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biosensis® proNGF *Rapid*™ ELISA Kit: Mouse, Rat

Catalogue Number: BEK-2236-1P/2P

For the quantitative determination of mouse or rat proNGF in cell culture supernatants, cell lysates and brain tissue extracts only if used as directed.

Please refer to the Sample Preparation Section for specific use instructions for each substrate application.

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

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1. Intended Use

The purpose of this kit is the quantitative determination of mouse or rat proNGF in cell culture supernatants, cell lysates and brain tissue extracts only if used as directed. This kit has also been successfully used on mouse urine (Ryu JC *et al.*, 2018) and rat urine samples (Mossa A *et al.*, 2021), but has not been tested for other sample applications. Non-validated samples may contain immunoglobulin's and other components that potentially interfere with this ELISA assay. Biosensis does not assume responsibility if this kit is used for unintended purposes.

This ELISA kit includes a mouse proNGF protein standard and has been tested on rat brain tissue and rat PC12 cell lysates. The antibodies used in the kit detect mouse and rat proNGF equally well due to high degree of homology (96%) based on amino acid sequence. In the absence of a true rat proNGF standard, however, results for rat may be expressed as "mouse proNGF equivalents".

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

2. Introduction

The full-length nerve growth factor protein proNGF is a homodimer that belongs to the neurotrophin family of growth factors that regulate neuronal proliferation and differentiation. ProNGF can be cleaved to mature NGF, which leads to opposing effects on cell apoptosis (proNGF via p75-sortilin complex) and cell survival (mature NGF via TrkA receptor). Overexpression of proNGF is linked to invasion of breast cancer cells (Demont *et al.*, 2012) and nerve infiltration in prostate cancer (Pundavela *et al.*, 2014) making proNGF a strong biomarker candidate. While the actions of mature NGF have been studied extensively, the roles of the pro-form have been more difficult to unravel, partly due to lack of methods for sensitive and specific detection of proNGF.

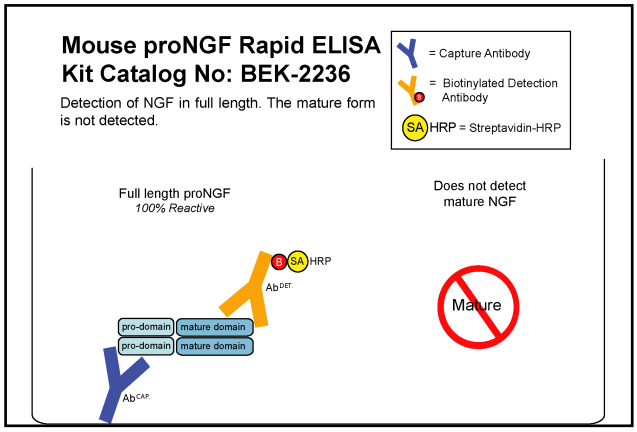
As for many other protein targets, studying the role of proNGF in animal models helps to better understand its actions in humans. The Biosensis Mouse/Rat proNGF *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the quantification of mouse and rat full-length proNGF protein in less than 4 hours.

This kit contains a recombinant mouse proNGF standard expressed in *E.coli* and consists of a pre-coated anti-proNGF capture antibody, a biotinylated anti-NGF 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 proNGF present in samples and protein standards.

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

This ELISA kit shows only 20% reactivity with human proNGF and is therefore not suitable to quantify human proNGF. No cross-reactivity was observed with mature mouse NGF and full-length proBDNF when spiked into assay buffer. Mature NGF did not interfere with proNGF quantification in rat brain homogenates. Absence of mature NGF intereference has also been independently validated by Mossa A *et al.*, 2021.

The antibodies used in this ELISA kit bind epitopes within the pro-domain (capture) and mature domain (detection) of the protein, thus this ELISA assay does not detect the pro-domain peptide.



