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.

Table of Contents

1. Intended Use .......................................................................................................................... 2
2. Introduction ............................................................................................................................ 2
3. Materials Provided and Storage Conditions .......................................................................... 3
4. Equipment Required but Not Supplied .................................................................................. 3
5. Before You Start..................................................................................................................... 3
6. Sample Preparation ............................................................................................................... 3
7. Preparation of Mature BDNF Standard .................................................................................. 7
8. Other Reagents and Buffer Preparation ............................................................................... 7
9. Assay Procedure .................................................................................................................. 7
10. Technical Hints ................................................................................................................... 8
11. Calculation of Results ......................................................................................................... 8
12. Typical Data ....................................................................................................................... 9
13. Informational References .................................................................................................... 14
14. Specific References ............................................................................................................. 14
15. Other Information ............................................................................................................... 16
Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments ........ 17
Appendix B: Tissue Lysate Preparation Procedures Acid-Extraction of BDNF ............................... 19
Appendix C: Tissue Lysate Preparation Procedures RIPA Buffer ............................................... 20
Appendix D: Troubleshooting Guide .......................................................................................... 21
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 quantification of BDNF in human CSF samples please contact us sales@biosensis.com

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

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 and therefore recognize the mature and pro- form of BDNF. However, the full-length proBDNF protein is detected much less efficiently 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.
Protocol for Quantification of Human, Mouse and Rat Mature BDNF

3. Materials Provided and Storage Conditions

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

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

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 (slgA) 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](http://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](http://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 slgA blocker 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 pg/mL if required. This might be particular useful for samples with expected very low BDNF concentrations.

Also, with unknown samples of all types it is highly recommended to run some BDNF 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 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
Protocol for Quantification of Human, Mouse and Rat Mature BDNF

- Add 3 parts of neutralization buffer (0.1 mol/L phosphate buffer; 0.1 mol/L KH₂PO₄ + 0.1 mol/L Na₂HPO₄, 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)

Optimization 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

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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 and anti-coagulant used for collection (Tsuchimine et al., 2014). Thus to prevent sample variation, strict adherence to consistent sample preparation procedures among samples and study groups are required.

**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 ng/mL or even higher in some samples (Weaver et al., 1998). Secretory IgA can interfere with sandwich ELISA assays, falsely elevating sample OD readings and thus causing false-positive results.

Biosensis has developed a proprietary 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. Suplementing 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

**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 on normal, pooled human CSF demonstrated accurate readings with a minimum required sample dilution of 1:2 – 1:4, 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. For higher sensitivity applications, the dilution series can be extended to 3.9 pg/mL, with 250 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. If using a standard curve range between 3.9 – 250 pg/mL, the reconstituted QC sample needs to be further diluted 1:2 with diluent. This will provide a QC sample within 88 – 163 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. 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.

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 (or 88 – 163 pg/mL when using the extended standard curve range, see Section 7) 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 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:**

<table>
<thead>
<tr>
<th>BDNF / [pg/mL]</th>
<th>OD 450 nm</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>3.068</td>
<td>0.052</td>
<td>0.030</td>
<td>1.68%</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>2.147</td>
<td>0.019</td>
<td>0.011</td>
<td>0.90%</td>
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<tr>
<td>125</td>
<td>1.507</td>
<td>0.013</td>
<td>0.007</td>
<td>0.85%</td>
<td></td>
</tr>
<tr>
<td>62.5</td>
<td>0.902</td>
<td>0.024</td>
<td>0.014</td>
<td>2.70%</td>
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</tr>
<tr>
<td>31.3</td>
<td>0.546</td>
<td>0.010</td>
<td>0.006</td>
<td>1.91%</td>
<td></td>
</tr>
<tr>
<td>15.6</td>
<td>0.304</td>
<td>0.010</td>
<td>0.006</td>
<td>3.29%</td>
<td></td>
</tr>
<tr>
<td>7.8</td>
<td>0.183</td>
<td>0.003</td>
<td>0.002</td>
<td>1.66%</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>0.060</td>
<td>0.003</td>
<td>0.002</td>
<td>5.62%</td>
<td></td>
</tr>
</tbody>
</table>

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 150% of blank OD value).

**Assay Precision**

Two human serum samples were assayed in triplicates on 3 different days.

<table>
<thead>
<tr>
<th>BDNF / [ng/mL]</th>
<th>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>SEM</td>
</tr>
<tr>
<td>Day 1</td>
<td>25.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Day 2</td>
<td>26.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Day 3</td>
<td>24.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Day 1</td>
<td>24.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Day 2</td>
<td>25.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Day 3</td>
<td>22.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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

Linearity of Dilutions 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.

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.

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.

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

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Recovery of BDNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>56%</td>
</tr>
<tr>
<td>1/2.5</td>
<td>87%</td>
</tr>
<tr>
<td>1/5</td>
<td>100%</td>
</tr>
</tbody>
</table>

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.

OD values of serial diluted brain extract 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.

