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

Catalogue Number: BEK-2217-1P/2P

For the quantitative determination of proBDNF in cell culture supernatants, cell lysates, human serum, human citrate plasma and tissue extracts only if used as directed.

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

This ELISA kit now includes a Quality Control sample for even better performance.

*See page 2 regarding the use of this kit for mouse and rat samples.

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

Table of Contents

1. Intended Use

The purpose of this kit is the quantitative determination of proBDNF in cell culture supernatants, cell lysates, human serum, human citrate plasma and tissue extracts only if used as directed. This kit has not been tested for other sample applications. Biosensis does not assume responsibility if this kit is used for unintended purposes. Non-validated samples may contain immunoglobulins and other components that potentially interfere with this ELISA assay.

Due to a high degree of amino acid sequence homology, mouse and rat proBDNF can be quantified and expressed as human proBDNF equivalents. Internal Biosensis validation suggests that the use of the human standard provided in this kit will provide estimates that are identical, or close, to the actual levels of rat and mouse proBDNF present in rodent samples.

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

2. Introduction

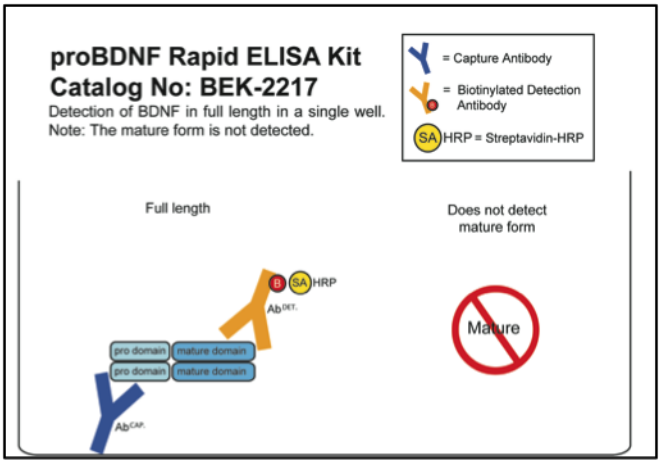
Brain-derived neurotrophic factor (BDNF) and its uncleaved precursor molecule proBDNF play important roles in a variety of physiological functions, for instance mediating neuronal survival and apoptosis, maintaining synaptic plasticity and regulating synaptic transmission. Altered levels of proBDNF and BDNF have been observed in blood and linked to a variety of neurodegenerative diseases. However, specific detection and quantification of proBDNF vs. mature BDNF is required in order to delineate the individual roles of the precursor and mature forms of BDNF.

The Biosensis proBDNF *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the specific, fast and reliable quantification of proBDNF in less than 4 hours. This kit consists of a pre-coated polyclonal anti-proBDNF 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 proBDNF present in samples and protein standards.

This proBDNF ELISA kit employs a recombinant, cleavage-resistant human proBDNF standard produced by Biosensis and validated against externally available proBDNF proteins.

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

The capture antibody used in this ELISA kit binds to epitopes within the pro-domain of proBDNF. Thus, this ELISA detects the full-length form of proBDNF and does not quantify mature BDNF.



3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
proBDNF 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
Heterophilic antibody Blocker BL-004-500*	1 vial	2 vials
Recombinant human proBDNF standard	2 x 1100 pg	4 x 1100 pg
Quality Control (QC) Sample	2 Vials (see vial for amount)	4 vials (see vial for amount)
proBDNF 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	

*Sample diluent additive for quantification of proBDNF in human serum and citrate plasma; refer to Section 6 for further details.

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 standard 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
Assay Diluent A, with BL-004-500 as additive	2 months at 2-8°C

Note:

- Do not freeze the streptavidin-HRP conjugate
- Do not use assay diluents from other ELISA kits

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 proBDNF in cell lysates, cell culture supernatants, serum, citrate-plasma and tissue extracts. 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. 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 proBDNF in samples, it is important to perform several dilutions of the sample to allow the proBDNF concentration to fall within the range of the proBDNF standard curve (15.6-1000 pg/mL). Also, with unknown samples of all types it is highly recommended to run some proBDNF spike- and recovery control tests over a short range of dilutions using our standard to help evaluate the particular sample

performance in the assay. Spike-recovery experiments that follow a reasonably linear progression and achieve a spiked recovery of 80-120% of spiked value demonstrates that the subject samples are performing acceptably in the assay. Failure of spiked recovery samples 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.

