biosensis® Mature BDNF *Rapid*™ ELISA Kit: Human, Mouse, Rat

Catalogue Number: BEK-2211-1P/2P

For the quantitative determination of mature BDNF in serum, plasma (EDTA and citrate), pig serum, cell culture supernatants, cell lysates, brain extracts, human milk and sheep CSF only if used as directed.

Please refer to the Sample Preparation Section for specific use instructions for each substrate application, in particular blood samples, human milk and CSF.

For research use only, not for use in clinical and diagnostic procedures.

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1. Intended Use

The purpose of this kit is the quantitative determination of mature BDNF in serum, plasma (EDTA and citrate), cell culture supernatants, cell lysates, brain extracts and human milk only if used as directed. This kit has also been successfully tested by researchers on sheep CSF and pig serum. This kit has not been tested for other sample applications. Non-validated samples may contain immunoglobulins and other components that potentially interfere with this ELISA assay. Biosensis does not assume responsibility if this kit is used for unintended purposes.

Please see our sample guidelines for information on BDNF measurements in specific substrates, because not all of the samples tested naturally contain detectable levels of mature BDNF. For human blood samples, consistent sample preparation is critical, and we highly recommend to review studies published by Tsuchimine *et al*., 2014* and Gejl *et al.*, 2019* before assaying BDNF content in human blood. For quantification of BDNF in human CSF samples please contact us sales@biosensis.com

For research use only. Not for diagnostic and clinical purposes.

*See section 13 for detailed references

2. Introduction

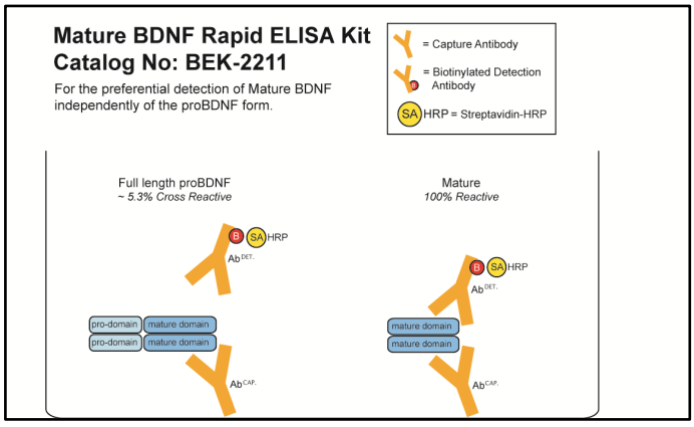
Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family of growth factors that play an important role in a variety of physiological functions, for instance mediating neuronal survival and apoptosis, maintaining synaptic plasticity and regulating synaptic transmission. Altered BDNF levels in the central nervous system and blood are implicated in a variety of neurodegenerative diseases such as amyotrophic lateral sclerosis, neuropathic pain and Alzheimer's Disease.

The Biosensis Mature BDNF *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the preferential quantification of mature BDNF in less than 3 hours. This kit consists of a pre-coated mouse monoclonal anti-mature BDNF capture antibody, a biotinylated anti-mature BDNF detection antibody and horseradish peroxidase (HRP)-conjugated streptavidin. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the concentration of mature BDNF present in samples and protein standards.

This Mature BDNF ELISA kit employs a recombinant human mature BDNF standard approved by the World Health Organization (WHO, www.nibsc.org). The amino acid sequence of BDNF is identical for human, mouse, rat and a number of other species. This kit therefore is suitable to measure mature BDNF in all these species and uses the same antibodies and antigen.

The provided Quality Control (QC) sample serves as a positive control with a defined concentration range of BDNF protein, formulated in a stabilized buffer solution and designed to assure assay performance.

The antibodies used in this ELISA kit bind epitopes within the mature domain of the protein. However, the full-length proBDNF protein is detected much less efficiently in the ELISA showing a cross-reactivity of approximately 5.3% in weight (m/v) concentration (12.1% in molar concentration), whereas 100% of the mature form of BDNF is quantified. At typical human serum dilutions, proBDNF does not interfere with mature BDNF quantification. Please refer to our Technical Note #5 for further information on ELISA assay validation for mature BDNF detection and quantification, and assessment of proBDNF cross-reactivity.



3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
Mature BDNF antibody coated 96 well microplate	12 x 8 wells	24 x 8 wells
Assay diluent A (1x)	2 x 25 mL	4 x 25 mL
Recombinant human mature BDNF standard	2 x 1000 pg	4 x 1000 pg
Quality Control (QC) Sample	2 Vials (see vial label for amount)	4 Vials (see vial label for amount)
Mature BDNF detection antibody (100x)	1 x 110 µL	2 x 110 µL
Streptavidin-HRP (100x)	1 x 110 µL	2 x 110 µL
Wash buffer (10x)	1 x 33 mL	2 x 33 mL
TMB substrate (1x)	1 x 11 mL	2 x 11 mL
TMB stop solution (1x)	1 x 11 mL	2 x 11 mL
Plate sealer	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard and QC sample	Use on same day; aliquot unused protein to prevent multiple freeze-thaw cycles and store at -20°C for 2 weeks
Diluted detection antibody and HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer (1x)	2 weeks at 2-8°C

Note:

- Do not freeze the streptavidin-HRP conjugate
- Do not use kit components from other ELISA kits
- Assaying mature BDNF in human milk requires the use of secretory IgA (sIgA) blocker BL-001-1250 for accurate BDNF quantification; this sample diluent additive can be purchased as an add-on reagent, please refer to www.biosensis.com

4. Equipment Required but Not Supplied

- Single- and multi-channel pipettes capable of delivering 10-1000 µL volumes
- Plastic and glass ware for sample collection, sample preparation and buffer preparation
- Plate shaker
- Microplate reader capable of reading absorbance at 450 nm

5. Before You Start….

- Read the entire protocol to familiarize yourself with the assay procedure; a tutorial about the use of *Rapid*™ ELISA kits is available online at: https://www.youtube.com/watch?v=7EOuc9qYL0E
- Bring the microplate and all reagents and solutions to room temperature before starting the assay
- Remove only the required amount of TMB substrate from the fridge to prevent multiple warm-ups and cool downs; keep the TMB substrate in the dark
- Remove the number of strips required and return unused strips to the pack and reseal
- Centrifuge all kit components to collect reagents to the bottom of tubes before use
- The stop solution provided with this kit is an acid; wear protective equipment when handling
- Please visit our Technical Notes section at www.biosensis.com for further information and helpful hints on ELISA-related topics

Careful experimental planning is required to determine the total number of samples that can be assayed per plate. Refer to Appendix A for help in determining the number of microplates required to conduct an ELISA assay experiment.

6. Sample Preparation

The assay diluent provided in this kit is suitable for measuring mature BDNF in serum, plasma, cell lysates, cell culture supernatants, brain extracts, human milk and sheep CSF. Note that human milk requires the addition of a sIgA blocker (cat# BL-001-1250).

See the following sample preparation guidelines for specific substrate preparation recommendations and sample dilution instructions. For brain extracts, please also see information provided in Appendix B and C. Final working pH of any assay sample should be near neutral (pH 6.8-7.5) for best results, adjust with mild acid or base as needed.

For unknown concentrations of mature BDNF in samples, it is important to perform several dilutions of the sample to allow the BDNF concentration to fall within the range of the BDNF standard curve (7.8-500 pg/mL). The low detection limit of this assay allows to shift the lowest standard from 7.8 pg/mL to 3.9 or 1.95 pg/mL if required. This might be particular useful for samples with expected very low BDNF concentrations. Please refer to Appendix D for step-by-step instructions on standard curve preparation, QC dilution and TMB incubation times for an extended standard curve.

Also, with unknown samples of all types it is highly recommended to run some validation experiments. This should include dilutional linearity/parallelism experiments, and spike- and recovery control tests at least at the minimum required dilution (MRD) for each sample. Parallelism and linearity of diluted samples with the standard curve demonstrates accurate quantification, as well as 80-120% recovery of spiked BDNF. Failure of these essential validation experiments indicates that sample buffer, preparation and dilution and or blocking procedures will need to be optimized by the end user for consistent, accurate results with this ELISA assay. Please refer to our Technical Note #1 (ELISA Assay Validation) for further details.

Cell Culture Supernatants

- Remove particulates by centrifugation (10,000 x g for 5 minutes)
- Analyze immediately or store at -20°C to -80°C in aliquots to prevent multiple freeze thaw cycles
- Samples with high FBS or proteinaceous content will require to be diluted with plain culture medium and/or Assay Diluent A for best results. Also note, bovine mature BDNF is identical in sequence to human, mouse or rat (and many other mammals) and will thus be detected in this assay, thus appropriate serum free, cell-free controls must be used for accurate detection.
- Rodent cell cultures typically produce very low amounts of free BDNF into the surrounding and collectable supernatants so detection levels may be low in standard culture conditions.
- In our experience, in human cultures, either naturally derived for derived from ISP culture, endogenous BDNF is rapidly taken up by neighboring cells and not released into the supernatants, thus detection levels in human cell culture supernatants may be low.

Cell Lysates

Cell lysis and protein extraction can be accomplished by a variety of methods (eg., chemical or mechanical). This ELISA kit has been tested on PC12 cells lysed with a modified RIPA buffer and by freeze-thaw cycles in a hypotonic salt buffer or acid-extraction buffer. The sample preparation procedures given here are guidelines only and they are based upon our in-house testing. Actual user preparations and testing procedures must be optimized for experimental conditions. It is expected that sample lysates will need to be diluted with Assay Diluent A prior to running the assay with RIPA buffers. In our testing a dilution of at least 1:4 is necessary, with higher dilutions performing more consistently than lower dilutions.