3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
proNGF antibody coated 96 well microplate	12 x 8 wells	24 x 8 wells
Assay diluent D (1x)	2 x 25 mL	4 x 25 mL
Recombinant mouse proNGF standard	2 x 20 ng	4 x 20 ng
Quality Control (QC) Sample	2 Vials (see vial label for amount)	4 Vials (see vial label for amount)
Mouse NGF detection antibody (100x)	1 x 110 µL	2 x 110 µL
Streptavidin-HRP (100x)	1 x 110 µL	2 x 110 µL
Wash buffer (10x)	1 x 33 mL	2 x 33 mL
TMB substrate (1x)	1 x 11 mL	2 x 11 mL
TMB stop solution (1x)	1 x 11 mL	2 x 11 mL
Plate sealer	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard and QC sample	Use within 1 hour of reconstitution; remaining standard may be aliquoted and reused if stored at -20°C for up to 1 week; vortex after thawing
Diluted detection antibody and HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer (1x)	2 weeks at 2-8°C

Notes:

- ProNGF degrades rapidly; prepare samples accordingly and do not freeze-thaw
- 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 proNGF in cell lysates, cell culture supernatants and brain extracts (TRIS- or RIPA-extracts). Note that the ELISA kit antibodies are not compatible with acid-extraction procedures.

See the following sample preparation guidelines for specific substrate preparation recommendations and sample dilution instructions. Final working pH of any assay sample should be near neutral (pH 7.0-8.0) for best results, adjust with mild acid or base as needed.

For unknown concentrations of proNGF in samples, it is important to perform several dilutions of the sample to allow the proNGF concentration to fall within the range of the proNGF standard curve (0.156-10 ng/mL). Also, with unknown samples of all types it is highly recommended to run some validation experiments. This should include

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

Cell Culture Supernatants

- Remove particulates by centrifugation (10,000 x g for 5 minutes)
- Analyze immediately or store at -20°C to -80°C in aliquots to prevent multiple freeze thaw cycles
- Samples with high FBS or proteinaceous content will require to be diluted with plain culture medium and/or Assay Diluent D for best results. Appropriate serum free, cell-free controls must be used for accurate detection.

Note:

- proNGF is readily processed into its mature form in presence of various proteases, thus the addition of a protease inhibitor cocktail is recommended
- Quantification of low levels of proNGF in cell culture supernatants may require concentrating the supernatant in ultrafiltration devices with filters of appropriate molecular weight cut-off (MWCO)

Cell Lysates

Cell lysis can be accomplished by a variety of methods (eg., chemical or mechanical). This ELISA kit has been tested on PC12 cells lysed with RIPA buffer or by freeze-thaw cycles in a hypotonic salt 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 D 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.

RIPA-mediated Cell Lysis

- Harvest whole cells from cell culture flask after washing off cell culture medium with ice-cold PBS
- Lyse cells 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
- The addition of a protease inhibitor cocktail to RIPA buffer just prior to cell lysis is highly recommended
- Lyse the cells on ice for 30 minutes

Cell Lysis by Freeze-Thawing

- 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)
- The addition of a protease inhibitor cocktail to cell lysis buffer and PBS is highly recommended

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 at least 1/10 with Assay Diluent D to quantify proNGF

Note: As an alternative, freeze whole cell pellets and store at -80°C for cell lysis at later time-point.

Brain Tissue Extracts

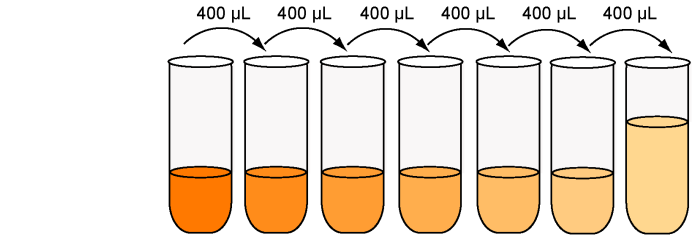
There are several published protocols on the extraction of neurotrophins from brain tissue, however internal testing and validation assays suggests RIPA or a neutral buffer (eg., TRIS pH 7.2-8.0) with mechanical cell membrane disruption (eg., bead-based) to be the preferred methods. Acid-extraction is not recommended to quantify proNGF with this kit. Typical dilutions with Assay Diluent D are 1:4 – 1:10 for increased assay accuracy, but higher dilutions might be required for samples with high levels of proNGF. See Appendix B for detailed sample preparation protocols for RIPA and TRIS-based buffers.

