ELISA PRODUCT INFORMATION & MANUAL

Melatonin ELISA Kit (Colorimetric)

NBP2-62160

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

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Please read entire booklet before proceeding with the assay.

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INTRODUCTION

The Melatonin ELISA Kit (Colorimetric) is a complete kit for the determination of melatonin in human, mouse, rat and other sample types. The detection is independent of species, with the use of a simple, rapid extraction protocol. Please read the complete kit insert before performing this assay.

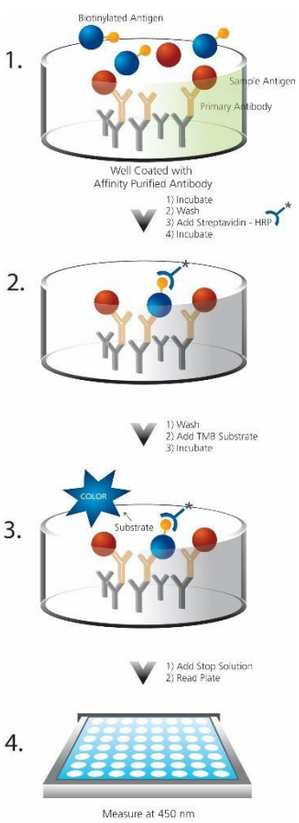
This assay allows for the easy and precise measurement of melatonin in multiple matrices with a rapid time-to-answer. Using this kit, changes in Melatonin levels can be measured in two hours, with a high level of reproducibility, specificity, and accuracy. The levels measured align with published, and the sensitivity of the assay is such that serum and plasma levels can be measured with extraction.[1]. This assay is independent of species, and results have been obtained from animal and fruit matrices. Melatonin (N-acetyl-5-methoxytryptamine) is produced in the pineal gland and is a major component of the regulation of the circadian rhythm. In typical individuals, it is present at higher concentrations during the night, and contributes to the normal sleep-wake cycle[2].

Melatonin is produced from tryptophan through a number hydroxylation and methylation steps. It is metabolized in the liver, and excreted in urine as 6-hyrdoxymelatonin and 6-sulfatoxymelatonin[5]. The Melatonin ELISA Kit (Colorimetric) shows no cross reactivity with any of the major melatonin metabolites or precursors.

Melatonin and metabolites of melatonin, have demonstrated antioxidant and free radical scavenging potential. There is also evidence that they play a role in the regulation of reactive oxygen protein pathways. Published research shows a possible correlation to melatonin levels and the prevalence of certain cancers.[6]

Saliva can be used to measure melatonin levels. Saliva, compared to serum or plasma, is relatively easy to obtain and does not contain many of the interfering substances present in serum and plasma. Melatonin in saliva is representative of the levels circulating throughout the body, although the exact proportion is still not fully quantified with published values ranging from 20 – 50 % of serum values[4]. New areas of research have grown around studying melatonin and the effect on libido, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and other hormone pathways[6]. The role in cancer, sleep cycle, aging, and other areas are also growing in interest, with particular focus on melatonin receptors and the bioavailability of circulating melatonin with the increased use of melatonin as a dietary supplement[3,5]. The effect on individuals that work late night shifts, and people with decreased ability to perceive light, is also of important as normal day and night cues are not present and the regulation of melatonin levels is affected.[2,6]

PRINCIPLE



1. Samples or standards are added to wells coated with a goat anti-mouse IgG antibody. A monoclonal antibody specific to free melatonin and a solution of a biotin labeled melatonin tracer are added to the wells.
2. The plate is incubated. During this incubation, the antibody binds to melatonin in the sample or to the tracer in a competitive manner.
3. The plate is washed, leaving only bound melatonin and bound tracer on the plate. A solution of Horseradish Peroxidase conjugated Streptavidin (SA-HRP) is added, which binds to the biotinylated tracer. The plate is then incubated.
4. Once the incubation is complete, excess SA-HRP is washed out and TMB substrate solution is added and incubated. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is inversely proportional to the level of melatonin in the sample.

MATERIALS SUPPLIED

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

1. **Goat anti-Mouse IgG Microtiter Plate, One Plate of 96 Wells**
   A plate using break-apart strips coated with a goat anti-mouse IgG antibody.
2. **Assay Buffer 10, 50ml**
   Tris buffered saline containing detergents.
3. **Melatonin Standard, 5,000ng**
   One vial containing 5,000 ng of lyophilized melatonin standard.
4. **Melatonin Tracer (100X), 60µl**
   100X concentrated solution of biotin labeled Melatonin tracer.
5. **Melatonin Antibody, Lyophilized**
   Lyophilized melatonin monoclonal antibody.
6. **Stabilizer (50X), 1ml**
   50X concentrated stabilizer for the addition to standards and samples in Assay Buffer 10 dilutions.
7. **Melatonin Conjugate, 20ml**
   A solution of Streptavidin-conjugated Horseradish Peroxidase.
8. **Wash Buffer Concentrate (20X), 25ml**
   One bottle containing 20X Tris buffered saline containing detergent.
9. **TMB Substrate, 25ml**
   A solution of 3,3’5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide.
10. **Stop Solution 2, 10ml**
    A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic**
11. **Clear Plate Sealer, 3 each**
12. **Melatonin ELISA Assay Layout, 1 each**

Protect from light

Stop solution is caustic. Keep tightly capped.