RIPA-mediated Cell Lysis

- Harvest whole cells from cell culture flask after washing off cell culture medium with ice-cold PBS
- Resuspend cell pellet in ice-cold RIPA buffer (50 mM Tris, 150 mM sodium chloride, 2 mM EDTA, 1% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4-8.0); do not add reducing agents such as DTT or β-mercaptoethanol
- Lyse the cells on ice for 30 minutes

Cell Lysis by Freeze-Thawing in Hypotonic Salt Buffer

- Harvest whole cells from cell culture flask after washing off cell culture medium with ice-cold PBS
- Resuspend cell pellet in ice-cold, hypotonic 5 mM phosphate cell lysis buffer (pH 6.8-7.5)
- Freeze cells in liquid nitrogen; wear appropriate protective equipment!
- Thaw cells at room temperature or 37°C
- Repeat for a total of 3 freeze-thaw cycles
- Add 3 parts of isotonic PBS solution (eg., 0.75 mL if the cell lysis was done in 0.25 mL buffer)

Cell Lysis by Freeze-Thawing in Acid-Extraction Buffer

- Harvest whole cells from cell culture flask after washing off cell culture medium with ice-cold PBS
- Resuspend cell pellet in ice-cold acid-extraction buffer (50 mmol/L sodium acetate, 1 mol/L NaCl, 0.1% Triton X100, pH 4.0)
- Freeze cells in liquid nitrogen; wear appropriate protective equipment!
- Thaw cells at room temperature or 37°C
- Repeat for a total of 3 freeze-thaw cycles
- Incubate lysate for 15-20 minutes at room temperature
- Add 3 parts of neutralization buffer (0.1 mol/L phosphate buffer; 0.1 mol/L KH_2PO_4 + 0.1 mol/L Na_2HPO_4, pH 7.6)

After cell lysis:

- Centrifuge cells for 15 minutes at 21,000 x g at 2-8°C
- Measure total protein concentration (eg., BCA or Bradford protein assay)
- Aliquot the supernatant into useful aliquots and store at -80°C; prevent multiple freeze-thaw cycles
- Dilute cell lysates with sample diluent to quantify mature BDNF
    - Use **Assay Diluent A** as sample diluent for cell lysates prepared with RIPA buffer (minimum required sample dilution is 1/5) and lysates prepared by freeze-thawing in hypotonic salt buffer. Check that the final sample pH is at least pH 6.8.
    - Use **Acid-Extraction Sample Diluent** (1 part acid-extraction buffer + 3 parts neutralization buffer) as sample diluent for cell lysates prepared by acid-extraction. Check that the final sample pH is pH 6.8-7.2.

General Notes – Cell Lysates

- The addition of a protease inhibitor cocktail to buffers used in protein extraction just prior to cell lysis is highly recommended
- Re-extraction of the pellet may yield higher recoveries of endogenous BDNF as demonstrated by Kolbeck *et al.* (1999) for tissue homogenates
- As an alternative, freeze whole cell pellets and store at -80°C for cell lysis at later time-point
- Please refer to Appendix B for more details about acid-extraction of BDNF

Serum

- Allow the serum to clot in a serum separator tube for 1 hour at room temperature; incubation can be extended to overnight at 2-8°C. Note: Longer clotting times can increase serum levels in certain samples such as human because of leakage from platelets.
- Centrifuge at approximately 1,000 x g for 15 minutes
- Analyze the serum immediately or aliquot and store frozen at -20°C to -80°C
- For human serum samples, a minimum dilution of 1/50 – 1/100 with Assay Diluent A is required.
- For rodent samples dilutions of 1/5 – 1/20 is recommended as rodent samples have markedly lower BDNF levels.
- For pig serum, sample dilutions of 1/2 – 1/10 are required to account for very low levels of BDNF present in pig serum (Rault *et al.*, 2017)

Optiminization of dilutions for these and other species are recommended for best results.

Plasma

- Collect plasma using citrate or EDTA as anticoagulant; internal validation suggests citrate as the preferred anticoagulant, because EDTA-treated plasma samples demonstrated higher interference in some samples at low dilution, and thus require higher sample dilutions; heparin-treated plasma samples have not been tested as yet
- Centrifuge for 15 min at 2-8°C at 1,500 x g within 30 minutes of collection

- For eliminating the platelet effect we suggest further centrifugation for 10 min at 2-8°C at 10,000 x g
- It is recommend to analyse samples immediately or aliquot and store samples at -20°C to -80°C, thawing only once.
- Dilute human citrate plasma samples at least 1/10 to 1/20 with Assay Diluent A in order to measure mature BDNF concentrations. For human EDTA-plasma, a minimum required dilution of 1/20 to 1/40 is recommended. For samples that show high interference, a higher dilution is recommended for better accuracy. Rodent and other species plasma samples will need optimization of the dilution for best results, but dilutions are typically low. This kit is not recommended for determining BDNF in pig plasma (Rault *et al.*, 2017)

Serum and Plasma Additional Notes

- Acid treatment for serum and plasma samples is typically not required
- Avoid freeze-thaw cycles of blood samples
- Unlike human blood, mature BDNF levels in rodent blood are low (rat) to undetectable (mouse) under physiological conditions (Klein, AB *et al.*, 2011; Radka, SF *et al.*, 1996), thus care should be taken when using this kit to measure BDNF in rodent blood samples
- Because of the release of BDNF from platelets, serum and plasma BDNF levels in humans will vary depending on temperature, storage time, anti-coagulant used for collection, and centrifugation strategy. **Thus, to prevent sample variation, strict adherence to consistent sample preparation procedures among samples and study groups are required. We highly recommend to review studies published by Tsuchimine *et al*., 2014 and Gejl *et al.*, 2019.**

Brain Tissue Extracts

Several protocols have been published in literature using their respective sample diluent for BDNF detection from animal and human tissue. This BDNF ELISA kit is compatible with a range of sample diluents. However, based on our own experience we recommend following the protocol developed by Y.-A. Barde and published by Kolbeck *et al.* (1999). This protocol allows measuring total mature BDNF concentrations in acid-treated tissue homogenates as demonstrated by Uys *et al*. (2016). See **Appendix B** and **Appendix C** for a detailed sample preparation protocols for acid extracted and RIPA based sample preparation guidelines.

Human Milk

Human milk contains natural high levels of sIgA protein, reported to be 1 mg/mL or even higher in some samples (Weaver *et al.*, 1998). Secretory IgA can interfere with sandwich ELISA assays, falsly elevating sample OD readings and thus causing false-positive results.

Biosensis has developed a proprietrary sIgA blocker (BL-001-1250) which has been tested to reduce or eliminate sIgA cross-reactivity in this ELISA assay, thus ensuring accurate BDNF quantification. Supplementing Assay Diluent A with this HA blocker is therefore highly recommended.

BL-001-1250 is available for purchase separately. One vial of BL-001-1250 is sufficient to prepare 25 mL of human milk sample diluent for one 96-well ELISA plate. This sample diluent is only used to dilute milk samples and BDNF protein standard. Do not use Assay Diluent A enriched with BL-001-1250 to dilute the detection antibody or streptavidin-HRP conjugate.

Preparation of Sample Diluent for Milk Samples

- Spin one vial of BL-001-1250 briefly and reconstitute with 1 mL of Assay Diluent A; mix by vortex and let stand at room temperature for 15 minutes
- Add 1 mL of reconstituted HA blocker to 24 mL Assay Diluent A; use this buffer as sample diluent for human milk samples only, and to prepare the BDNF calibration curve in parallel

Human Milk Preparation

- Collect whole milk and centrifuge for 15 minutes at 10,000 x g and 2-8°C
- Carefully remove the top lipid layer and discard
- Collect the aqueous part of the human milk and add to a clean tube and re-centrifuge as above; repeat centrifuge step for a total of 3 times
- Analyze milk immediately or aliquot and store samples at -20°C to -80°C; avoid repeated freeze-thaw cycles. Note: If frozen aliquots are > 6 months old, on thawing re-centrifuge samples for 15 minutes at 10,000 x g and

2-8°C. Carefully remove the top lipid layer and discard and collect the aqueous part of the milk for testing
- Dilute human milk at least 1:4 – 1:8 with human milk sample diluent, containing sIgA blocker BL-001-1250

Important: Expected endogeneous levels of BDNF in human milk are low and may be lower than the Limit of Detection (LOD) for this ELISA assay. Human milk was validated for this ELISA assay by using pooled human milk.

Cerebrospinal Fluid (CSF)

Researchers have used this kit successfully on sheep CSF, with a minimum required sample dilution of 1:2 – 1:4.

Limited testing has been done on human CSF. While spike- and recovery and dilutional linearity testing with spiked recombinant BDNF on normal, pooled human CSF demonstrated accurate readings with a minimum required sample dilution of 1:2 – 1:4, endogeneous BDNF levels were below the detection limit of this ELISA assay. Using this ELISA assay on a larger sample cohort will require further validation for optimal performance.

Note: High-quality CSF samples are required for best results. Contamination of CSF fluid with blood will cause inaccurate results due to interference of blood factors.