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

<table>
<thead>
<tr>
<th>BDNF / [ng/mL]</th>
<th>Total protein* / [mg/mL]</th>
<th>BDNF / [ng / mg extracted protein]</th>
<th>BDNF / [ng / g brain]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.67</td>
<td>1.30</td>
<td>2.82</td>
<td>36.6</td>
</tr>
</tbody>
</table>

* 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC12 Lysate (RIPA)</td>
<td>1/5</td>
<td>106</td>
</tr>
<tr>
<td>PC12 Lysate (Freeze-Thaw in hypotonic buffer)</td>
<td>1/5</td>
<td>101</td>
</tr>
<tr>
<td>PC12 Lysate (Freeze-thaw in acid-extraction buffer)</td>
<td>1/5</td>
<td>117</td>
</tr>
</tbody>
</table>

* Mean of 2 assays

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.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>pg BDNF / mg total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/5</td>
<td>64.5</td>
</tr>
<tr>
<td>1/10</td>
<td>84.0</td>
</tr>
<tr>
<td>1/20</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>pg BDNF / mg total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/25</td>
<td>153.7</td>
</tr>
<tr>
<td>1/125</td>
<td>150.0</td>
</tr>
<tr>
<td>1/625</td>
<td>196.3</td>
</tr>
</tbody>
</table>

Average 166.7
Quantification of BDNF in Human Milk - Effect of BL-001-1250 on sIgA Cross-Reactivity

The ability of 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.

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 parallelism between calibration curve and diluted BDNF in human milk (A), as well as 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.

<table>
<thead>
<tr>
<th>CSF Dilution</th>
<th>% BDNF conc. of previous dilution</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>-</td>
<td>85%</td>
</tr>
<tr>
<td>1:4</td>
<td>100%</td>
<td>85%</td>
</tr>
<tr>
<td>1:8</td>
<td>109%</td>
<td>93%</td>
</tr>
<tr>
<td>1:16</td>
<td>105%</td>
<td>97%</td>
</tr>
<tr>
<td>1:32</td>
<td>105%</td>
<td>105%</td>
</tr>
</tbody>
</table>

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 (VEGF165) tested at 25 ng/mL in assay buffer.

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). This further demonstrates that proBDNF does not interfere in this ELISA assay and concentrations obtained are due to measurement of mature BDNF alone.

13. Informational References


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

14. Specific References


Perricos A et al., 2017. Increased serum levels of mBDNF in women with minimal and mild endometriosis have no predictive power for the disease. Exp Biol Med (Maywood) [Epub ahead of print]. Application: Human serum.


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.


Protocol for Quantification of Human, Mouse and Rat Mature BDNF


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) as published by researchers from Professor Y.-A. Barde’s laboratory. Our ELISA assay has been validated using this protocol.

Acid-Extraction Buffer

50 mmol/L sodium acetate, 1 mol/L NaCl, 0.1% Triton X100, add 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

0.1 mol/L phosphate buffer, pH 7.6. For instance, weigh in the following amounts per 1 L:
KH₂PO₄ (MW 136.09 g/mol): 2.72 g
Na₂HPO₄ (MW 141.96 g/mol): 2.84 g

Acid-Extraction Sample Diluent

Mix acid-extraction buffer and incubation/neutralization buffer at a ratio of 1 to 3 (e.g., 1 mL of extraction buffer and 3 mL of incubation/neutralization buffer). Check pH of solution is approximately pH 6.8 to 7.2.

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 (e.g., 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 (e.g., 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. Check diluted sample’s pH is near neutral before using them in the ELISA.
13. Results can then be reported as ng BDNF/mg total soluble protein or g wet weight if tissue sample is large enough

Note: Biosenss 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: 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).

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>High background (blank OD &gt; 0.30)</td>
<td>Insufficient washing</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>Excessive concentration of detection antibody and/or HRP-conjugate</td>
<td>Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume.</td>
</tr>
<tr>
<td></td>
<td>Increased incubation time and temperature</td>
<td>Please follow incubation times as stated in the protocol and perform incubations at room temperature</td>
</tr>
<tr>
<td></td>
<td>Contamination</td>
<td>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</td>
</tr>
<tr>
<td>Low absorbance readings</td>
<td>Concentration of BDNF in the sample is lower than the detection limit of this assay</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>Insufficient antibody or insufficient HRP-conjugate</td>
<td>Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume</td>
</tr>
<tr>
<td></td>
<td>Reagents expired</td>
<td>Ensure correct storage of the kit and do not use the kit beyond its expiry date</td>
</tr>
<tr>
<td>Low absorbance readings</td>
<td>Decreased incubation times and temperature</td>
<td>Please follow incubation times as stated in the protocol and warm reagents to room temperature before use</td>
</tr>
<tr>
<td></td>
<td>Microplate wells dried out</td>
<td>Do not leave microplate wells without solution for an extended time</td>
</tr>
<tr>
<td></td>
<td>Wash buffer not diluted</td>
<td>Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution</td>
</tr>
<tr>
<td></td>
<td>Stop solution not added</td>
<td>Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm</td>
</tr>
<tr>
<td>Standard OD values above plate reader limit</td>
<td>Excessive incubation with TMB substrate solution</td>
<td>Reduce incubation time by stopping the reaction at an earlier time-point</td>
</tr>
<tr>
<td>Sample OD values above standard curve range</td>
<td>BDNF concentration in sample is too high</td>
<td>Perform a serial dilution of your sample to obtain a reading that falls within the standard curve</td>
</tr>
<tr>
<td>High coefficient of variations (CV)</td>
<td>TMB and stopping solution not added consistently</td>
<td>Add TMB and stopping solution to the wells in the same order and speed</td>
</tr>
<tr>
<td></td>
<td>Inconsistent pipetting</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>Insufficient mixing of reagents</td>
<td>Briefly vortex to mix solutions before pipetting into the wells</td>
</tr>
<tr>
<td></td>
<td>Bottom of the plate is dirty</td>
<td>Clean the bottom of the plate before reading the plate</td>
</tr>
</tbody>
</table>