Reagents require separate storage conditions.

Avoid repeated freeze/ thaw cycles.

STORAGE

All of the components of this kit (except antibody and standard) are stable at 4°C until the kit's expiration date. The melatonin standard and antibody must be stored at -20ºC until the kits expiration date. Freeze any unused portions of reconstituted standard and 10X antibody at -20 º C. Avoid repeated freeze thaw cycles.

OTHER MATERIALS NEEDED

1. Deionized or distilled water
2. Precision pipets for volumes between 5µl and 1,000µl
3. Repeater pipet for dispensing volumes between 50µl and 200µl
4. Disposable containers for diluting concentrates, tracer, and antibody solutions.
5. Graduated cylinders
6. A microplate shaker
7. Adsorbent paper for blotting
8. Microplate reader capable of reading at 450 nm
9. Glass tubes for preparing standards and small dilutions.
10. Ethyl Acetate for solution phase extraction of samples.
11. SpeedVac™ (vacuum centrifuge with vapor trap) for drying extracted samples. Alternatively, drying under a stream of inert gas is acceptable.

SAMPLE HANDLING

Samples should be stored at or below -20°C to avoid loss of bioactive analyte. Avoid repeated freeze/ thaw cycles.

This assay is suitable for measuring melatonin in a number of matrices. Human, mouse and rat, serum and plasma and porcine serum have been validated for use, but other species may also work. Human saliva, and fruit (banana and plum) have also been validated in this assay. Other matrices, not presented in this manual, should be validated by the researcher using the extraction and assay protocol supplied.

Please refer to the section titled “Sample Matrix Properties” for validation data and minimum required dilution range for matrices confirmed for use with this kit. However, due to variation in samples, further dilution *may* be required. Users must determine the optimal dilution(s) for their samples and experiments.

The protocol for testing fruit is similar to other samples, but some alterations to the protocol may be required to produce the best extract. Because of the great range in consistency in plant materials, the researcher should use great care in producing the cleanest possible sample for assay. To do this, freezing, freeze drying, sonication, or homogenization in the presence of the 1X Stabilizer may be necessary.

Saliva samples can range in viscosity. It is suggested that the investigator take great care in collection and storage of saliva samples. A general protocol for the clean capture of saliva suitable for assay is provided.

SERUM / PLASMA EXTRACTION

Many sample types will contain materials that will interfere with accurate determination of melatonin levels. It is recommended that all samples be extracted using the protocol provided. Extraction also provides a way to concentrate matrices with low levels of melatonin.

The solution phase extraction protocol provided uses Ethyl Acetate, followed by reconstitution in 1X Stabilizer. Using this procedure you can concentrate up to 750µl of sample up to 7- fold. The level of enrichment is dependent on the sample and the requirements of the researcher.

<u>Solution Phase Extraction of Melatonin</u>

1. Add sample (i.e. Serum, plasma, saliva) to tube.
    a. Make sure all precipitates and solids have been removed by centrifugation prior to addition. For serum and plasma, a 1000g spin for 5 – 10 minutes at 4°C is sufficient.

Extracted samples are most stable after drying.

Once dried, the pellets can be stored at -20°C for a month.

b. Add an equal volume of cold Ethyl Acetate (typical extraction is between 500 – 750µl sample and 500 – 750µl Ethyl Acetate).

2. Vortex gently. Allow layers to separate on ice, in a covered ice bucket for three minutes. Vortex once more and incubate on ice for two minutes.

3. Spin at 1000g for 10 minutes at 4°C.

4. Carefully remove the organic layer to a new tube.

   For Ethyl Acetate this will be the top layer, with a fairly solid interface, with the aqueous portion on the bottom. It is better to remove less with higher purity than to contaminate the sample with aqueous or interface material. Remove as much as you can without contamination, usually 95% of the original volume.

5. Use a SpeedVac™, with condenser, apply no heat, and dry the sample completely. The dried extract will be a clear to off-white pellet. Some color may be present in certain samples (some fruits, or serums). If no SpeedVac™ is available, drying under a stream of inert gas is acceptable.