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 buffer for best results. Also note, bovine proBDNF shares a high degree of sequence homology to human proBDNF (and many other mammals) and may be detected in this assay, thus appropriate serum free, cell-free controls must be used for accurate detection.

Note:

- proBDNF is readily processed into its mature form in presence of various proteases, thus the addition of a protease inhibitor cocktail may be required
- Quantification of low levels of proBDNF in cell culture supernatants may require concentrating the supernatant in ultrafiltration devices with filters of 10 kDa molecular cut-off or lower.

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 C6 cells lysed with a RIPA buffer. The sample preparation procedures given here are suggested 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.

- 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
- 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
- Alternatively, freeze whole cell pellets, store at -80°C and lyse before use for analysis at later time-point

General Notes

- The addition of a protease inhibitor cocktail to buffers used in protein extraction just prior to cell lysis is highly recommended

Notes:

- For most accurate results it is good practice to run samples spiked with a known amount of proBDNF in parallel. Expected recoveries are 80 – 120 % of spiked proBDNF, values outside this range indicate interference issues with that particular sample
- The sample preparation protocols are provided as a guide only! ProBDNF is readily processed into its mature form by enzymatic cleavage. Thus, sample preparation may require the addition of protease inhibitors such as aprotinin to improve detection and recovery results from some samples.

Human Serum and Citrate-Plasma

It is strongly recommended to dilute all human serum and citrate-plasma samples in a Sample Diluent specifically designed to reduce or eliminate heterophilic antibody (HA) interferences. The addition of HA blocker BL-004-500 to Assay Diluent A is strongly recommended to avoid matrix interferences and reporting false positive or false negative results.

Sample Diluent Preparation for Human Serum and Citrate Plasma with HA Blocker BL-004-500:

- Reconstitute HA blocker BL-004-500 in 1 mL of Assay Diluent A to give an IgG concentration of 500 µg/mL, mix gently by vortex
- Add the 1 mL of reconstituted BL-004-500 to 24 mL of Assay Diluent A to a final volume of 25 mL. Use this Sample Diluent to prepare all human serum, citrate plasma and protein standard dilutions; do not use Assay Diluent A enriched with HA blocker to dilute detection antibody and HRP-conjugate
- Non-human serum and citrate plasma samples typically do not require the addition of BL-004-500 to Assay Diluent A

Serum Collection and Sample Dilutions:

- Allow the serum to clot in a serum separator tube (about 30 minutes to 4 hours) at room temperature
- 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
- Dilute serum samples in Sample Diluent (above) to measure proBDNF concentrations in human serum

Testing with a limited number of normal human serum samples indicate a minimum required sample dilution with Sample Diluent of 1/10 or higher to avoid matrix interferences and achieve acceptable recoveries of spiked proBDNF.

Citrate Plasma Collection and Sample Dilutions:

- Collect citrate plasma and centrifuge for 15 min at 2-8°C at 1,500 x g within 30 minutes of collection; EDTA and heparin-treated plasma samples have not been tested as yet
- For eliminating the platelet effect we suggest further centrifugation for 10 min at 2-8°C at 10,000 x g
- Analyze immediately or aliquot and store samples at -20°C to -80°C
- Testing a limited number of plasma samples indicates that a minimum dilution of 1/5 - 1/10 is required with Assay Diluent A containing BL-004-500

Note

- Acid treatment of serum and citrate plasma samples is usually not required
- For most accurate results it is good practice to run samples spiked with a known amount of proBDNF in parallel. Expected recoveries are 80 – 120 % of spiked proBDNF, values outside this range indicate interference issues with that particular sample
- The sample preparation protocols are provided as a guide only. ProBDNF is readily processed into its mature form by enzymatic cleavage. Thus, sample preparation may require the addition of protease inhibitors such as aprotinin to improve detection and recovery results from some samples.