7. Preparation of proNGF Standard

- Reconstitute the lyophilized antigen standard with 1 mL of the **same diluent used for preparing sample dilutions**
- Label the vial with the reconstituted proNGF standard as “20 ng/mL”; vortex and let stand for 15 minutes
- Dilute the 20 ng/mL proNGF standard 1:2 (eg., 400 µL of 20 ng/mL standard + 400 µL diluent); label this tube “10 ng/mL”
- **Note:** 10 ng/mL is the highest concentration of the proNGF standard curve

In order to generate a proNGF standard curve, perform a 1:2 serial dilution down to 0.156 ng/mL. The volumes used for the dilution series depends on the number of repeats per proNGF concentration. For triplicate measurement (100 µL per well) of each proNGF standard concentration, we recommend this procedure:

1. Label 6 tubes with “5 ng/mL”, “2.5 ng/mL”, “1.25 ng/mL”, “0.625 ng/mL”, “0.313 ng/mL” and “0.156 ng/mL”, respectively
2. Aliquot 400 µL of the diluent into each tube
3. Take 400 µL from the “10 ng/mL” tube and transfer to the “5 ng/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 “0.156 ng/mL” is prepared and mixed well



proNGF (ng/mL)	10	5	2.5	1.25	0.625	0.313	0.156
Final volume in vial (µL)	400	400	400	400	400	400	800

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 mouse proNGF Standard curve**. This will provide a QC sample within 1.75 – 3.25 ng/mL.
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent D; **do not use cell culture medium to dilute these reagents** 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 proNGF standards, QC sample, samples and blank (sample 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 2 hours
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 4
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 10-25 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 proNGF 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 10-25 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.

11. Calculation of Results

1. Average the readings for each proNGF standard concentration, blank and sample
2. Plot a standard curve with the proNGF standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the proNGF 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 proNGF in the QC sample. An observed concentration within the range of 1.75 – 3.25 ng/mL indicates acceptable assay performance
6. Perform a regression analysis to calculate the concentration of proNGF in the samples. Multiply the result by the sample dilution factor

Manual Plate Reading

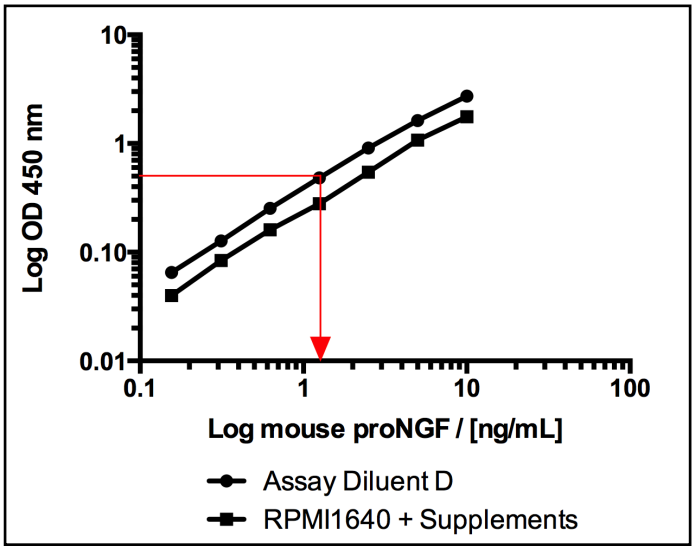
The relative OD_{450} = (the OD_{450} of each well) – (the OD_{450} of Zero well).