6. Suspend the pellet in 100 - 250µl of 1X Stabilizer

   As an example, with a starting volume of 750µl (with 700µl removed for purity margin), and a suspension volume of 100µl, so sample has been concentrated 7-fold. Be sure to include any concentration or dilution factor in your final sample calculations. At a minimum, reconstitution volume will need to be enough to pipette required sample volume into each well for the assay. Final reconstitution volume will need to be optimized for each sample population for the degree of concentration or dilution of sample may vary.

Protect from light

7. If samples are to be assayed immediately, keep on ice after suspension and perform assay. Dried samples can be stored at -20 degrees for a month. Once suspended, assay the sample as soon as possible, if needed, freeze the suspended sample at -20 degrees, and assay within one week.

NOTE: Sample loss may be possible during extraction. However, most samples validated for this assay yielded a near 100% recovery as demonstrated in the sample recovery table. If extraction efficiency is a concern and is to be accounted for then sample recovery post-extraction can be included in sample handling optimization by the end user by comparing spike vs. non-spiked samples and determining the rate of recovery. Extraction efficiency can be determined in a number of ways, but an example procedure is provided in Appendix II of this insert.

SALIVA COLLECTION & EXTRACTION

Contamination of Saliva samples with food, blood, or other materials will greatly affect the returned results.

The following is a recommended protocol for collecting and processing saliva samples for use in the assay.

Note: Do not eat or drink anything with dyes or color 30 minutes prior to collection as this may affect results. Collect under dimmest possible lighting, as light can have a marked effect on melatonin levels.

Saliva Collection Protocol

1. Rinse mouth 3 times with water.
2. Place collection tube to mouth and let saliva pool in the mouth. Salivary production can be stimulated by chewing on Parafilm™ or dental wax.
3. Let the pooled saliva flow into the tube by gravity. Do not forcibly propel saliva into tube as this increases viscosity and additional mouth proteins that can interfere with the assay.
4. When at least 0.5 – 1 ml has been collected, cap the tube and protect from light. Keep the solution frozen or on ice until time to assay.
5. If samples are to be assayed within a few days, short term storage at 4°C in glass tubes (protected from light) should be acceptable. Longer term storage should be by freezing at -20˚C in glass tubes.
6. If samples are overly viscous, vortex, and spin at 1000g for 3 -5 minutes. If sample is still to viscous, recollection may be required.
7. Once the saliva is frozen and thawed, assay as soon as possible. Saliva contains melatonin at a level that is approximately 20 - 50% of serum levels. Saliva can be collected easily, and simplifies measurements at multiple time points.

8. Some saliva samples (base levels, daytime levels or typical low phase), will have too little melatonin to be measured. For these samples it is necessary to extract and concentrate to a higher degree than samples collected during the night.
9. For consistency of results, it is suggested that the researcher extracts all saliva samples with Ethyl Acetate prior to assay. This will allow for more accurate control, and longer storage of the sample.

Parafilm is a trademark of Bemis Company, Inc.

See Appendix I, for sample saliva time course experiment.

FRUIT SAMPLE EXTRACTION & DATA

For assaying fruit, the preparation of a well-extracted homogenate is required. The protocol below works well for softer fruits (bananas, plums, cherries, etc.). Harder fruits, or more fibrous plant materials, may require sonication, liquid nitrogen freezing or freeze drying and suspension to create a proper sample for ELISA.

1. Weigh out 500mg of fruit, or an amount that occupies approximately 500µl of space.
2. Add 125µl of 1X Stabilizer, and use a mini pestle to homogenize to a smooth consistency.
3. Add an equal volume (approximately 750µl) of cold Ethyl Acetate and vortex. Use mini pestle if needed to homogenize further.
4. Set sample on ice for 3 minutes. Vortex again, and incubate on ice an additional 2 minutes, then spin at 1000g for 10 minutes.
5. Remove the organic layer to a fresh tube and dry to completeness.
6. Store dry pellet at -20 degrees until needed, or proceed directly to assay.
7. To assay, suspend pellet in 125 - 250µl of 1X Stabilizer, and assay at least two dilutions, in duplicate, to make sure that your sample values are in the range of the assay.

Using this protocol, bananas and red plums were assayed. The results are presented in the table below along with published values acquired using RIA, HPLC and GC/MS methods.

Data from Fruit Tested		
Sample	**ng / gram**	**Published Results***
Banana	0.6 ng / gram	0.5 ng / gram
Red Plum	16 ng / gram	5 – 20 ng / gram

* Results for typical values for fruit were obtained from data published in Hattori, et al. 1995, Dubbels et al, 1995 and Burkhardt et al., 2001.

SAMPLE MATRIX PROPERTIES

If reagents or procedures other than those provided are used for this assay, the end-user must validate all non-provided materials and protocols.

Linearity

Extracted serum and EDTA plasma samples, saliva, and fruit were serially diluted 1:2 in 1X Stabilizer. Samples were run in the assay and compared to the standard curve.