Brain Tissue Extracts

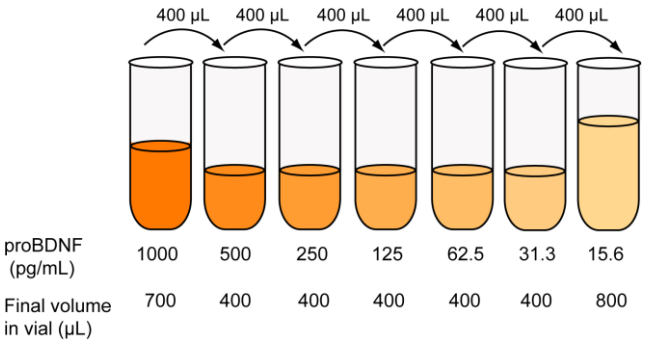
This proBDNF ELISA kit has been shown to be compatible with RIPA-based extraction methods (Riffault *et al.*, 2016). See **Appendix B f**or a detailed sample preparation protocol for RIPA-mediated tissue extraction.

7. Preparation of proBDNF Standard

- Reconstitute the lyophilized antigen standard with 1.1 mL of the **same diluent used for preparing sample dilutions**
- Label the vial with the reconstituted proBDNF standard as 1000 pg/mL
- Vortex and let stand for 15 minutes.

Perform a 1:2 serial dilution down to 15.6 pg/mL. The volumes used for the dilution series depends on the number of repeats per proBDNF concentration. For triplicate measurement (100 µL per well) of each proBDNF standard concentration, we recommend this procedure:

1. Label 6 tubes with “500 pg/mL”, “250 pg/mL”, “125 pg/mL”, “62.5 pg/mL”, “31.3 pg/mL” and “15.6 pg/mL”, respectively
2. Aliquot 400 µL of the diluent into each tube except the tube labeled “1000 pg/mL”
3. Take 400 µL from the “1000 pg/mL” tube and transfer to the “500 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 “15.6 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 proBDNF standard curve**.
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent A; **do not use culture medium or assay diluent A enriched with HA blocker**; 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 proBDNF standards, QC sample, samples and blank (assay diluent only) to the pre-coated microplate wells
2. If available, include a 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 90 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 60 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 3
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 10-15 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 C 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 proBDNF 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 10-15 minutes is usually sufficient to obtain a very sensitive standard curve. 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 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.

11. Calculation of Results

1. Average the readings for each proBDNF standard concentration, blank and sample
2. Plot a standard curve with the proBDNF standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the proBDNF 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 proBDNF in the QC sample. An observed concentration within the range of 350 – 650 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

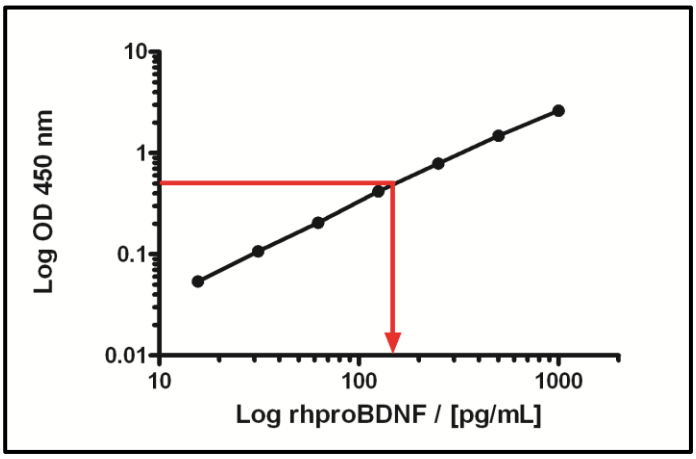
The relative OD_{450} = (the OD_{450} of each well) – (the OD_{450} of Zero well).

1. The **standard curve** can be plotted as the relative OD_{450} of each proBDNF standard solution (Y-axis) vs. the respective known concentration of the proBDNF 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 proBDNF 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 proBDNF in the unknown sample. In the exemplary standard curve on the right, a sample with OD_{450} = 0.5 reads as 160 pg/mL proBDNF (red line). If the samples measured were diluted, multiply the concentrations from interpolation (see step 1) with the dilution factor to obtain the actual proBDNF 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 proBDNF 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 above example graph, proBDNF 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 proBDNF standards are summarized in the following table:

	OD 450 nm		
proBDNF / [pg/mL]	**Mean**	**SD**	**CV**
1000	2.623	0.046	1.67 %
500	1.484	0.007	0.46 %
250	0.789	0.005	0.57 %
125	0.418	0.001	0.19 %
62.5	0.205	0.008	2.41 %
31.3	0.107	0.003	1.47 %
15.6	0.054	0.003	1.59 %
Blank	0.000 (0.115)	0.001	0.43 %

SD: standard deviation; CV: coefficient of variation

Limit of Detection

This proBDNF ELISA kit typically detects a minimum of 16 pg/mL proBDNF (defined as 150% of blank value).