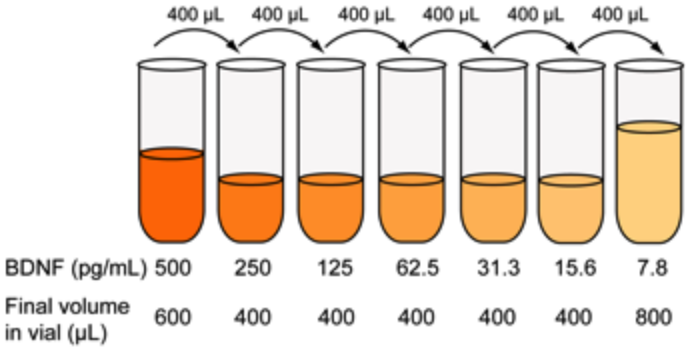
7. Preparation of Mature BDNF Standard

The instructions given in this section relate to the normal assay range of 7.8 – 500 pg/mL, with 500 pg/mL being the highest concentration of the mature BDNF standard curve.

- Reconstitute the lyophilized antigen standard with 1 mL of the **same diluent used for preparing sample dilutions**
- Label the vial with the reconstituted mature BDNF standard as “1000 pg/mL”; vortex and let stand for 15 minutes
- Dilute the 1000 pg/mL mature BDNF standard 1:2 (eg., 500 µL of 1000 pg/mL standard + 500 µL diluent); label this tube “500 pg/mL”

In order to generate a BDNF standard curve, perform a 1:2 serial dilution down to 7.8 pg/mL. The volumes used for the dilution series depends on the number of repeats per BDNF concentration. For triplicate measurement (100 µL per well) of each BDNF standard concentration, we recommend this procedure:

1. Label 6 tubes with “250 pg/mL”, “125 pg/mL”, “62.5 pg/mL”, “31.3 pg/mL”, “15.6 pg/mL” and “7.8 pg/mL”, respectively
2. Aliquot 400 µL of the sample diluent into each tube
3. Take 400 µL from the “500 pg/mL” tube and transfer to the “250 pg/mL” tube
4. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex
5. Repeat steps 3 and 4 for each consecutive concentration until the last tube “7.8 pg/mL” is prepared and mixed well



8. Other Reagents and Buffer Preparation

- Quality Control (QC) sample: Reconstitute the lyophilized sample with 1 mL of the **same diluent used for preparing the Mature BDNF Standard curve**. This will provide a QC sample within 175 – 325 pg/mL.
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent A; **do not use** culture medium, Assay Diluent A enriched with blocker or other buffers and prepare enough volume to add 100 µL per well
- Wash buffer (10x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly and dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

9. Assay Procedure

All steps are performed at room temperature (20-25°C, 70-75°F).

1. Add 100 µL of diluted mature BDNF standards, QC sample, samples and blank (sample diluent only) to the pre-coated microplate wells
2. If available, include sample-specific negative and positive control sample in the assay procedure
3. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 45 minutes
4. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 µL per well). See the technical hints section for a detailed description of the washing procedure
5. Add 100 µL of the detection antibody (1x) into each well
6. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
7. Discard the solution inside the wells and wash as described in step 4
8. Add 100 µL of the 1x streptavidin-HRP conjugate into each well
9. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
10. Discard the solution inside the wells and wash as described in step 4
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 4-8 minutes without shaking in the dark.
12. Stop the reaction by adding 100 µL of the stop solution into each well. Visible blue color will change to yellow. Read the absorbance at 450 nm on a plate reader. **Note:** Color will fade over time; hence, we recommend plate to be read within 5 minutes after adding the stop solution or no longer than 30 minutes after addition
13. See Appendix D for a troubleshooting guide when unexpected difficulties are encountered

* RCF= 1.12 x Orbit Radius x $(rpm/1000)^2$

10. Technical Hints

1. Do not perform dilutions within the well
2. At least duplicate measurements for each standard and sample dilution is recommended
3. Dilute samples to a BDNF concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
4. Avoid touching the inside surface of the wells with the pipette tip
5. Proper emptying and washing the plate is crucial for low background and non-specific binding. For manual plate washing, we recommend the following procedure:
    a. To remove liquid from the wells, place the plate on the palm of the hand and quickly invert the plate over the sink. Forcefully move the arm downwards and stop abruptly to force the liquid out of the wells. When done correctly, this technique should prevent liquid from getting onto the fingers or onto the outside of the microplate wells
    b. Blot and forcefully tap the microplate against clean paper towels for 3-5 times
    c. Wash the wells by pipetting 200 µL of wash buffer into each well and empty the wells as described in step a-b)
    d. Repeat this procedure for a total of 5 times
6. Complete removal of liquid from the wells at each step is essential for reliable results. However, avoid letting the wells dry out at any time
7. Add TMB and the stop solution to the wells in the same order
8. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading
9. Stopping the TMB reaction after 5 minutes is usually sufficient to obtain a very sensitive standard curve. For incubation times with an extended standard curve refer to Appendix D. However, TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm (0.351 G) and perform all incubations at room temperature (20-25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of

4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.

10. TMB incubation times can vary, and assay sensitivity can be affected if color development is stopped too early. In order to determine the optimal time to stop the TMB incubation, blue color development should be monitored at **650 nm**. Addition of Stop Solution will convert blue into yellow color, with an ~2.5-3X increase in OD at **450 nm**. Once the highest standard reaches an OD of ~1.2 – 1.3 at 650 nm, immediately stop the reaction by adding Stop Solution. Note that before taking a 650 nm measurement, the plate should be briefly and gently shaken by hand to homogenise the blue TMB reaction product within the wells for most accurate readings.

11. Calculation of Results

1. Average the readings for each BDNF standard concentration, blank and sample
2. Plot a standard curve with the BDNF standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the BDNF standards are adjusted for background absorbance, then subtract the blank value from the OD_{450} of the samples as well
4. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression analysis
5. Perform a 4-PL regression analysis to calculate the concentration of mature BDNF in the QC sample. An observed concentration within the range of 175 – 325 pg/mL indicates acceptable assay performance
6. Perform a 4-PL regression regression analysis to calculate the concentration of mature BDNF in the samples. Multiply the result by the sample dilution factor

Manual Plate Reading

The relative OD_{450} = (the OD_{450} of each well) – (the OD_{450} of Zero well and/or Blank well).

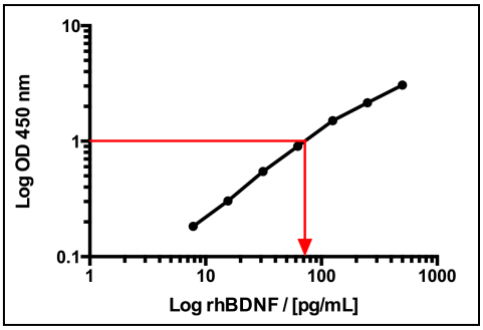
1. The **standard curve** can be plotted as the relative OD_{450} of each BDNF standard solution (Y-axis) vs. the respective known concentration of the mature BDNF standard solution (X-axis). The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding OD_{450}. The greater the concentration of target protein in the solution, the higher the OD_{450}.
2. **Determine concentration of target protein in unknown sample:** The Mature BDNF protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of BDNF in the unknown sample. In the exemplary standard curve below, a sample with OD_{450} = 1 reads as 80 pg/mL mature BDNF (red line). If the samples measured were diluted, multiply the concentrations from the interpolation with the dilution factor to obtain the actual BDNF concentration in the sample.

12. Typical Data

Standard Curve

Standard curves are provided for demonstration only. A standard curve has to be generated for each mature BDNF ELISA assay.

In addition, we recommend measuring common samples when performing multiple assays across several days in order to normalize the standard curve numbers between the various runs.



In the example graph on the left, BDNF standards were run in triplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 5 minutes.

Typical optical densities and coefficient of variations for diluted BDNF standards are summarized in the following table:

	OD 450 nm			
BDNF / [pg/mL]	Mean	SD	SEM	CV
500	3.068	0.052	0.030	1.7%
250	2.147	0.019	0.011	0.9%
125	1.507	0.013	0.007	0.9%
62.5	0.902	0.024	0.014	2.7%
31.3	0.546	0.010	0.006	1.9%
15.6	0.304	0.010	0.006	3.2%
7.8	0.183	0.003	0.002	1.7%
Blank	0.060	0.003	0.002	5.6%

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Limit of Detection

This Mature BDNF ELISA kit detects a minimum of 2 pg/mL BDNF in assay buffer (defined as BDNF concentration at 150% of blank OD value). Refer to Appendix D for extending the standard curve for lower sensitivity applications.

Assay Precision

Several experiments were performed to assess assay reproducibility.

In experiment 1, two human serum samples were assayed in triplicates on 3 different days by 1 operator.

		BDNF / [ng/mL]			Intra-Assay	Inter-Assay
		Mean	SD	SEM	CV	CV
1	Day 1	25.8	1.3	0.8	5.0%	3.6%
	Day 2	26.1	1.5	0.9	5.7%	
	Day 3	24.4	0.9	0.5	3.7%	
2	Day 1	24.9	0.4	0.2	1.6%	7.2%
	Day 2	25.1	1.8	1.0	7.2%	
	Day 3	22.0	0.5	0.3	2.3%	

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

In experiment 2, ten serum samples were assayed by one operator to obtain **inter-plate CV**. Three plates of the same manufactured plate batch were randomly selected and samples run on one day. Samples were assayed at 2 dilutions in triplicates. Sample dilutions were chosen to cover the entire range of the BDNF reference standard curve. Serum BDNF values for diluted samples ranged from 14.0 pg/mL to 416 pg/mL.