Dilutional Linearity, %					
Dilution	**Extracted Human Serum**	**Extracted Human Plasma**	**Extracted Human Saliva**	**Extracted Banana**	**Extracted Plum**
Neat	100	100	100	100	100
1:2	107	97	118	97	90
1:4	100	107	130	114	96
1:8	91	100	---	---	---

Dilutional Linearity, %					
Dilution	**Extracted Mouse Serum**	**Extracted Mouse Plasma**	**Extracted Rat Serum**	**Extracted Rat Plasma**	**Extracted Porcine Serum**
Neat	100	100	100	100	100
1:2	104	136	101	97	86
1:4	98	159	100	103	116
1:8	---	136	102	107	98

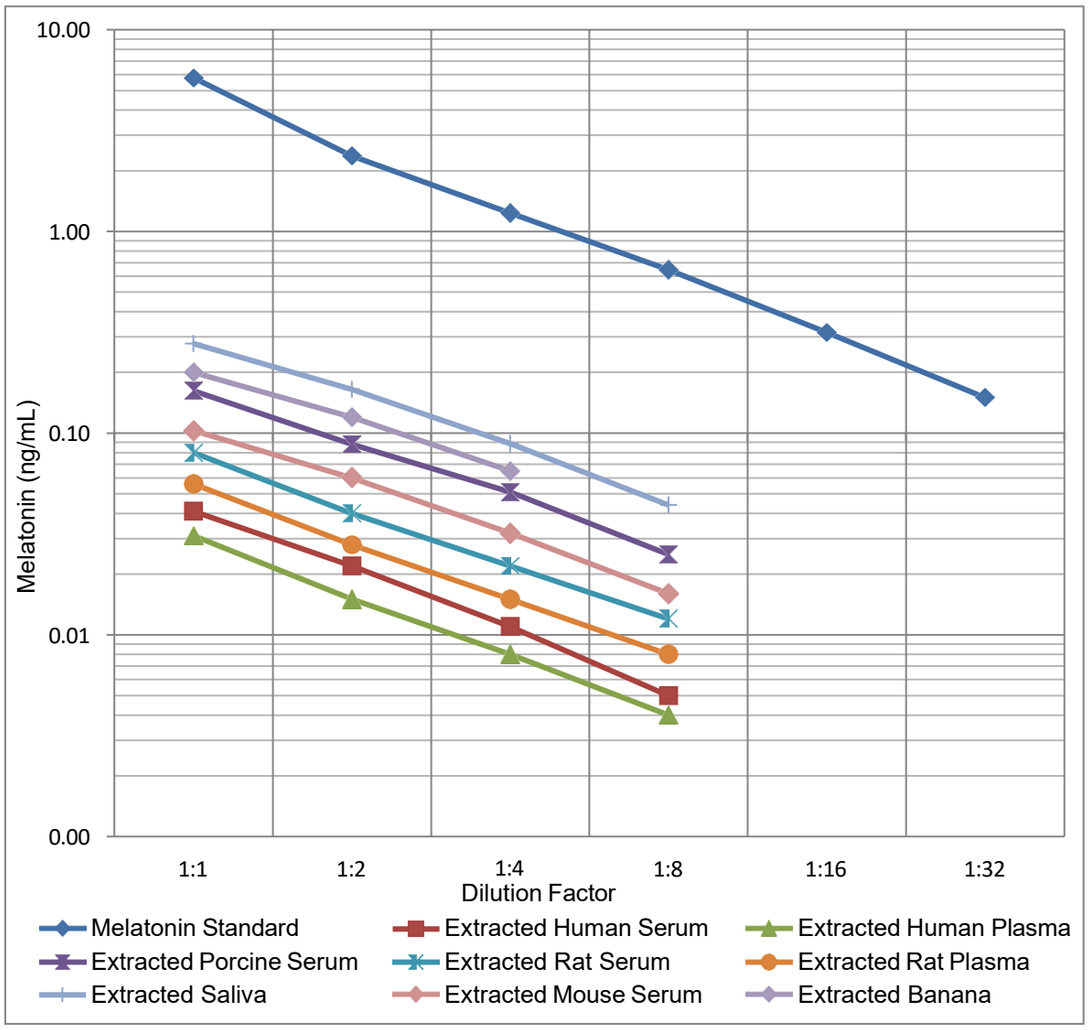
Spike and Recovery

Purified melatonin was spiked at three concentrations into the minimum recommended dilution of human, rat, mouse, porcine and fruit matrices. Matrix background was subtracted from the spiked values and the average percent recovery for each matrix at the minimum required dilution is presented below. These results show the tested matrices at the minimum recommended dilution have no obvious interference with the Melatonin ELISA assay.