Quantification and Recovery in Cell Lysates

C6 cell lysates (10 x 10^6 cells) were prepared in RIPA buffer (1 mL) according to Section 5 and proBDNF quantified at 1:10 dilution with Assay Diluent A.

	proBDNF / [pg/mL]
Assay 1	680
Assay 2	729

500 pg/mL proBDNF were spiked into RIPA buffer at different dilutions and recovery of proBDNF determined. The table below shows that the assay antibodies are compatible with RIPA buffer.

Dilution	*Recovery of BDNF*
1/2.5	76%
1/5	94%
1/50	94%

Quantification of proBDNF in Human Serum (1)

Three normal serum samples were assayed for proBDNF content, in presence and absence of HA blocker BL-004-500.

Sample	Dilution	proBDNF (ng/mL)		
		- Blocker	+ Blocker	Mean (+ Blocker)
1	1:10	1.70	0.35	0.37
	1:20	0.90	0.39	
2	1:10	3.40	0.67	0.61
	1:20	1.78	0.54	
3	1:10	0.20	< LOD	< LOD
	1:20	< LOD	< LOD	

Without the addition of BL-004-500, sample 1 and 2 show high apparent levels of proBDNF, which change upon sample dilution. In presence of HA blocker, false-positive OD readings are reduced and proBDNF concentrations are determined accurately.

Quantification of proBDNF in Human Serum (2)

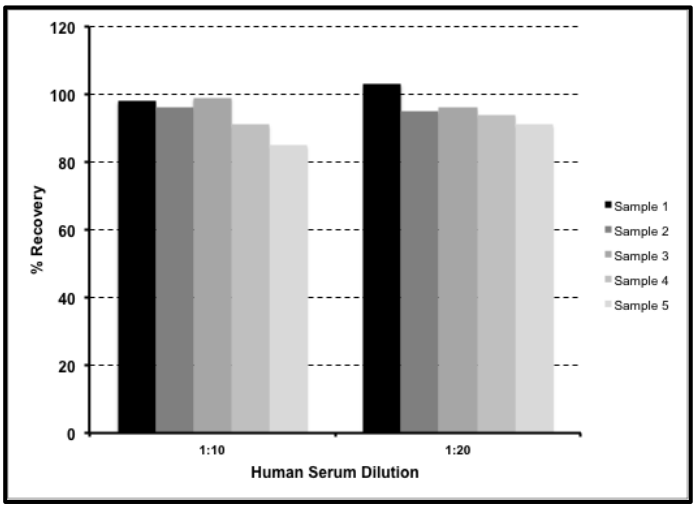
Seven normal serum samples were assayed for proBDNF content using the optimized sample diluent containing HA blocker BL-004-500.

Sample	Dilution	proBDNF (ng/mL)	
			Mean
1	1:10	< LOD	< LOD
	1:20	< LOD	
2	1:10	< LOD	< LOD
	1:20	< LOD	
3	1:10	< LOD	< LOD
	1:20	< LOD	
4	1:10	< LOD	< LOD
	1:20	< LOD	
5	1:10	< LOD	< LOD
	1:20	< LOD	
6	1:10	< LOD	< LOD
	1:20	< LOD	
7	1:10	0.34	0.33
	1:20	0.32	

Overall, the proBDNF content in 10 human serum samples ranged from not detectable (< LOD) to 0.61 ng/mL.

Recovery of proBDNF in Human Serum

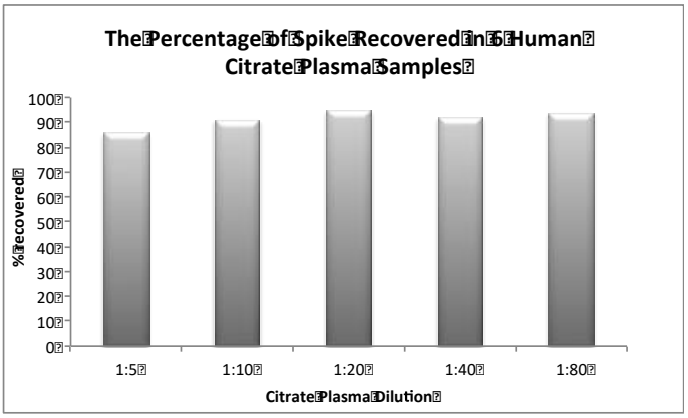
Five normal human serum samples were diluted 1:10 and 1:20 with the optimized serum sample diluent and spiked with 200 ng/mL of the supplied proBDNF protein standard.