Serum	Plate 1 (ng/mL)	Plate 2 (ng/mL)	Plate 3 (ng/mL)	Mean (ng/mL)	CV (%)
1	13.7	13.7	13.5	13.6	0.8
2	12.0	12.0	11.7	11.9	1.3
3	7.5	7.8	7.4	7.6	2.8
4	1.19	1.24	1.14	1.19	4.1
5	1.18	1.23	1.13	1.18	4.2
6	28.3	27.5	28.1	28.0	1.6
7	25.6	24.4	25.9	25.3	3.1
8	21.5	21.1	21.9	21.5	1.8
9	24.4	24.6	25.3	24.7	1.9
10	21.2	20.4	21.2	20.9	2.2

The average inter-plate CV was 2.4% (0.8 – 4.2%), demonstrating excellent reproducibility across coated plates from one manufactured batch.

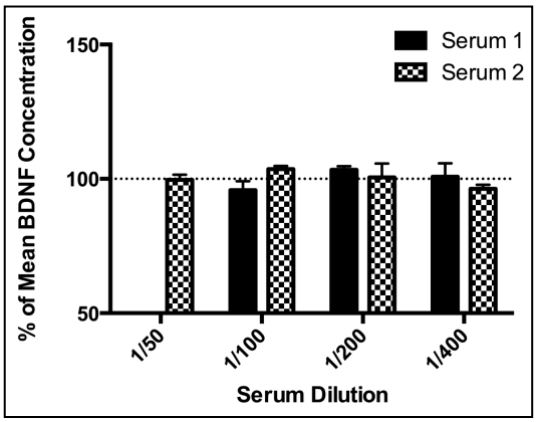
In experiment 3, **inter-assay precision** was assessed by assaying ten serum samples by one operator on 3 days, using 2 different batches of coated plates.

Serum	Assay 1 (ng/mL)	Assay 2 (ng/mL)	Assay 3 (ng/mL)	Mean (ng/mL)	CV (%)
1	12.1	13.2	13.6	13.0	6.1
2	11.4	12.0	11.9	11.8	2.8
3	7.7	7.7	7.6	7.7	1.1
4	1.10	1.00	1.19	1.10	8.8
5	1.00	1.00	1.18	1.06	9.9
6	26.8	28.3	28.0	27.7	2.8
7	23.8	25.4	25.3	24.8	3.6
8	18.7	19.7	21.5	20.0	7.1
9	22.9	23.1	24.7	23.6	4.3
10	19.9	19.4	20.9	20.1	3.8

The average inter-assay CV was 5.0% (range: 1.1 – 9.9%), demonstrating excellent precision of serum BDNF values across plates and assay days.

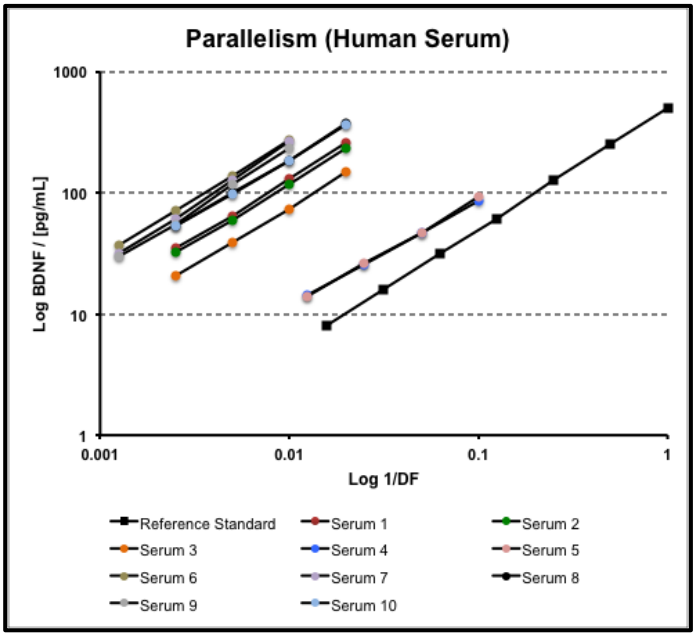
Assay Accuracy (Linearity of Dilutions/Parallelism of Serum Samples)

Two human serum samples were each diluted 1/50 – 1/400 with the assay diluent provided in this kit and assayed in triplicates. A 4-PL regression analysis was carried out to determine mean BDNF levels in serum.



The mature BDNF concentrations in human serum varied between 95.8–103.6% of the mean BDNF concentration within the dilution range.

In another experiment, 10 serum samples were assayed at four dilutions ranging from 1/10 to 1/80 to assess parallelism with the BDNF reference standard.



BDNF concentrations ranged from 1.0 – 28.3 ng/mL in these 10 human serum samples. The inter-dilution Coefficient of Variation (CV) for each sample ranged from 3.4 – 11.9 % (mean: 5.8%), demonstrating excellent parallelism and thus accurate quantification of serum BDNF.

Recovery in Human Serum

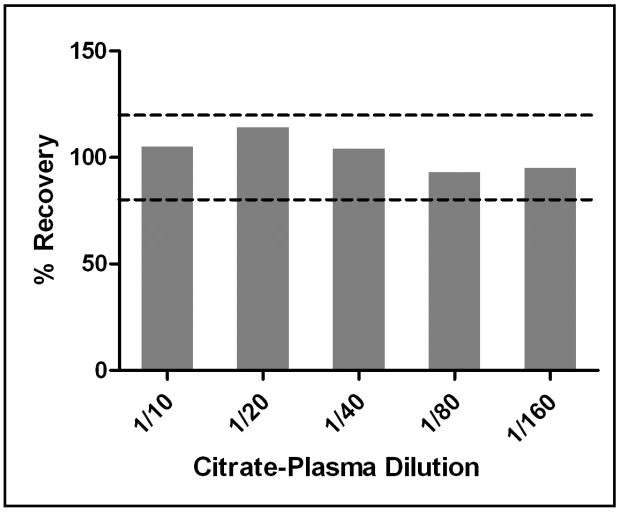
Two human serum samples were each spiked with 3 different levels of BDNF (20, 50, 100 pg/mL) at a sample dilution of 1/100. No matrix interference was observed.

Quantification of Mature BDNF in Human Plasma

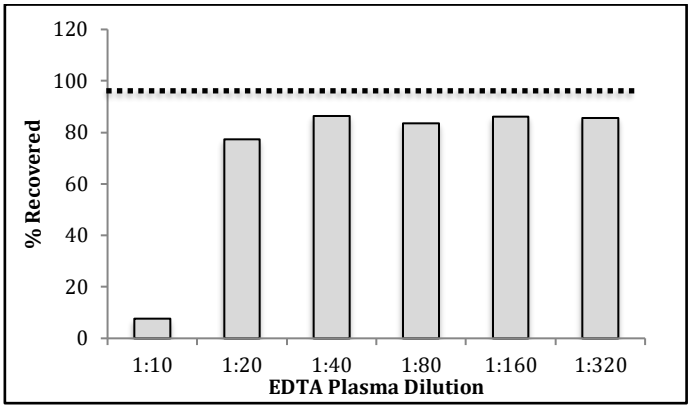
Six citrate-treated human plasma samples were assayed on six days. Plasma samples showed dilutional linearity (98%-117%) between sample dilutions of 1/10 to 1/160. The CV of diluted plasma for each sample ranged from 5.7 – 10.0% (mean: 7.7%), demonstrating accurate BDNF quantification in human citrate-plasma.

Citrate-Plasma Sample	Dilution	BDNF / [ng/mL]			% of Previous Dilution
			Mean	CV %	
1	1/10	2.63	2.86	10.0	-
	1/20	2.77			105
	1/40	3.18			115
2	1/10	3.21	3.37	7.9	-
	1/20	3.23			101
	1/40	3.68			114
3	1/10	2.02	2.17	7.8	-
	1/20	2.13			105
	1/40	2.35			110
4	1/40	2.44	2.62	6.1	-
	1/80	2.69			110
	1/160	2.74			102
5	1/40	2.68	2.87	5.7	-
	1/80	2.96			110
	1/160	2.97			100
6	1/40	2.68	2.97	8.4	-
	1/80	3.14			117
	1/160	3.08			98

Spiking of BDNF (100 pg/mL) gave acceptable recoveries in the range of 93%-114%.



One EDTA-treated human plasma sample was spiked with exogenous BDNF then diluted 2-fold and assayed in triplicate. The ELISA was repeated on different occasions for each dilution (n = 2-6 assays).



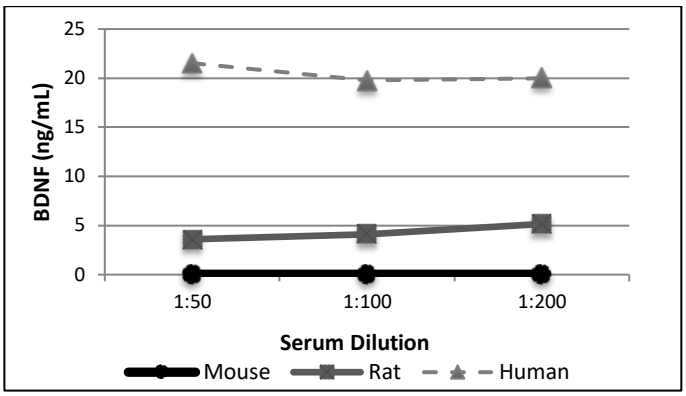
The results of the above figure show that ≥ 1:20 dilution is required for human EDTA plasma to achieve acceptable recoveries of the BDNF spike (between 80-86%).

For quantification of endogenous BDNF in human EDTA plasma, plasma was diluted in Assay Diluent A (1/20 to 1/320) and assayed in triplicate wells on four separate occasions. The mean concentration of mature BDNF in human EDTA plasma was 11 ng/mL +/- 0.75 ng/mL (mean +/- SEM) .