1. The **standard curve** can be plotted as the relative OD_{450} of each proNGF standard solution (Y-axis) vs. the respective known concentration of the proNGF 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 proNGF 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 proNGF in the unknown sample (red line). If the samples measured were diluted, multiply the concentrations from interpolation (see step 1) with the dilution factor to obtain the actual proNGF 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 proNGF ELISA assay. In addition, we strongly 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, mouse proNGF was either reconstituted in Assay Diluent D or complete cell culture medium (RPMI1640 with 10% FBS and antibiotics) and serial dilutions perfomed using each diluent. Note that OD readings, background readings and sensitivity will vary when using different diluents.

Typical optical densities when using Assay Diluent D as sample diluent and coefficient of variations for diluted standards are summarized in the following table:

	OD 450 nm	
proNGF / [ng/mL]	**Mean**	**CV**
10	2.57	4.7%
5	1.70	6.5%
2.5	1.10	3.8%
1.25	0.66	3.1%
0.625	0.44	2.1%
0.313	0.35	2.7%
0.156	0.26	1.9%
Blank	0.20	3.5%

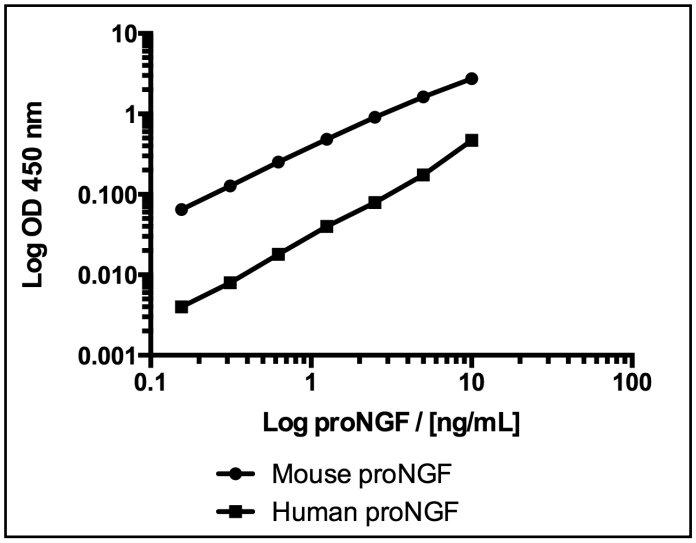
CV: coefficient of variation

Limit of Detection

This proNGF ELISA kit detects typically less than 50 pg/mL mouse proNGF (defined as concentration at blank OD_{450} plus 3x standard deviation, n=10).

Specificity

This proNGF ELISA assay detects mouse proNGF and rat proNGF based on experiments completed with rat PC12 cells and rat brain extracts, though the precise differiental reactivity between these species has not been assessed. The human form of proNGF shows a reactivity of only 20% in this ELISA assay when tested in assay buffer.



No cross-reactivity was observed for mature mouse NGF and proBDNF tested at 25 ng/mL in assay buffer. No interference of spiked mature nerve growth factor (NGF) was observed in rat brain samples.

Due to the nature of capture and detection antibodies used, the pro-peptide is not detected.

Intra- and Inter-Assay Precision

Complete cell culture medium (RPMI1640 with 10% FBS and antibiotics) was spiked with 0.5 ng/mL (Sample 1) and 2 ng/mL (Sample 2) mouse proNGF. Samples were assayed (n=16) according to the provided protocol by 2 different operators.

Sample	Operator	proNGF / [ng/mL]		Intra-Assay	Inter-Assay
		Mean	SD	CV	CV
1	A	0.520	0.033	6.3%	6.7%
	B	0.512	0.037	7.2%	
2	A	2.142	0.086	4.0%	4.0%
	B	2.089	0.075	3.6%	

SD: standard deviation; CV: coefficient of variation

The average Intra-Assay Coefficient of Variation (CV) was found to be 5.3% (range: 3.6-7.2%), the Inter-Assay CV was ≤ 6.7%.