Sample	Spike Concentration, ng/mL	% Recovery	Minimum Recommended Dilution
Human Serum Extracted	2	102	Neat
	1	83	
	0.5	124	
Human EDTA Plasma Extracted	2	101	Neat
	1	90	
	0.5	112.2	
Rat Serum Extracted	1	89.7	Neat
	0.5	99.6	
	0.25	112.4	
Mouse Serum Extracted	1	90.9	1:2
	0.5	105.8	
	0.25	115.6	
Porcine Serum Extracted	3	102.8	1:2
	0.6	107.8	
	0.12	112.5	
Human Saliva Extracted	5	83.3	1:2
	2.5	98.7	
	1.25	108.4	
Banana Extracted	5	100.9	1:2
	2.5	115.7	
	1.25	87.6	
Plum Extracted	5	100.1	1:2
	2.5	108.0	
	1.25	96.4	

Parallelism

To assess parallelism, human serum and EDTA plasma samples, human saliva, porcine along with other matrix samples were serially diluted in 1X Stabilizer. The samples were then run in the assay. The melatonin concentration in each sample was determined from the standard curve. Concentrations were plotted as a function of sample dilution. Parallelism of the curves demonstrates that the standard binding characteristics are similar enough to allow the accurate determination of native melatonin levels in diluted samples matrices of animal and plant origin.



REAGENT PREPARATION

It is important to use glass tubes for preparation of the standard curve.

NOTE: ALL SAMPLE AND STANDARD DILUTIONS SHOULD BE PREPARED IN GLASS. ANTIBODY AND TRACER DILUTIONS CAN PREPARED IN POLYPROPYLENE.

1. 1X Stabilizer Solution
For use in Sample/Standard Dilutions

Dilute the 50X Stabilizer Solution to 1X in Assay Buffer 10 to create the 1X Stabilizer. This solution should be made fresh for each assay run and is used to reconstitute the lyophilized standard and for preparing the standard curve dilutions. It is also needed for the suspension and dilution of all samples. To prepare 5ml of 1X Stabilizer, dilute 100µl of 50X Stabilizer into 4900 µl of Assay Buffer 10.

2. Wash Buffer

Prepare the Wash Buffer by diluting 25ml of the supplied concentrate with 475ml of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

Protect from light

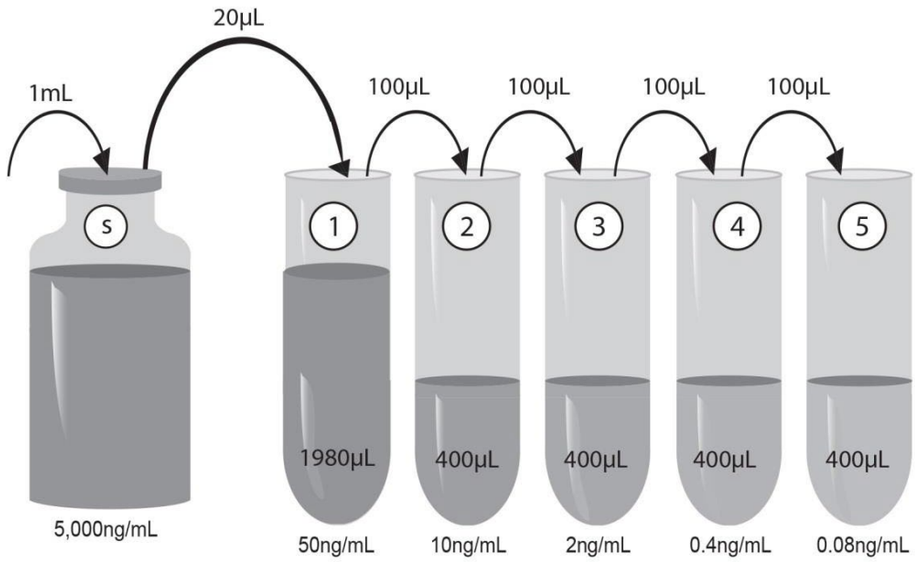
3. Melatonin Standard Curve

Suspend the lyophilized Standard by adding 1ml of 1X Stabilizer to the vial. Vortex, and store on ice until needed. The suspended standard is 5,000 ng/ml of Melatonin. To prepare the standard curve, dilute the 5000 ng/ml stock, 1:100 to create the first point, 50ng/ml, then perform 5-fold serial dilutions for 4 additional points.

Standard curve preparation:

1. Label 5 glass tubes 1-5.
2. To tube 1 add 1980 µL of 1X Stabilizer, to tubes 2 -5 add 400µl of 1X Stabilizer.
3. Transfer 20µl of reconstituted standard (5,000ng/ml) to tube #1, this is your 50 ng/ml standard.
4. Mix tube #1, and with a new tip, transfer 100µl to tube #2. This is 100 µl plus 400µl 1X Stabilizer, or a 1:5 dilution. The final concentration is 10 ng/ml.
5. Mix tube #2, and with a new pipet tip, transfer 100µl, to tube #3. Repeat this for tubes 4 and 5.

