proBDNF ELISA Kit

Enzyme Immunoassay for the quantification of proBDNF in Human and rat cell culture supernatants and cell lysates; human serum and plasma (EDTA, citrate) and Rat tissue extracts

Catalog number: ARG81240

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INTRODUCTION

BDNF is a member of the nerve growth factor family. It is induced by cortical neurons, and is necessary for survival of striatal neurons in the brain. Expression of this gene is reduced in both Alzheimer's and Huntington disease patients. This gene may play a role in the regulation of stress response and in the biology of mood disorders. Multiple transcript variants encoding distinct isoforms have been described for this gene. [provided by RefSeq, Jan 2009]

During development, BDNF promotes the survival and differentiation of selected neuronal populations of the peripheral and central nervous systems. Participates in axonal growth, pathfinding and in the modulation of dendritic growth and morphology. Major regulator of synaptic transmission and plasticity at adult synapses in many regions of the CNS. The versatility of BDNF is emphasized by its contribution to a range of adaptive neuronal responses including long-term potentiation (LTP), long-term depression (LTD), certain forms of short-term synaptic plasticity, as well as homeostatic regulation of intrinsic neuronal excitability. [UniProt]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for proBDNF has been pre-coated onto a microtiter plate. Standards or samples are pipetted into the wells and any proBDNF present is bound by the immobilized antibody. After washing away any unbound substances, a biotin-conjugated antibody specific for mature BDNF is added to each well and incubate. Following a washing to remove unbound substances, streptavidin conjugated to Horseradish Peroxidase (HRP) is added to each

microplate well and incubated. After washing away any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and color develops in proportion to the amount of proBDNF bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450nm ±2nm. The concentration of proBDNF in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8°C. Use the kit before expiration date.

Expiration date: twelve months at 4°C.

Component	Quantity	Storage information
Antibody-coated microplate	8 X 12 strips	4°C. Unused strips should be sealed tightly in the air-tight pouch.
Standard (Lyophilized)	2 X 1.1 ng/vial	4°C
Control (350 - 650 pg/mL)	2 vial	4°C
Assay diluent	2 X 25 ml (Ready to use)	4°C
Antibody conjugate concentrate (100X)	1 vial (110 µl)	4°C
HRP-Streptavidin concentrate (100X)	1 vial (110 µl)	4°C
Blocker	1 vial	4°C
10 X Wash Buffer	33 ml	4°C
TMB substrate	11 ml (Ready to use)	4°C (Protect from light)
STOP solution	11 ml (Ready to use)	4°C

Plate sealer	5	Room temperature

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Pipettes and pipette tips
- Deionized or distilled water
- 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.
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- Reconstituted standard and Control sample should be used on same day. The reconstituted standard should be aliquoted and store at-20°C for up to 2 weeks. Avoid repeated freeze-thaw cycles.
- To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, a pilot experiment using standards and a small number of samples is recommended.
- The TMB Color developing agent should be colorless and transparent before using.
- Ensure complete reconstitution and dilution of reagents prior to use.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.

- If crystals are observed in the 10X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- Do not let strips dry, as this will inactivate active components in wells.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.
- Avoid using reagents from different batches.
- 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.
- All materials should be equilibrated to room temperature (RT, 20-25°C) before use.
- Avoid touching the inside surface of the wells with the pipette tip.
- Add TMB and the stop solution to the wells in the same order.
- Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading.
- Samples contain azide cannot be assayed.

SAMPLE COLLECTION & STORAGE INFORMATION

- The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.
- ProBDNF is readily processed into its mature form by enzymatic cleavage. Thus, sample preparation may require the addition of protease inhibitors such as protease inhibitor cocktail or protease inhibitor cocktail plus aprotinin to improve detection and recovery results from some samples.
- The Assay Diluent provided in this kit is suitable for measuring proBDNF in cell lysates, cell culture supernatants, human serum, citrate and EDTA plasma, and tissue extracts. Blood samples (serum/plasma) require the addition of a heterophilic antibody blocking reagent (Blocker) to minimize matrix interference.

Cell Culture Supernatants –

- Remove particulates by centrifugation for 5 min at 10,000 x g at 4°C. Collect the supernatants and assay immediately or aliquot & store samples at -20°C to -80°C. Avoid repeated freeze-thaw cycles.

- Protease inhibitors such as **protease inhibitor cocktail or protease inhibitor cocktail plus aprotinin is recommended** to be used to prevent ProBDNF degradation.

- 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.

- 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 and NSC34 cells lysed with a RIPA buffer (Please refer to MATERIALS REQUIRED BUT NOT PROVIDED).

- 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 prior to running the assay with RIPA buffers. In our testing a dilution of at least 1:10 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.

- Add protease inhibitor cocktail (add additional aprotinin is recommended) in RIPA buffer just prior to cell lysis. Resuspend cell pellet in ice-cold RIPA buffer, do not add reducing agents such as DTT or β-mercaptoethanol in the buffer.