Spike- and Recovery 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. Lysates were diluted 1/5-1/40 in Assay Diluent D to measure endogenous levels of proNGF. Samples were also spiked with 0.4 ng/mL mouse proNGF and recovery calculated in relation to the same spike in Assay Diluent D. As comparison, the same spike was performed in RIPA buffer only with protease inhibitors.

Sample	Dilution	Recovery %	ng proNGF / mg extracted protein
RIPA only	1/5	95	N/A
	1/10	98	
	1/20	81	
	1/40	82	
PC12 Lysate (RIPA)	1/5	40	11.0
	1/10	76	
	1/20	85	
	1/40	93	
PC12 Lysate (Freeze-thaw)	1/5	37	10.9
	1/10	74	
	1/20	83	
	1/40	105	

This data shows that the proNGF ELISA is compatible with RIPA buffer when diluted 1/5 with Assay Diluent D. In cell lysates, lower recoveries are observed likely related to interference from sample constituents.

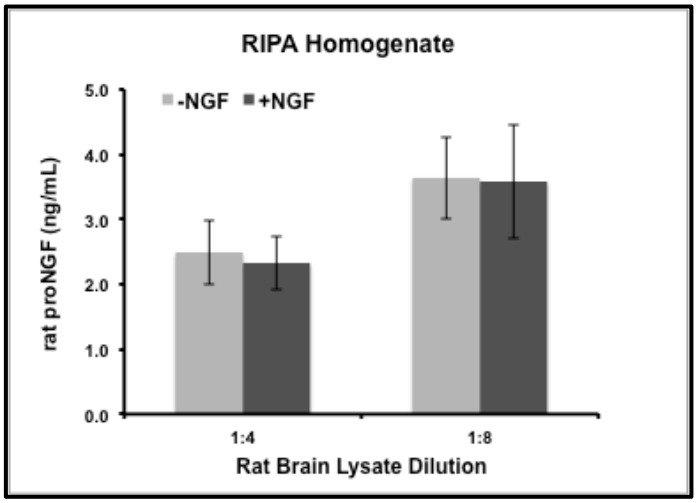
Thus, the mimumum required dilution for cell lysates is 1/10 with values improving at higher dilutions. We suggest to perfom spike- and recovery for other sample types and sample preparation methods to assess accuracy of proNGF quantifications.

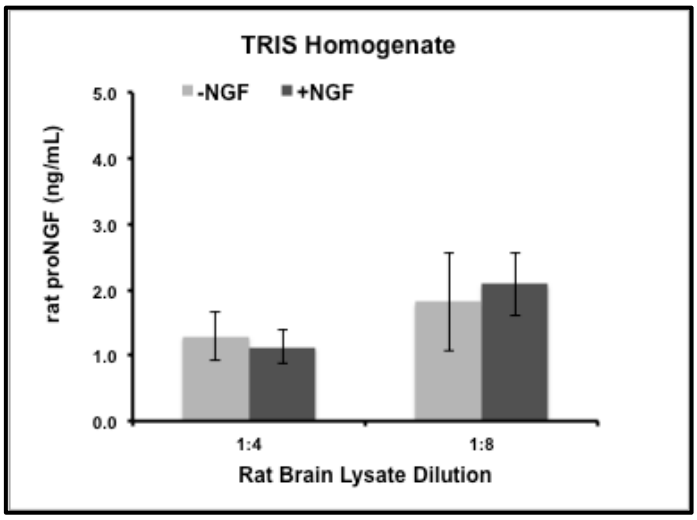
Quantification of proNGF in Rat Brain Homogenate

Rat brain tissue (100-300 mg) was homogenized in RIPA or TRIS buffers at a 10:1 ratio of buffer volume over tissue net weight, according to the procedure outlined in Appendix B. Tissue was homogenized using a bead homogenizer.