A diagram of the dilution series is presented below



Diluted standards should be used within 60 minutes of preparation. Discard any unused standard dilutions.

4. Melatonin Tracer

Dilute the 100X Tracer to 1X in Assay Buffer 10, vortex briefly, and place on ice until needed. Do not reuse diluted tracer solutions, prepare fresh daily. Store the unused 100X concentrate at 4°C.

5. Melatonin Antibody

Reconstitute the lyophilized melatonin antibody in 600µl Assay Buffer 10 to create a 10X solution. Dilute to 1X in Assay Buffer 10 before running the assay. Store the unused reconstituted 10X concentrate at -20°C. It is stable for 3 freeze-thaw cycles.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove unneeded wells and return them, with the desiccant, to the plate bag and seal. Store the unused strips in the original bag at 4°C.

1. Add 100µl of the 1X Stabilizer to the B0 (0 ng/ml standard) wells and 150µl to the NSB wells.
2. Add 100µl of standards #1 through #5 into the appropriate wells.
3. Add 100µl of the samples into the appropriate wells.
4. Add 50µl of the 1X melatonin tracer to all wells except for the blank.

5. Add 50µl of the 1X melatonin antibody to all wells except for the NSB and blank.
6. Seal with plate sealer and incubate at room temperature (RT) on a plate shaker for 1 hour at ~500rpm*. **See note.**
7. Empty the contents of the wells and wash by adding full well volume, ~ 400µl, of wash solution. Repeat the wash 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Add 200µl of the melatonin conjugate solution to each well except the blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500rpm*.
10. Wash as above (Step 7).
11. Add 200µl of TMB substrate solution into each well. Seal the plate and incubate for 30 minutes at RT on a plate shaker at ~500rpm*.
12. Add 50µl of the stop solution into each well.
13. After zeroing the plate reader against the blank, read optical density at 450 nm. If the plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

*** The plate shaker speed was based on a BellCo Mini Orbital Shaker (model no. 7744-08096). The actual speed of the plate shaker should be such that the liquid in the plate wells mixes thoroughly, but does not splash out of the well.**

CALCULATION OF RESULTS

The concentration of melatonin can be calculated as follows:

1. Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average NSB OD

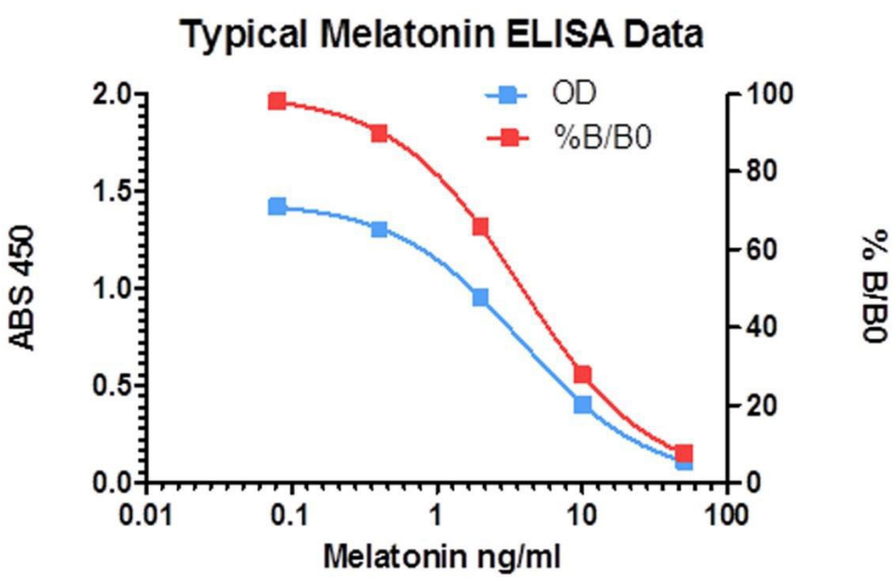
2. Using data analysis software, plot the Average Net OD for each standard versus melatonin concentration in each standard. We recommend that the data be handled by a software package utilizing a 4 parameter logistic (4PL) curve fitting program.

   Samples with concentrations outside of the standard curve range will need to be reanalyzed using alternative dilution.

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results.

Sample	Optical Density (450nm)	Melatonin (ng/ml)	%B/Bo
NSB	0.070	**n/a**	
Bo	1.450	**0**	**100.00**
S1	0.112	**50.0**	**7.70**
S2	0.402	**10.0**	**27.75**
S3	0.956	**2.00**	**65.97**
S4	1.305	**0.40**	**89.99**
S5	1.421	**0.08**	**98.11**



PERFORMANCE CHARACTERISTICS

Specificity

The specificity of the assay was determined by assay of potential cross reactants in the kit at a concentration multiple logs higher than the high standard and then measuring in the assay. The cross reactivity is designated as the amount calculated divided by the amount in the assay.