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

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot & store samples at -20°C to-80°C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g. within 30 minutes of collection. Collect the plasma. For eliminating the platelet effect we suggest further centrifugation for 10 min at 2-8°C at 10,000 x g. Collect the supernatants and assay immediately or aliquot and store samples at -20°C to-80°C. Avoid repeated freeze-thaw cycles.

Note for Serum/plasma samples:
- Serum and plasma sample should be diluted with Serum/Plasma Sample Dilution Buffer (which with blocker, please refer the REAGENT PREPARATION) to reduce or eliminate heterophilic antibody (HA) interferences.
- Normal human serum and citrate-plasma should be diluted at least 1:10 dilution, and EDTA-plasma sample might be diluted at least 1:20 dilution to

avoid matrix interferences and achieve acceptable recoveries of spiked proBDNF.

- Do not use haemolytic, icteric or lipaemic specimens.
- Do not use acid treatment of serum and plasma samples
- Samples containing sodium azide should not be used in the assay.
- 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 or protease inhibitor cocktail to improve detection and recovery results from some samples.

Tissue Extracts-

This proBDNF ELISA kit has been tested on rat brain homogenate using a RIPA-based extraction method. Mouse brain tissue has not been tested as yet.

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

(It is recommended to do pre-test and recovery testing for all type of samples to determine the suitable dilution factor).

REAGENT PREPARATION

- Serum/Plasma Sample Dilution Buffer:

  - Reconstitute Blocker in 1 mL of Assay Diluent to give an IgG concentration of 500 µg/mL, mix gently by vortex.

  - Add the 1 mL of reconstituted Bloker to 24 mL of Assay Diluent to a final volume of 25 mL, mix well.

  - Use this Serum/Plasma Sample Diluent to prepare all human serum, citrate and EDTA plasma samples, and also protein standard dilutions when testing blood samples.

  - Do not use Serum/Plasma Sample Dilution Buffer to dilute detection antibody and HRP-conjugate.

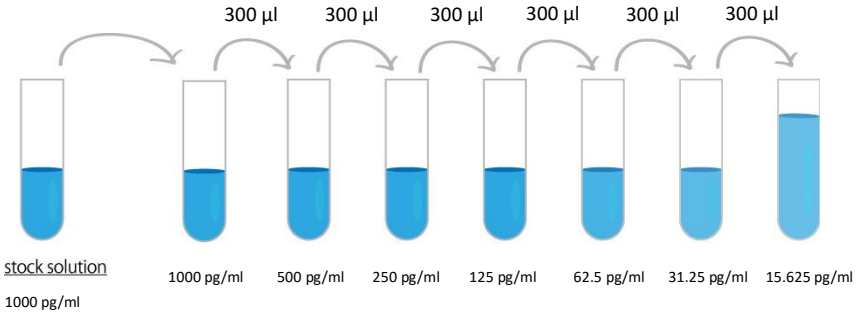
  - This Serum/Plasma Sample Dilution Buffer is stable for two weeks at 2°C to 8°C.

- 1X Wash Buffer: Dilute 10X wash buffer with ultrapure water to yield 1X wash buffer. The diluted 1X wash buffer is stable for two weeks at 2°C to 8°C.
- Control sample: Reconstitute the lyophilized Control with 1 mL of the same diluent used for preparing the proBDNF standard curve. (Assay Diluent for cell lysates, cell culture supernatants, and tissue extracts; Serum/Plasma Sample Dilution Buffer for serum and plasma samples). Accepted concentration range: 350 – 650 pg/mL
- 1X Antibody conjugate and 1X HRP-Streptavidin Solution: It is recommended to prepare this reagent immediately prior to use and use it within 2 hours after preparation. Dilute 100X antibody conjugate

concentrate or 100X HRP-Streptavidin Solution concentrate into Assay Diluent to yield 1X working solution. (e.g. 10 µl of 100X antibody conjugate concentrate + 990 µl of Assay Diluent) The diluted 1X Antibody conjugate and 1X HRP-Streptavidin Solution can be store at 2°C to 8°C up to two weeks.

- Sample: If the initial assay found samples contain proBDNF higher than the highest standard, the samples can be diluted with Assay Diluent for cell lysates, cell culture supernatants, and tissue extracts and diluted with Serum/Plasma Sample Dilution Buffer for serum and plasma samples and then re-assay the samples. For the calculation of the concentrations this dilution factor also has to be taken into account. The sample must be well mixed with the diluent buffer before assay.
  (It is recommended to do pre-test to determine the suitable dilution factor).
- Standards: Reconstitute the standard with 1.1ml of the same diluent as sample dilution (Assay Diluent for cell lysates, cell culture supernatants, and tissue extracts; Serum/Plasma Sample Dilution Buffer for serum and plasma samples) to yield a stock concentration of 1000 pg/ml. Allow the stock standard to sit for at least 15 minutes with gentle agitation to make sure the standard is dissolved completely before making serial dilutions. The diluent only serves as zero standard (0 pg/ml), and the rest of the standard serial dilution can be diluted with Standard/ Sample diluent as according to the suggested concentration below: 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, 15.625 pg/ml.
  Note: The reconstituted standard solutions are best used within 2 hours.