The recovery of spiked proBDNF ranged from 85% - 103%. In summary, the use of BL-004-500 is strongly recommended to accurately determine proBDNF concentrations in human serum, with a minimum required dilution of 1:10.

Recovery of proBDNF Spike in Human Citrate Plasma

Six human citrate plasma samples were spiked with proBDNF (250 pg/mL) and then diluted 1/5 or 1/10 in Assay Diluent enriched with BL-004-500. The samples were then further diluted 2-fold and assayed in the proBDNF ELISA. Results are shown as the mean of six separate human citrate plasma samples at each dilution in the following table.



The recovery of the spikes was between 77-115% in the human citrate plasma diluted in sample diluent containing the heterophilic antibody blocker BL-004-500.

Quantification of proBDNF in Human Citrate Plasma

Six human citrate plasma samples were assayed for proBDNF content using the optimized sample diluent containing HA blocker BL-004-500. The mean of the proBDNF concentration detected in the six samples is shown in the following table.

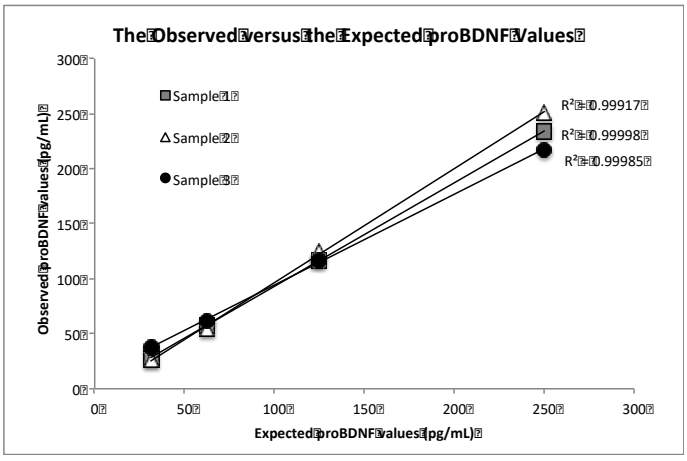
Sample	*proBDNF (pg/mL)*
1	< LOD
2	< LOD
3	765
4	< LOD
5	< LOD
6	< LOD

< LOD: Below the limit of detection

The proBDNF concentration detected in the six human citrate plasma samples ranged from, below the limit of detection (< LOD) in the ELISA to 765 pg/mL.

Linearity of Dilution of Human Citrate Plasma

A spike of proBDNF (250 pg/mL) was added to three separate human citrate plasma samples at a 1:5 dilution in sample diluent containing the Biosensis heterophilic antibody blocker # BL-004-500 (Section 6). These samples were then serially diluted (1:5 – 1:80) in sample diluent. The observed or measured proBDNF values were plotted against the expected proBDNF values.



The above graph demonstrates linearity of dilution across the tested dilution range in citrate-plasma samples. In

summary, spike-recovery and linearity of dilution experiments show that proBDNF concentrations can be accurately determined in human citrate-plasma, if samples are diluted at least 1:5 with Assay Diluent A enriched with HA blocker BL-004-500.

Specificity

The capture antibody used in this ELISA kit binds to epitopes within the pro-domain of proBDNF. Thus, this ELISA detects the full length and potentially truncated form of proBDNF.

A cross-reactivity of 2% in weight concentration (0.9% in molar concentration) has been observed for mature BDNF assayed at 25 ng/mL (893 pmol/L) in Assay Diluent A.

Due to a high degree of sequence homology, this human proBDNF ELISA kit cross-reacts with the mouse and rat form of proBDNF. Other species have not yet been tested, but cross-reactivity with a wide range of mammalian forms of proBDNF is expected.