Analyte	Cross Reactivity
Tryptophan	< 0.01 %
Serotonin	< 0.01 %
N-Acetyl Serotonin	0.05 %
6-Hydroxy Melatonin	< 0.01 %
6-Sulfatoxy Melatonin	< 0.01 %
3-Methoxy Tryptamine	0.03 %
5-Methoxy Tryptamine	< 0.01 %

Sensitivity

The sensitivity or limit of detection of the assay is 162 pg/mL. This was determined by interpolation at 2 standard deviations away from the net OD of a total of 20 zero standard replicates. Data was collected from 12 standard curves.

Intra-assay precision was determined by assaying 20 replicates of two controls containing Melatonin in a single assay.

Intra-assay precision	
ng/mL	**%CV**
5.74	4.31
0.45	13.74

Inter-assay precision was determined by measuring controls of varying Melatonin concentrations in 12 separate assays.

Inter-assay precision	
ng/mL	**%CV**
5.98	7.36
0.58	17.54

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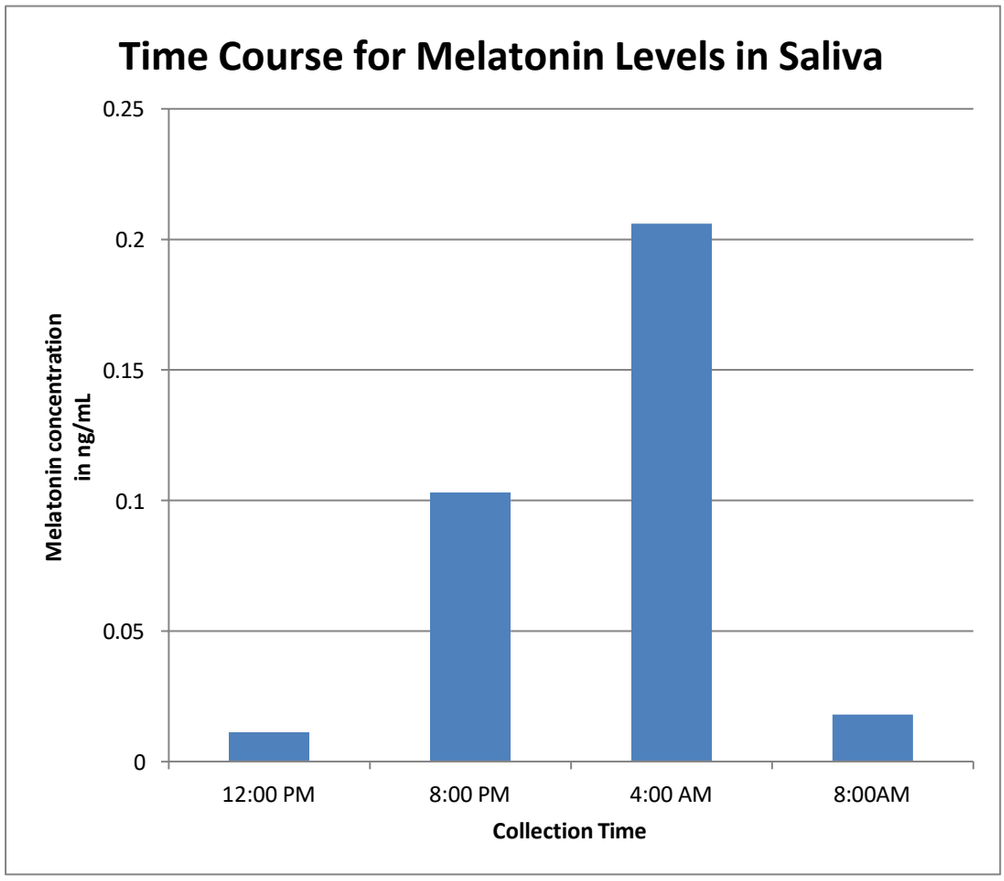
APPENDIX I: SALIVA SAMPLE DATA

The data presented below is from a single individual, collected using the protocol above, and assayed using the Melatonin ELISA Kit (Colorimetric). The resulting data was graphed and fit to a 4-parameter curve. The amounts present at each time point were extrapolated, adjusted for dilution and plotted versus time. The amount of melatonin can vary greatly among individuals, and this is one example that demonstrates how changes over time can be measured using this ELISA. This data should not be interpreted as a typical salivary melatonin level, as many environmental, dietary, and personal factors will affect the level measured.

This experiment shows the normal rise and fall of melatonin levels during the course of the day, and this kit's ability to measure the biological relevant amounts.



