam. 1 Avg.		**0.801**		**10.2**	**2.0**
Sample 2		0.392		35.5	
Sample 2		0.411		32.8	
Sam. 2 Avg.		**0.401**		**34.2**	**5.6**

ED_{20} = 20.2 ng/ml ED_{50} = 5.3 ng/ml ED_{80} = 1.6 ng/ml

Figure 1: **Example of a Standard Curve**

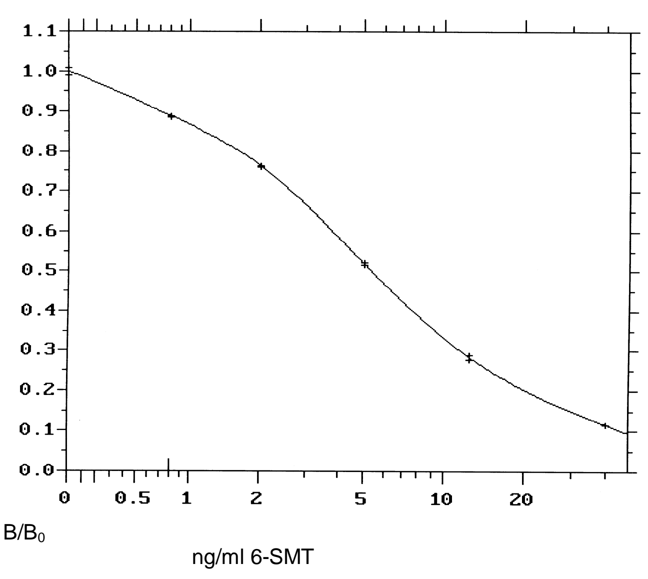


Table 4: **Intra-Assay Precision**

Urine Sample, diluted 1:200	Mean (ng/ml)	S.D. (ng/ml)	C.V. (%)
1	3.09	0.30	9.7
2	11.29	0.70	6.2
3	34.65	1.82	5.3
Mean			7.1

Table 5: **Inter-Assay Precision**

Urine Sample diluted 1:200	Mean (ng/ml)	S.D. (ng/ml)	C.V. (%)
5	1.58	0.28	17.4
6	1.54	0.24	15.3
7	2.03	0.27	13.2
8	3.16	0.30	9.6
9	10.62	0.79	7.5
10	32.10	2.71	8.4
Mean			11.9

Table 6: **Dilution Linearity/Parallelism**

Sample	Basic Value (ng/ml)	Dilution Factor	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
11	29.7	1:200	29.7	---	----
		1:400	15.2	14.8	102
		1:800	7.3	7.4	98
		1:1600	3.7	3.7	100
		1:3200	2.0	1.9	110
12	26.7	1:50	26.7	--	--
		1:100	13.3	13.4	100
		1:200	6.4	6.68	96
		1:400	3.2	3.34	96
		1:800	1.5	1.67	90
		1:1600	0.77	0.83	92
13	16.9	1:12.5	16.9	--	--
		1:25	7.5	8.50	89
		1:50	3.8	4.23	89
		1:100	2.1	2.12	97
		1:200	1.2	1.06	112
Mean					97.8

Table 7: **Spiking Recovery**

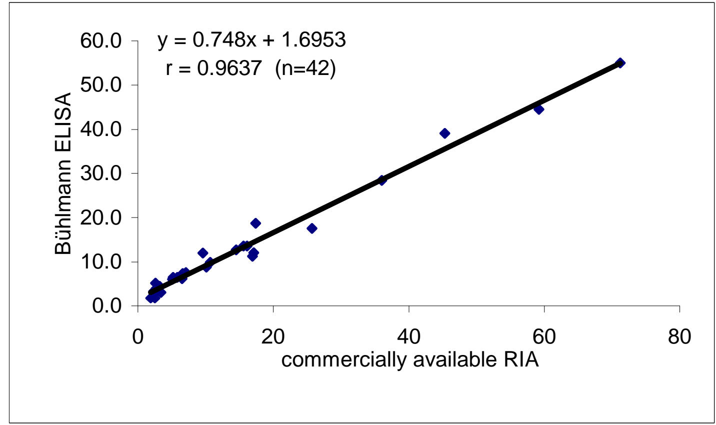
Sample	Basic Value (ng/ml)	Spiked with (ng/ml)	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
14	0.88	0.5	1.38	1.43	104
		1.0	1.88	1.89	100
		2.0	2.88	3.53	123
		4.0	4.88	3.76	77
		8.0	8.88	8.58	97
		16.0	16.88	17.32	103
		32.0	32.88	28.05	85
15	6.3	0.5	6.8	6.20	91
		1.0	7.3	6.87	94
		2.0	8.3	7.90	95
		4.0	10.3	10.25	100
		8.0	14.3	17.78	124
		16.0	22.3	28.23	127
		32.0	38.3	38.24	100
16	4.6	0.5	5.1	4.01	79
		1.0	5.6	5.15	92
		2.0	6.6	7.58	115
		4.0	6.6	10.44	122
		8.0	12.6	14.82	118
		16.0	20.6	25.75	125
		32.0	36.6	35.76	98
Mean					119

Table 8: **Specificity**

6-Sulfatoxymelatonin………………….	100 %
N-Acetyl-Serotonin Sulfate:……………	0.01 %
Melatonin:………………………………	0.007 %
6-Hydroxymelatonin:……………………	0.001 %
Related compounds as follows:……….	< 0.001 %

5-Sulfatoxy-N-Acetylserotonin, 5-Glucuronide-N-Acetylserotonin, N-Acetylserotonin, 6-Glucuronidemelatonin, 5-Methoxyindole Acetic Acid, Tryptophan, N-Acetyltryptophan, 5-Methoxytryptophan, 5-Hydroxytryptophol, N-Acetyltryptamine, N-Methyltryptamine, 5-Hydroxytryptamine, 5-Methoxytryptamine.