13. Specific References

Riffault B *et al.*, 2016. *Pro-Brain-Derived Neurotrophic Factor (proBDNF)-Mediated p75NTR Activation Promotes Depolarizing Actions of GABA and Increases Susceptibility to Epileptic Seizures.* Cereb. Cortex. [Epub ahead of print]. **Application: Rat cortical and hippocampal RIPA tissue extracts.**

Hashimoto T *et al.*, 2016. *Effect of mirtazapine versus selective serotonin reuptake inhibitors on benzodiazepine use in patients with major depressive disorder: a pragmatic, multicenter, open-label, randomized, active-controlled, 24-week trial*. Ann Gen Psychiatry. 15(27). **Application: Human serum**.

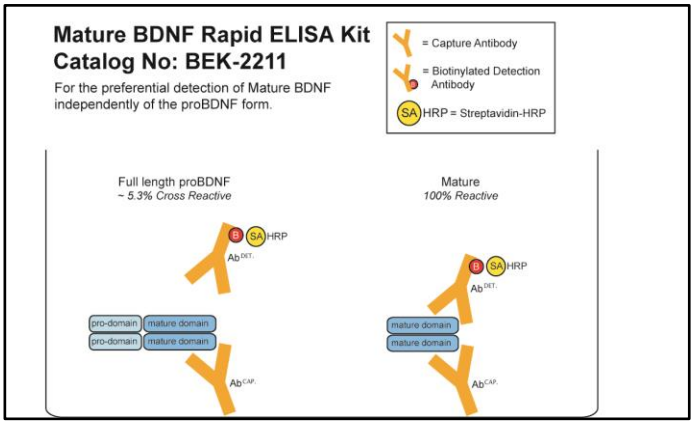
Niimi M *et al.*, 2016. *Role of Brain-Derived Neurotrophic Factor in Beneficial Effects of Repetitive Transcranial Magnetic Stimulation for Upper Limb Hemiparesis after Stroke*. PLos One. 11(3):e0152241. **Application: Human serum**.

Stary CM *et al.*, 2015. *Astrocytes Protect against Isoflurane Neurotoxicity by Buffering pro-brain-derived Neurotrophic Factor*. Anesthesiology. 123(4):810-9. **Application: Rat tissue culture supernatants (neuron and astrocyte cell cultures).**

Riffault B et al., 2014. *Pro-Brain-Derived Neurotrophic Factor Inhibits GABAergic Neurotransmission by Activating Endocytosis and Repression of GABAA Receptors.* J Neurosci. 34(4):13516-34. **Application: Rat tissue culture supernatants (hippocampal cultures).**

14. Related Products

Biosensis offers the Mature BDNF *Rapid*™ ELISA kit (Cat# BEK-2211-1P/2P) in order to quantify mature BDNF with only minimal cross-reactivity of proBDNF full-length protein.



Heterophilic antibody (HA) blocker cat# BL-004-500 has been specifically developed to reduce or eliminate HA interference when measuring proBDNF in serum and citrate-plasma. This blocker is available for purchase separately. Please visit our website www.biosensis.com for a full range of BDNF/proBDNF research reagents.

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

Standard curve, blank and controls:

- Standard (1000 pg/mL. 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 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 samples) 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 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 samples on the "standard curve plate" (the plate containing the standard curve, controls and blanks) and a full 96 test samples on the "test only plate" for a total available number of 174 tests 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 test samples, duplicate measurement at one single dilution
How many wells and plates do I need for sixty test samples at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 samples, 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 test samples. In summary: 60 samples, 4 tests per sample, 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 test sample 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 test samples, tested in duplicate (two draws per sample, 1 dilution, 4 wells per test, 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 test samples tested in duplicate at two dilutions (two draws per test, 2 dilutions, 8 tests/wells per test, total of 480 tests/wells).

2-Plate Optional Single Control Set Option:

If running 60 test samples 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 test samples, 2 draws per sample 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 test samples.

For the two dilutions per sample, sixty test samples, 2 draws per sample 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 test samples.

Appendix B: 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 C: Troubleshooting Guide

This proBDNF 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 proBDNF in the sample is lower than the detection limit of this assay	Confirm that the assay protocol was carried out correctly by either spiking a known amount of proBDNF into your sample and/or check that the QC sample value falls within the expected proBDNF 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	ProBDNF 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