Overall, the results of our internal testing demonstrate that citrate and EDTA anticoagulants are suitable for collecting human plasma for testing in this BDNF ELISA kit, with citrate-plasma showing better recoveries at lower sample dilutions.

Quantification of Mature BDNF in Mouse, Rat & Human Serum

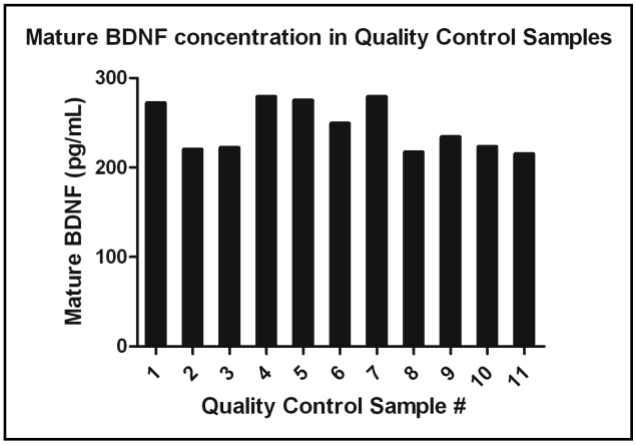
Serum samples of each mouse, rat and human were assayed in duplicate on two separate days for their BDNF content. The following graph shows the concentrations of BDNF detected in mouse, rat and human sera.



The above figure shows that no mature BDNF was detected in mouse serum. In contrast, 4.5 ng/mL mature BDNF was found in rat serum and around 20 ng/mL in human serum. These results correlate with published data in that inter-species serum samples vary in their mature BDNF concentration (Klein *et al*., 2011 and Radka *et al*., 1996). Note that these results have been adjusted for the dilution factor.

Concentration of Mature BDNF in Quality Control Samples

Eleven Quality Control samples were each reconstituted in 1 mL Assay Diluent A and assayed in triplicate by 2 different operators on 2 different days. The mature BDNF concentration was determined with a 4-PL regression analysis and found to be 244 ± 8 pg/mL BDNF (mean ± SEM).



Recovery in RIPA Buffer

250 pg/mL BDNF were spiked into RIPA buffer at different dilutions and recovery of BDNF determined.

Dilution	Recovery of BDNF
Undiluted	56%
1/2.5	87%
1/5	100%

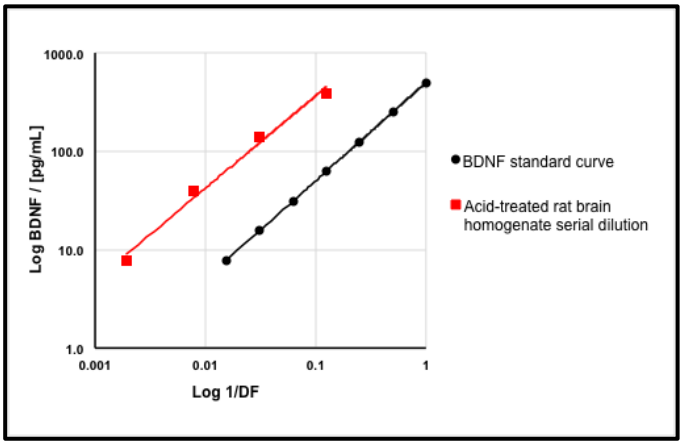
This demonstrates that the Mature BDNF ELISA is compatible with RIPA buffer.

Quantification of BDNF in Acid-Extracted Rat Brain Homogenate

Fifty-three milligram of normal rat cerebellum were homogenized in 530 µL acid-extraction buffer (weight/volume ratio of 10:1) according to the procedure outlined in Appendix B. Tissue was homogenized using a bead homogenizer.

The brain sample was assayed for BDNF content at 1:8 – 1:512 dilution (4-fold dilution series) in acid-extraction sample diluent. Each diluted sample was spiked with 100 pg/mL mature BDNF standard to assess recovery.

Sample dilutions showed parallelism to the BDNF calibration curve, demonstrating accurate quantification of BDNF in acid-extracted rat brain extracts.



Excellent recovery of spiked BDNF further demonstrated that this ELISA assay yields accurate results in acid-extracted brain tissue.

Sample	Dilution	Recovery*
Rat brain extract	1/8	99%
	1/32	119%
	1/128	103%
	1/512	107%

* Mean of 2 assays

The following table summarizes BDNF values obtained from acid-extracted rat cerebellum:

BDNF / [ng/mL]	Total protein* / [mg/mL]	BDNF / [ng / mg extracted protein]	BDNF / [ng / g brain]
3.67	1.30	2.82	36.6

* Determined by Bradford assay

Quantification and Recovery of Mature BDNF in PC12 Cell Lysates

PC12 cells (5.2 x 10^6 cells) were lysed with either RIPA buffer or by performing 3 freeze-thaw cycles in 5 mM phosphate (pH 6.8) or acid-extraction buffer. Lysates were diluted 1/5-1/40 as outlined in the Sample Preparation Section. Protein extracts were also spiked with 100 pg/mL exogenous BDNF and recovery calculated in relation to the same spike in sample diluent.

Sample	pg BDNF / mg extracted protein
PC12 Lysate (RIPA)	437.3
PC12 Lysate (Freeze-Thaw in hypotonic buffer)	*Not detected*
PC12 Lysate (Freeze-thaw in acid-extraction buffer)	201.4

Sample	Dilution	Recovery
PC12 Lysate (RIPA)	1/5	106%
	1/10	113%
	1/20	118%
	1/40	112%
PC12 Lysate (Freeze-Thaw in hypotonic buffer)	1/5	101%
	1/10	118%
	1/20	116%
	1/40	109%
PC12 Lysate (Freeze-thaw in acid-extraction buffer)	1/5	117%
	1/10	124%
	1/20	120%
	1/40	115%

Biosensis recommends performing spike- and recovery experiments with cell lysates to assess accuracy of the results.

Quantification of Mature BDNF in NSC34 Cell Lysates

NSC34 cells were lysed in RIPA buffer with protease inhibitors. Total protein concentration was determined by Bradford Assay.

Dilution	pg BDNF / mg total protein
1/5	64.5
1/10	84.0
1/20	*Not detected*
Average	74.3

Quantification of Mature BDNF in Human Platelet Lysates

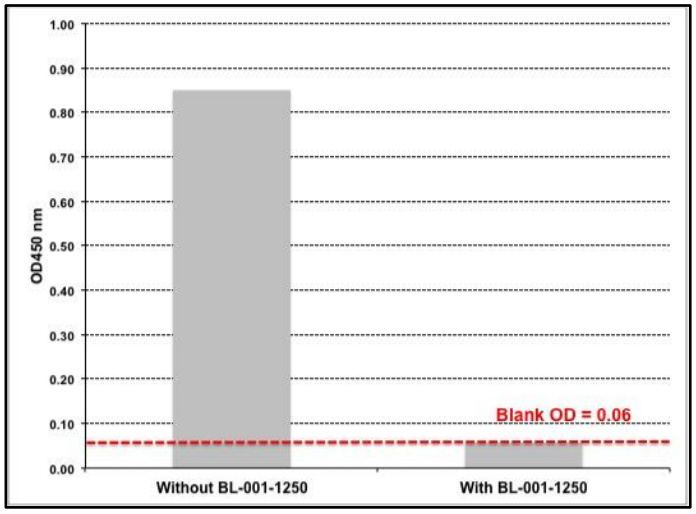
Pooled normal human platelets were lysed by repeated freeze-thaw cycles and BDNF quantified. Total protein concentration was determined by Bradford Assay.

Dilution	pg BDNF / mg total protein
1/25	153.7
1/125	150.0
1/625	196.3
Average	166.7

Quantification of BDNF in Human Milk - Effect of BL-001-1250 on sIgA Cross-Reactivity

The ability of immunoassay blocker BL-001-1250 to reduce sIgA cross-reactivity was demonstrated in two experiments:

(1) Purified sIgA protein at 250 µg/mL was spiked into assay buffer with and without BL-001-1250. The sIgA concentration was chosen to mimic a 1:4 dilution of a theoretical sample containing high concentrations of 1 mg/mL sIgA.



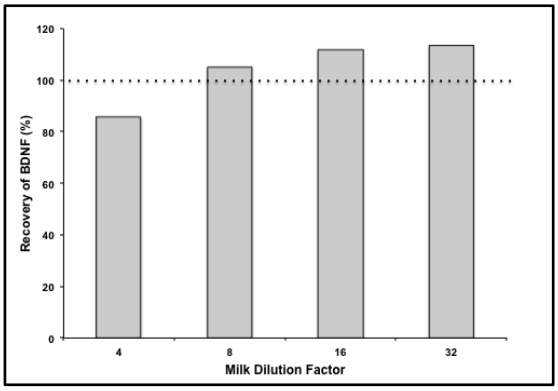
(2) Pooled, normal human milk was assayed for BDNF content, diluting milk in either Assay Diluent A without, or with BL-001-1250.

Sample Dilution	BDNF (pg/mL), dilution corrected	
	Without BL-001-1250	With BL-001-1250
1:4	211	0
1:8	130	0
1:16	0	0
1:32	0	0

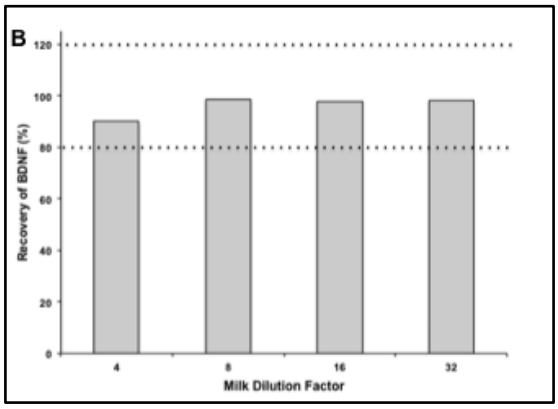
Data shows that BL-001-1250 effectively reduces false-positive OD readings due to sIgA cross-reactivity to background levels.