APPENDIX II: EXTRACTION EFFICIENCY PROTOCOL

Extraction efficiency is a calculation that is performed in order to determine how well you are able to recover a targeted molecule post-extraction of a biological sample or solution. Extraction efficiency is calculated as the net amount of spiked material that is recovered post-extraction, divided by the amount of spiked material detected in the assay buffer. This number is usually expressed as a percentage. Required for this extraction efficiency determination is a single representative sample which will need to be split into two equal aliquots as well as a non-extracted Assay Buffer Control.

1. Pipet an equal volume of a sample into fresh tubes labeled as “+spike” and “-spike.”

2. Pipet the same volume of Assay Buffer into a fresh tube labeled as “Assay Buffer Control.”

3. Spike the “+ spike” tube and the “Assay Buffer Control” tube with a known concentration of the targeted molecule using the standard provided in the kit. Generally speaking, it is best to pick a concentration that will fall around the middle of the curve unless you plan to account for dilution/concentration of the sample post-extraction in which case a higher/lower concentration should be chosen. Be sure to add the same volume of assay buffer to the “-spike” tube so that it will be the same volume as the “+spike” and the “Assay Buffer Control” tube.

4. Cap and store the “Assay Buffer Control” containing the spiked standard at or below -20°C. Note: This control will not be put through the extraction protocol so will not be needed until the assay is to be run.

5. Extract the “+spike” sample and the “-spike” sample according to the extraction procedure.

6. Dry down completely and reconstitute the samples as directed. Note: If you reconstitute in the starting volume of the sample there will be no need to adjust for a dilution/concentration factor of the original sample. At a minimum, you will need to reconstitute in enough volume to run the assay.

7. Thaw the “Assay Buffer Control” tube. Run the reconstituted samples (+spike and -spike) and “Assay Buffer Control” in the assay with a standard curve diluted in Assay Buffer.

8. Calculate the concentration of the “+spike” sample and the “-spiked” samples as well as the “Assay Buffer Control.” Note: If you dilute or concentrate the sample when reconstituting then the appropriate dilution or concentration factor will need to be taken into account when calculating the target molecule concentration.

9. Calculate the net spiked standard that was detected after the extraction by subtracting the “-spike” extract concentration from the “+spike” extract concentration. Doing this will remove the endogenous level detected in the sample leaving only the recovered spike concentration.

Net Spike Concentration =
(“+spike” concentration) – (“-spike” concentration)

10. The recovered spike concentration is divided by the “Assay Buffer Control” concentration and multiplied by 100 to give the extraction efficiency percentage.

% Extraction Efficiency =
(Net Spike Concentration / Assay Buffer Control Concentration) * 100

Melatonin ELISA Kit (Colorimetric) LAYOUT SHEET
for use with Novus Biologicals Catalog No. NBP2-62160

- **TABLE FOR MAKING STANDARDS 1- 5**

Std.	1X Stabilizer Solution Vol. (µl)	Suspended Standard Vol. Added (µl)	Final Melatonin Conc. (ng/ml)
1	1980	20, stock	50
2	400	100, Std. 1	10
3	400	100, Std. 2	2
4	400	100, Std. 3	0.4
5	400	100, Std. 4	0.08

- **ASSAY PROTOCOL FLOW CHART: Vol. Added (µL)**

Well ID:	Blank A1, B1	NSB C1-D1	Bo E1-F1	Standards G1-H2	Samples A3-H12
1 X Stabilizer Solution	–––	150µl	100µl	–––	–––
Standards or Samples	–––	–––	–––	100µl	100µl
Melatonin Tracer	–––	50µl	50µl	50µl	50µl
Melatonin Antibody	–––	–––	50µl	50µl	50µl
Incub. 1 hour @ RT, shaking & sealed	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Asp. & Wash 3 x Full well vol. (~400µl)	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Conjugate	–––	200µl	200µl	200µl	200µl
Incub. 30 min. @ RT, shaking & sealed	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Asp. & Wash 3 x Full well vol. (~400µl)	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
TMB Substrate	200µl	200µl	200µl	200µl	200µl
Incub. 30 min. @ RT, shaking, sealed	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒
Stop Solution 2 (1N HCl)	50µl	50µl	50µl	50µl	50µl
Read OD at 450 nm	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒	⇒⇒⇒⇒

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MELATONIN PLATE LAYOUT:

A1 Blank	A2 Std 2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1 Blank	B2 Std 2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1 NSB	C2 Std 3	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1 NSB	D2 Std 3	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1 Bo	E2 Std 4	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1 Bo	F2 Std 4	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1 Std 1	G2 Std 5	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1 Std 1	H2 Std 5	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Kit Lot No. _____ Exp. Date _____ Date _____ Tech. _____

1st Incub.: Start Time _____ Temp. _____

End Time _____ Temp. _____

2nd Incub.: Start Time _____ Temp. _____

End Time _____ Temp. _____

3rd Incub.: Start Time _____ Temp. _____

End Time _____ Temp. _____

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