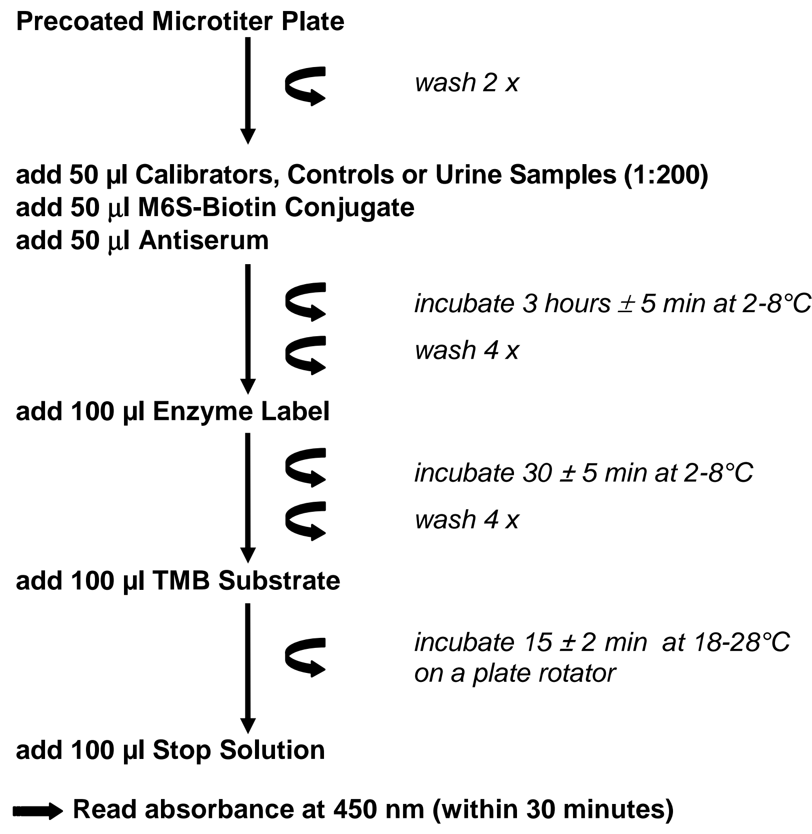
Figure 2: **Method Comparison**



APPENDIX II
REFERENCES

1. Markey, SP*, et al. The correlation between human plasma melatonin levels and urinary 6-hydroxymelatonin excretion*. Clin Chim Acta **150**, 221-5. (1985).
2. Bojkowski, CJ*, et al. Melatonin secretion in humans assessed by measuring its metabolite, 6-sulfatoxymelatonin*. Clin Chem **33**, 1343-8. (1987).
3. Brown, GM*, et al. Urinary 6-sulphatoxymelatonin, an index of pineal function in the rat*. J Pineal Res **10**, 141-7. (1991).
4. Klante, G*, et al. Creatinine is an appropriate reference for urinary sulphatoxymelatonin of laboratory animals and humans*. J Pineal Res **23**, 191-7. (1997).
5. Graham, C*, et al. Prediction of nocturnal plasma melatonin from morning urinary measures.* J Pineal Res **24**, 230-8. (1998).
6. Kripke, DF*, et al. Melatonin: marvel or marker?* Ann Med **30**, 81-7. (1998).
7. Garfinkel, D*, et al. Improvement of sleep quality in elderly people by controlled-release melatonin*. Lancet **346**, 541-4. (1995).
8. Peniston-Bird, JF*, et al. An enzyme immunoassay for 6-sulphatoxy-melatonin in human urine*. J Pineal Res **20**, 51-6. (1996).
9. Arendt, J*, et al. Immunoassay of 6-hydroxymelatonin sulfate in human plasma and urine: abolition of the urinary 24-hour rhythm with atenolol.* J Clin Endocrinol Metab **60**, 1166-73. (1985).
10. Aldhous, ME and Arendt, J. *Radioimmunoassay for 6-sulphatoxymelatonin in urine using an iodinated tracer.* Ann Clin Biochem **25**, 298-303. (1988).
11. Harthe, C*, et al. Direct radioimmunoassay of 6-sulfatoxymelatonin in plasma with use of an iodinated tracer.* Clin Chem **37**, 536-9. (1991).

6-SULFATOXYMELATONIN ELISA



TIME TO RESULT: 4 HOURS

Symbol	Explanation
(hourglass)	Use By
REF	Catalogue number
LOT	Batch code
Σ	Content sufficient for <n> tests
(i)	Consult Instructions for Use
(thermometer)	Temperature limitation
MP	Microtiterplate
BUF WASH 10X	Wash Bufer Concentrate (10x)

Symbol	Explanation
BUF INC	Incubation Buffer
CAL A - CAL F	Calibrator A -F
CONTROL L	Control Low
CONTROL H	Control High
Ab	Antiserum
BC	Biotin Conjugate
EL	Enzyme Label
SUBS TMB	TMB Substrate
SOLN STOP	Stop Solution

Printing Date
2016-01-18