Linearity and Recovery of BDNF in Human Milk

The effect of sample preparation on BDNF recovery was tested by spiking 1 ng/mL mature BDNF into whole human milk after sample preparation procedure, as outlined in Section 6. Prepared human milk was then assayed at 1:4 – 1:32 dilutions (n=2 independent assays). Recovery values ranged from 86-113% (mean = 104%) demonstrating that BDNF concentrations are not affected by sample preparation.



Sample buffer compatibility with human milk was validated by spiking known amounts of BDNF into 1:4 – 1:32 dilutions of human milk. The ELISA assay showed excellent recovery values (B) ranging from 90-98% across the dilution range. This demonstrates that a minimum dilution of 1:4 is required in order to assay BDNF in human milk accurately.



Mature BDNF levels in normal pooled human milk were lower than the lowest standard of the calibration curve. Individual milk samples, while not tested, may contain quantifiable levels of BDNF.

Linearity and Recovery of BDNF in Human CSF

Pooled human CSF sample was spiked with 250 pg/mL mature BDNF and serially diluted from 1:2 to 1:32. Recoveries ranged from 85 – 105% indicating that human CSF can be accurately measured. Endogenous mature BDNF was undetectable. It is expected that most normal human CSF samples will have BDNF concentrations below the detection limit of this assay.

CSF Dilution	% BDNF conc. of previous dilution	% Recovery
1:2	-	85%
1:4	100%	85%
1:8	109%	93%
1:16	105%	97%
1:32	105%	105%

Specificity

No cross-reactivity was observed or nerve growth factor (NGF), neurotrophin-3 (NT-3), NT-4/5, glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor ($VEGF_{165}$) tested at 25 ng/mL in assay buffer.

This Mature BDNF *Rapid*™ ELISA assay preferentially detects the mature BDNF isoform The reactivity of full-length proBDNF (0.125 ng/mL – 5 ng/mL) was determined in six independent assays using proBDNF proteins from four different sources (mammalian and bacterial, wild-type and mutated). The average cross-reactivity of proBDNF was found to be 5.3% ± 0.5% in weight (w/v) concentration, or 12.1% ± 1.2% in molar concentration (mean ± SEM). In an additional experiment, a mixture of 25 ng/mL mature BDNF and 5 ng/mL proBDNF in PBS was prepared and assayed in comparison to 25 ng/mL mature BDNF alone. The recovery of mature BDNF in the mature BDNF/proBDNF mixture was 103% (mean of 3 assays with duplicate samples at 1/100 and 1/200 dilutions).

Additional validation experiments were performed with spiked human serum, showing that at typical human serum dilutions, proBDNF does not interfere with mature BDNF quantification. Please refer to our Technical Note #5 for further details.

13. Informational References

Gejl *et al.* 2019, *Associations between serum and plasma brain-derived neurotrophic factor and influence of storage time and centrifugation strategy.* Sci. Rep. July;9(1):9655.

Uys *et al.* 2016, *The α2C-adrenoceptor antagonist, ORM-10,921, has antipsychotic-like effects in social isolation reared rats and bolsters the effects of haloperidol.* Prog Neuropsychopharmacol Biol Psychiatry. Nov;71:108-16.

Tsuchimine *et al.* 2014, *Preanalysis storage conditions influence the measurement of brain-derived neuotrophic factor levels in peripheral blood*. Neuropsychobiology. April;69(2): 83-8.

Klein *et al*. 2011, *Blood BDNF concentrations reflect brain-tissue BDNF levels across species.* Int J Neuropsychopharmacology. April;14(3):347-53.

Kolbeck R *et al.* 1999, *Brain-derived neurotrophic factor levels in the nervous system of wild-type and neurotrophin gene mutant mice.* J Neurochem. May;72(5):1930-8.

Weaver *et al.* 1998, *Human milk IgA concentrations during the first year of lactation.* Arch Dis Child. 78:235-239.

Radka *et al*. 1996, *Presence of brain-derived neurotrophic factor in brain and human and rat but not mouse serum detected by a sensitive and specific immunoassay.* Brain Res. Feb;706(1):122-130.

14. Specific References (Selection)

Nishiyama M *et al.* 2020. *Homostachydrine is a Xenobiotic Substrate of OCTN1/SLC22A4 and Potentially Sensitizes Pentylenetetrazole-Induced Seizures in Mice.* Neurochem Res. [Epub ahead of print]. **Application: Acid-extracted mouse hippocampal homogenates.**

Lorinczova HT *et al.* 2020. *Co-Administration of Iron and a Bioavailable Curcumin Supplement Increases Serum BDNF Levels in Healthy Adults.* Antioxidants (Basel). 9(8):E645. **Application: Human serum.**

Sartori A *et al.* 2019. *Interferon-beta, but not Glatiramer Acetate treatment induces gender-specific increase in BDNF serum levels in relapsing-remitting multiple sclerosis female patients.* Res. J Neuro N Disord. 1:5-18. Application: Human serum. **Please refer to our Technical Note #5 for validation experiments disproving the author's claim that the Biosensis Mature BDNF *Rapid*™ ELISA quantifies total BDNF!**

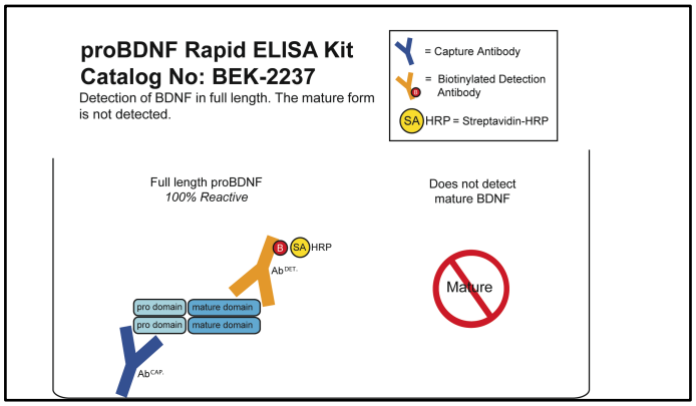
Rault JJ *et al.* 2017. *Brain-derived neurotrophic factor as an indicator of environmental enrichment effectiveness*. Report prepared for the Co-operative Research Centre (CRC) for High Integrity Australian Pork. https://www.porkcrc.com.au/wp-content/uploads/2017/11/1C-119-Final-Report-BDNF.pdf**Application: Pig serum.**

Polacchini A *et al.* 2015. *A method for reproducible measurements of serum BDNF: comparison of the performance of six commercial assays.* Sci Rep. 10;5:17989. **Application: Human serum.**

Please refer to our website for more product-specific references.

15. Other Information

We recommend the Biosensis proBDNF *Rapid*™ ELISA kit (Cat# BEK-2237) to quantify proBDNF independent of mature BDNF in human samples.



The BDNF ELISA is also available as CE-marked version (BEK-2211-CE) for diagnostic applications in the European Economic Area (EEA).

Please visit our website (www.biosensis.com) for a full range of BDNF-related research reagents.

Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments

Standard curve, blank and controls:

- Standard (500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL): 7 wells
- Blank (0 pg/mL): 1 well
- Control vial (either provided in the ELISA kit or the researcher): 1 well

Gives a total of 9 wells used per Standard/Control set.

We recommend that the standard curve, blanks, and controls be run on each separate plate for the most accurate calculations. Note that if two (or more) independent assays are run on one plate, each assay requires its own Standard/Control set. We also recommend that ALL samples (i.e. standards, controls, blanks and test subjects) be performed in duplicate at least.

Thus, for standards, blanks and controls, 9 x 2 = **18 standard wells are required per assay.** This leaves 96 wells – 18 wells = **78 sample wells per plate** for test/patient samples for EACH of our standard 1P ELISA kits run with a standard curve and controls.

1P kit: 78 experimental wells per 96 well ELISA plate experiment
2P kit: 156 experimental wells per 192 well ELISA plate experiment

The 2-Plate Optional Single Control Set:
Some researchers using our 2P kits run a single, duplicate standard curve, blank and control sample set (18 wells) for the entire 2-plate experiment. This allows 78 test/patient samples on the “standard curve plate” (the plate containing the standard curve, controls and blanks) and a full 96 test/patient samples on the “test only plate” for a total available number of 174 test/patients per 2 plate kit. (78 wells +96 wells = 174).

2P kit, single set of controls (in duplicate): 174 experimental wells per 192 well ELISA plate experiment

Example: 60 patient samples, duplicate measurement at one single dilution
How many wells and plates do I need for sixty patients at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 patients, x 2 draws each (e.g. before and after) = 120 stock samples
2. Factor in the number of repeat tests desired, in order to calculate the number of wells required. Biosensis recommends that ALL samples be run in duplicate at least.
3. Multiply the number of stock samples by the number of dilutions per sample. In this example, we will be testing EACH stock sample at a single dilution (i.e. 1:100) in duplicate, so 120 stock samples x 2 wells = 240 microplate wells required for sixty patients. In summary: 60 patients, 4 tests per patient, equals 240 total number of wells required.
4. Decide on the number of standards, blanks and controls run on each plate. We recommend standard, control and blank duplicates for each 96-well plate. Alternatively, one standard, blank and control duplicate set can be run on every 2 plates (2-Plate Optional Single Control Set option discussed above).