The stock standard solution should be aliquoted & stored at-20°C for up to 2 weeks. Avoid repeated freeze-thaw cycles.



Dilute proBDNF standard as according to the table below:

Standard	proBDNF Conc. (pg/ml)	µl of diluent	µl of standard
S7	1000 pg/ml	1100	(1000 pg/ml Stock)
S6	500 pg/ml	300	300 (S7)
S5	250 pg/ml	300	300 (S6)
S4	125 pg/ml	300	300 (S5)
S3	62.5 pg/ml	300	300 (S4)
S2	31.25 pg/ml	300	300 (S3)
S1	15.625 pg/ml	300	300 (S2)
S0	0	300	0

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-25°C) before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard proBDNF detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of proBDNF amount in samples. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add 100 µl of diluted samples, standards, and controls into wells.
3. (Optional) If available, include a negative and positive control sample in the assay procedure.
4. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate for 120 minutes at RT on a microplate shaker (140 rpm).
5. Aspirate each well and wash, repeating the process four times for a total five washes. Wash by filling each well with 1X Wash Buffer (200 µl) using a squirt bottle, manifold dispenser, or autowasher, keep the wash buffer in the wells for 1 min before remove. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels for 3-5 time. DO NOT let the wells completely dry at any time.
6. Add 100 µl 1X Antibody conjugate into each well. Cover wells and incubate for 30 minutes at RT on a microplate shaker (140 rpm).
7. Discard the solution inside the wells and wash as described in step 5.
8. Add 100 µl of 1X HRP-Streptavidin solution to each well. Cover wells and

incubate for 30 minutes at RT on a microplate shaker (140 rpm).

9. Discard the solution inside the wells and wash as described in step 5.
10. Add 100 µl of TMB substrate to each well. Incubate for 10-15 minutes at RT in dark without shaking.
11. Add 100 µl of Stop Solution to each well. The color of the solution should change from blue to yellow. Gently tap the plate to ensure thorough mixing.
12. Read the OD with a microplate reader at 450nm immediately. 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.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. The relative OD 450 = (the OD 450 of each well) – (the OD 450 of Zero well).

4. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

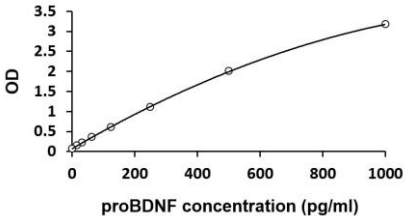
5. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

6. 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.

7. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of proBDNF ranged from 15.6- 1000 pg/ml. The mean MDD was 6 pg/ml (defined as 150% of blank value).

Specificity

This assay recognizes natural and recombinant full-length form of proBDNF. The assay antibodies are known to react with rat and mouse proBDNF, but the exact level of cross-reactivity of human with rodent proBDNF is unknown. In absence of a reliable rodent proBDNF protein standard, values for mouse or rat proBDNF may be reported as “human proBDNF equivalents”.

This ELISA kit detects the full length and potentially truncated form of proBDNF. Cross-reactivity mature BDNF (25 ng/mL) is < 0.3% (w/v).

Species Cross-Reactivity

Cross-reactivity with rodent proBDNF has been confirmed with rat brain, rat cell lysate and mouse cell lysate.

Recovery

A. Blood samples

	Human Serum	EDTA Plasma	Citrate Plasma
1/5	77%	49%	70%
1/10	83%	69%	81%
1/20	89%	85%	87%
1/40	97%	90%	98%

In summary, linearity-of-dilution and spike-recovery experiments in human blood samples show that proBDNF concentrations can be accurately determined in serum/plasma dilution buffer, and appropriate minimum required dilutions:

- Human serum: 1/10
- Human citrate-plasma: 1/10
- Human EDTA-plasma: 1/20

B. RIPA buffer

	RIPA
1/2.5	98%
1/5	99%
1/10	108%
1/40	100%

C. Cell lysate

	Rat C6 Cells lysate	Mouse NSC34 Cell lysate
1/5	65%	73%
1/10	81%	83%
1/20	89%	91%
1/40	92%	95%

proBDNF was detected in both cell lysate preparations demonstrating the detection of rodent proBDNF protein. A minimum required dilution of 1/10 is required, with higher dilutions performing more consistently as demonstrated by spike-recovery experiments.

Intra-assay and Inter-assay precision

The CV values of intra-assay was 2.5-6.7% and inter-assay was 1.5-6.4%.