Running the Test:
Running the 60 patient experiment with a standard curve, blanks and controls on *each* test plate, in duplicate (our recommended option):

Single Dilution per Test Sample:

(240 wells required) / (78 wells per plate available) = 3.077 plates required, or just over 3 plates, thus researcher will need to order **4 x 1-plate kits or 2 x 2-plate kits** (there will be unused wells) to ensure enough wells for the entire sixty patient sample, tested in duplicate (two draws per patient, 1 dilution, 4 wells per patient, total of 240 wells). The unused 8-well strips can be used for other assays later.

Two Dilutions per Test Sample:

If testing at two dilutions, (e.g. 1:50 & 1:100 per sample) and doing duplicates, then the number of wells/tests is doubled to 480 (e.g. 120 x 2 @ 1:50 = 240, PLUS 120 x 2 @1:100 = 480). Then the number of plates is determined by (480 wells required) / (78 wells per plate available) = 6.15 plates required, or just over 6 plates, thus the researcher will need to order **3 x 2-plate kits and 1 x 1-plate kit** to ensure the minimum number of wells for sixty patient samples tested in duplicate at two dilutions (two draws per patient, 2 dilutions, 8 tests/wells per patient, total of 480 patient tests/wells).

2-Plate Optional Single Control Set Option:

If running 60 patients with a single dilution, and a single duplicate standard curve, blank, and control sample for every two plates, then one has **174 available test wells per 2P kit**.

For the single dilution, sixty patients, 2 draws per patient experiment (240 tests), one would need (240/174) = 1.38 2-plate kits, or **2 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty patient samples.

For the two dilutions per sample, sixty patients, 2 draws per patient experiment (480 tests), one would need (480/174) = 2.76 2-plate kits, or **3 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty patient samples.

Appendix B: Tissue Lysate Preparation Procedures Acid-Extraction of BDNF

BDNF is bound to its receptors and chaperons within many tissues, which prevents its detection in any ELISA. Acid extraction protocols have therefore been developed to release this bound BDNF, which not only release the bound BDNF, but also precipitates the receptors. We recommend the following sample preparation protocol for measuring total BDNF concentrations in acid-treated samples. This protocol is based on Kolbeck *et al.* (1999).

Acid-Extraction Buffer

50 mmol/L sodium acetate (820 mg / 200 mL), 1 mol/L NaCl (11.7 g / 200 mL), 0.1% Triton X100 (200 µL / 200 mL), add glacial acetic acid until pH 4.0 is reached. Before use add one "Complete" or "Complete Mini" protease inhibitors cocktail tablet (Roche, cat. no. 11697498001 or 11836153001), to be used as recommended by the manufacturer.

Incubation/Neutralization Buffer

0.2 mol/L phosphate buffer, pH 7.6. For instance, weigh in the following amounts per 200 mL total buffer volume and adjust pH with concentrated NaOH solution (≥ 5 mol/L):
KH_2PO_4 (MW 136.09 g/mol): 5.44 g
Na_2HPO_4 (MW 141.96 g/mol): 5.68 g

Acid-Extraction Sample Diluent

Mix acid-extraction buffer and incubation/neutralization buffer at a ratio of 1 to 3 (eg., 1 mL of extraction buffer and 3 mL of incubation/neutralization buffer). Check pH of solution is approximately pH 6.6 to 7.0.

Protocol

1. Dissect brain structures, weigh tissue fragments and then freeze them rapidly in liquid nitrogen
2. For long term storage transfer the frozen tissue samples to -80°C
3. Re-suspend brain tissues in approximately 10 weight/volume-ratio of extraction buffer (for example, 100 µL extraction buffer for 10 mg tissue)
4. Sonicate the suspension to homogeneity on ice with a probe sonicator (e.g. Misonix Ultrasonic Liquid Processor)
5. Sonicate in short bursts (5-7 sec) to avoid excessive sample heating
6. Keep homogenates on ice for 30 min, then repeat sonication as well as incubation on ice
7. Centrifuge homogenates for 30 minutes at 10,000 – 20,000 x g and 4°C, then transfer clear supernatants into clean tubes and discard pellets
8. Measure total protein concentration (eg., BCA or Bradford protein assay)
9. These supernatants may be stored at -80°C and must be centrifuged again for 30 min at 10,000- 20,000 x g and 4°C immediately after thawing and before being neutralized and used in the ELISA.
10. Reconstitute the supplied BDNF standard with 1 mL of Acid-Extraction Sample Diluent; perform a 1:2 serial dilution as outlined in Section 6 ("Preparation of BDNF Standard").
11. Neutralization and preparation for ELISA: prepare a sample dilution with 1 part tissue extract and 3 parts of Incubation/Neutralization Buffer (eg., 50 µL tissue extract and 150 µL Incubation/Neutralization buffer); check that pH of sample is near neutral. Note that the neutralization step equals a sample dilution factor of 4 which needs to be considered when calculating BDNF concentrations in samples. The prepared, neutralized samples are now ready for the ELISA assay. Note: Freezing of prepared, neutralized, samples is not recommended. Typically, freezing of samples is best when protein concentrations are more concentrated, as in step 9.
12. If the BDNF concentration in the sample is out of the assay range (7.8-500 pg/mL) after performing the initial tests, dilute the solution prepared in step 11 further with Acid-Extraction Sample Diluent for best results. Do not use Assay Diluent A for acid-treated/neutralized samples.
13. Results can then be reported as ng BDNF/mg total soluble protein or g wet weight if tissue sample is large enough

Note: Biosensis recommends evaluating the recovery of BDNF when preparing tissue extracts. In order to conduct a recovery experiment, known amounts of BDNF standard are added to an aliquot of the brain tissue homogenates and assayed. Spike-recovery experiments allow comparison of technique and methods. Recoveries of 80-120% of spiked values are acceptable.

Appendix C: Tissue Lysate Preparation Procedures RIPA Buffer

The key with any RIPA extraction buffer is to provide enough detergent to solubilize the proteins, but not so much that it will interfere with subsequent uses, particularly native ELISAs where antibody binding is critical. We recommend the following RIPA extraction buffer, which has lower detergent amounts and has been successfully used in brain tissue extracts.

Biosensis In-house RIPA

50 mM Tris-HCL, 150 mM sodium chloride; 1.0% NP-40 or Triton X-100; 0.5% sodium deoxycholate; pH 7.5 to 8.0. (Note: 0.1% SDS is left out for ELISA samples, but can be added back for western or IP samples, which will be diluted before use). Complete proteinase inhibitor cocktails are added to this base buffer in all cases. Tissue samples should be homogenized in ice-cold RIPA buffer, and proper protein preparation procedures including lysates being kept on ice, etc. are required for consistent and best results.

Note: Biosensis recommends evaluating the recovery of BDNF when preparing tissue extracts. In order to conduct a recovery experiment, known amounts of BDNF standard are added to an aliquot of the brain tissue homogenates and assayed. Spike recovery experiments allow comparison of technique and methods. Recoveries of 80-120% of spiked values are acceptable.

It is recommended that tissue samples should be rapidly excised, weighed, and snap frozen in liquid nitrogen prior to storage at -80°C. We recommended using frozen samples within two weeks of freezing.

RIPA homogenates should be prepared in approximately 10 to 100 volumes of the homogenization buffer to tissue wet weight, but the most appropriate ratio needs to be determined by the user for each tissue. Typical ratios are 10-20 mg wet weight/100 µL of lysate, or 1 g tissue per 10 mL of lysis buffer. The tissue can be homogenized either via sonication or mechanical shearing or both (polytron). The homogenates are centrifuged at ~ 14,000 x g for 30 minutes. The resulting supernatants can be used for ELISAs. Check that sample pH is near 7.5-8.0 for best results.

Concentrated stock lysates should be divided into aliquots and frozen at -80°C and thawed and used only once. Lysate stability is fragile and should be used within two weeks for best results.

RIPA Buffer Tissue Lysis and Preparation

1. Sonicate the suspension to homogeneity on ice with a probe sonicator (e.g. Misonix Ultrasonic Liquid Processor). Sonicate in short bursts (5-7 sec) to avoid excessive sample heating
2. Keep homogenates on ice for 30 min, then repeat sonication as well as incubation on ice
3. Centrifuge homogenates for 30 minutes at 10,000 – 20,000 x g and 4°C, then transfer clear supernatants into clean tubes and discard pellets
4. Measure total protein concentration (e.g., BCA or Bradford protein assay)
5. These supernatants may be stored at -80°C and must be centrifuged again for 30 min at 10,000- 20,000 x g and 4°C immediately after thawing and before being used in the ELISA

RIPA Sample Dilution

Prepared, cleared, concentrated, lysates are typically diluted at least 1:5 with Biosensis Assay Diluent A before use in the ELISA. After dilution, check that sample pH is within 7.0 – 8.0 with pH paper for best results.

Note: The final sample dilution will vary depending upon the tissue and exact extraction method. Typically, 1:5-1:20 (w/v) dilution for many, but some tissues such as hippocampus can be greater (e.g. 1:300) in order for the assay values to be consistent and fall within the linear range of the assay. Thus, the optimal dilution needs to be determined for each experimental set and laboratory. Spike recovery experiments are highly recommended, see note above.

RIPA sample ELISA results can be reported as ng BDNF/mg total soluble protein or g wet weight if tissue sample is large enough.

Appendix D: Preparation of Standards for Extending the Standard Curve

For higher sensitivity applications, the dilution series can be extended to 3.9 or 1.95 pg/mL, with 250 pg/mL or 125 pg/mL respectively being the highest concentration of the mature BDNF standard curve.

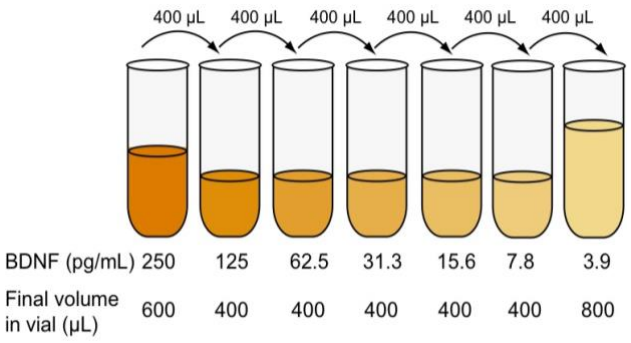
- Reconstitute the lyophilized antigen standard with 1 mL of the **same diluent used for preparing sample dilutions**
- Label the vial with the reconstituted mature BDNF standard as “1000 pg/mL”; vortex and let stand for 15 minutes

To extend the Standard curve to 3.9 pg/mL

1. Dilute the 1000 pg/mL mature BDNF standard 1:4 (eg., 250 µL of 1000 pg/mL standard + 750 µL diluent); label this tube “250 pg/mL”

   In order to generate a BDNF standard curve, perform a 1:2 serial dilution down to 3.9 pg/mL. The volumes used for the dilution series depends on the number of repeats per BDNF concentration. For triplicate measurement (100 µL per well) of each BDNF standard concentration, we recommend this procedure:

2. Label 6 tubes with “125 pg/mL”, “62.5 pg/mL”, “31.3 pg/mL”, “15.6 pg/mL”, “7.8 pg/mL” and “3.9 pg/mL”, respectively
3. Aliquot 400 µL of the sample diluent into each tube
4. Take 400 µL from the “250 pg/mL” tube and transfer to the “125 pg/mL” tube
5. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex
6. Repeat steps 3 and 4 for each consecutive concentration until the last tube “3.9 pg/mL” is prepared and mixed well.

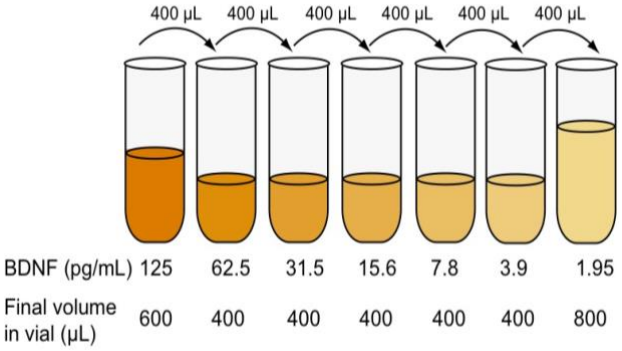


To extend the Standard curve to 1.95 pg/mL

1. Dilute the 1000 pg/mL mature BDNF standard 1:8 (eg., 125 µL of 1000 pg/mL standard + 875 µL diluent); label this tube “125 pg/mL”

   In order to generate a BDNF standard curve, perform a 1:2 serial dilution down to 1.95 pg/mL. The volumes used for the dilution series depends on the number of repeats per BDNF concentration. For triplicate measurement (100 µL per well) of each BDNF standard concentration, we recommend this procedure:

2. Label 6 tubes with “62.5 pg/mL”, 31.3 pg/mL”, “15.6 pg/mL”, “7.8 pg/mL”, “3.9 pg/mL” and “1.95 pg/mL”, respectively
3. Aliquot 400 µL of the sample diluent into each tube
4. Take 400 µL from the “125 pg/mL” tube and transfer to the “62.5 pg/mL” tube
5. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex
6. Repeat steps 3 and 4 for each consecutive concentration until the last tube “1.95 pg/mL” is prepared and mixed well



To dilute the QC into the range of the standard curve

- Reconstitute the lyophilized antigen QC with 1 mL of the **same diluent used for preparing sample dilutions.** Further perform a 1:4 dilution in sample diluent. This will provide a QC sample within the range of 22 – 41 pg/mL

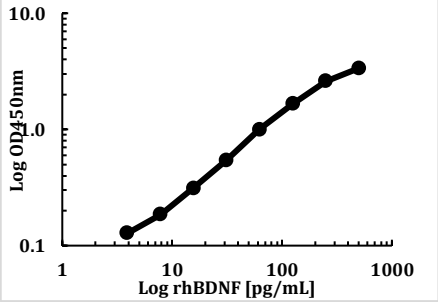
After the standards have been prepared follow the same procedure as described in Section 8 for the preparation of detection antibody and streptavidin-HRP. Run the assay as per the procedure described in Section 9.

Note: TMB incubation times can vary however they may need to be extended for up to 30 minutes when generating a higher sensitive ELISA. In order to determine the optimal time to stop the TMB incubation, blue color development should be monitored at **650 nm**. Addition of Stop Solution will convert blue into yellow color, with an ~2.5-3X increase in OD at **450 nm**. Once the highest standard reaches an OD of ~1.2 – 1.3 at 650 nm, immediately stop the reaction by adding Stop Solution. Note that before taking a 650 nm measurement, the plate should be briefly and gently shaken by hand to homogenise the blue TMB reaction product within the wells for most accurate readings.

Typical Standard Curve Data for 250 to 3.9 pg/mL Standard Curve

Standard curves are provided for demonstration only. A standard curve has to be generated for each mature BDNF ELISA assay.

In addition, we recommend measuring common samples when performing multiple assays across several days in order to normalize the standard curve numbers between the various runs.



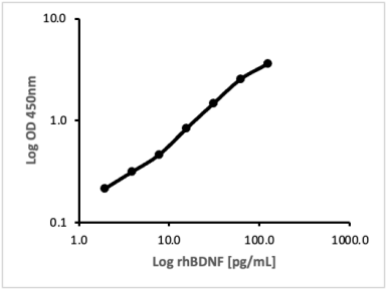
In the example graph above, BDNF standards were run in triplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 10 minutes.

Typical optical densities and coefficient of variations for diluted BDNF standards are summarized in the following table:

	OD 450 nm			
BDNF / [pg/mL]	**Mean**	**SD**	**SEM**	**CV**
250	2.608	0.035	0.020	1.3%
125	1.660	0.025	0.014	1.5%
62.5	0.997	0.018	0.010	1.8%
31.3	0.542	0.008	0.005	1.5%
15.6	0.313	0.002	0.001	0.6%
7.8	0.185	0.001	0.000	0.4%
3.9	0.128	0.003	0.002	2.5%
Blank	0.065	0.004	0.002	5.7%

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Typical Standard Curve Data for 125 to 1.95 pg/mL Standard Curve



In the example graph above, BDNF standards were run in triplicate using the assay diluent provided in this kit. The TMB reaction was stopped after **25** minutes.

Typical optical densities and coefficient of variations for diluted BDNF standards are summarized in the following table:

	OD 450 nm			
BDNF / [pg/mL]	**Mean**	**SD**	**SEM**	**CV%**
125	3.581	0.071	0.050	2.0
62.5	2.535	0.052	0.030	2.1
31.3	1.463	0.072	0.042	5.0
15.6	0.828	0.028	0.016	3.4
7.8	0.458	0.006	0.003	1.3
3.9	0.311	0.012	0.007	3.8
1.95	0.213	0.010	0.006	4.5
Blank	0.110	0.001	0.001	3.9

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Appendix E: Troubleshooting Guide

This Mature BDNF ELISA kit has been developed to deliver reproducible results when following the provided assay protocol. Please refer to the troubleshooting guide below when unexpected difficulties are encountered. If you require further assistance, talk to a scientist at Biosensis (biospeak@biosensis.com).

Problem	Cause	Solution
High background (blank OD > 0.30)	Insufficient washing	Make sure to perform 5 washes after each incubation step with sample, detection antibody and streptavidin-HRP conjugate. Follow the recommended manual washing procedure for optimal washing performance
	Excessive concentration of detection antibody and/or HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume.
	Increased incubation time and temperature	Please follow incubation times as stated in the protocol and perform incubations at room temperature
	Contamination	Ensure that all plastic- and glassware used is clean. Do not use the same pipette tip to transfer samples and blank solution into the wells
Low absorbance readings	Concentration of BDNF in the sample is lower than the detection limit of this assay	Confirm that the assay protocol was carried out correctly by spiking a known amount of BDNF into your sample and/or check that the QC sample value falls within the expected BDNF concentration range
	Insufficient antibody or insufficient HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume
	Reagents expired	Ensure correct storage of the kit and do not use the kit beyond its expiry date
Low absorbance readings	Decreased incubation times and temperature	Please follow incubation times as stated in the protocol and warm reagents to room temperature before use
	Microplate wells dried out	Do not leave microplate wells without solution for an extended time
	Wash buffer not diluted	Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution
	Stop solution not added	Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm
Standard OD values above plate reader limit	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
Sample OD values above standard curve range	BDNF concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
High coefficient of variations (CV)	TMB and stopping solution not added consistently	Add TMB and stopping solution to the wells in the same order and speed
	Inconsistent pipetting	Ensure pipettes are working correctly and are calibrated; review your pipetting technique; add standards and samples to the wells using a single-channel rather than a multi-channel pipette
	Insufficient mixing of reagents	Briefly vortex to mix solutions before pipetting into the wells
	Bottom of the plate is dirty	Clean the bottom of the plate before reading the plate