The brain sample was assayed for proNGF content at 1:4 and 1:8 dilution in Assay Diluent D, in presence and absence of a spike of mature NGF (5 ng/mL) to

determine the interference of mature NGF in the proNGF ELISA. The assay was repeated on 3 (RIPA) and 2 (TRIS) separate occasions (n=2-3), and results plotted in the following two graphs:





The above figures show the concentration of proNGF detected in rat brain after RIPA and TRIS buffer extraction. Mature NGF did not interfere with proNGF concentration determinations.

The amount of proNGF (ng per mg extracted protein) was found to be the same for both RIPA or TRIS extracted rat brain tissue, while the amount of proNGF (ng per g brain tissue) differed due to the extraction method used:

Buffer	*Protein (mg/mL)	proNGF (ng/mL)	proNGF (ng)	proNGF (ng/mg protein)	proNGF (ng/g brain)
RIPA	13.17	3.06	9.18	0.23	30.60
Tris	6.00	1.55	1.55	0.26	15.54

*Total Protein concentration (mg/mL) was determined using the Bradford assay

Overall, the results in the table and figures above show that proNGF can be detected in rat brain homogenates, using either RIPA or neutral buffer extraction methods. Mature NGF protein is not detected and does not interfere with the mouse/rat proNGF *Rapid*™ ELISA.

13. Informational References

Demont Y., Corbet C., *et al.*, Pro-nerve growth factor induces autocrine stimulation of breast cancer cell invasion through tropomyosin-related kinase A (TrkA) and sortilin protein. J Biol Chem. 2012, Jan 13; 287(3):1923-31.

Pundavela J., Demont Y., *et al.*, ProNGF correlates with Gleason Score and is a potential driver of nerve infiltration in prostate cancer. Am J Pathol. 2014, Oct 3; pii: S0002-9440(14)00494-5.

14. Specific References

Mossa A *et* al. (2021). Adaptation to partial urethral obstruction in healthy aging LOU rats and the role of nerve growth factor signaling pathway in the bladder. Exp Gerontol. [Epub ahead of print]. **Application: Rat urine.**

Sugimoto J *et al.* (2021). Fabry disease-associated globotriaosylceramide induces mechanical allodynia via activation of signaling through proNGF p75NTR but not mature NGF TrkA. Eur. J. Pharmacol. 895. **Application: Mouse tissue homogenate (RIPA).**

Mossa AH *et al.* (2020). Antagonism of proNGF or its receptor p75NTR reverses remodelling and improves bladder function in a mouse model of diabetic voiding dysfunction. Diabetologia. [Epub ahead of print]. **Application: Mouse bladder extracts (RIPA).**

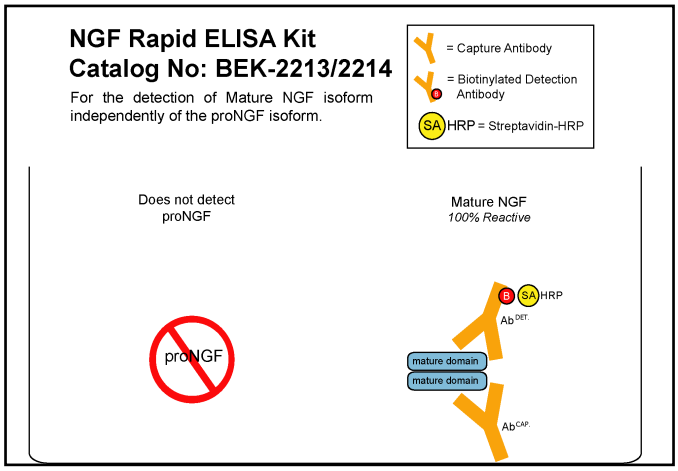
Ryu JC *et al.* (2018). Role of proNGF/p75 signaling in bladder dysfunction after spinal cord injury. J Clin Invest. [Epub ahead of print]. **Application: Mouse urine.**

15. Other Information

Biosensis offers a range of *Rapid*™ ELISA kits to quantify mature NGF or full-length proNGF in a variety of samples.

The Biosensis Human proNGF *Rapid*™ ELISA kit (Cat# BEK-2226) specifically detects the human form of proNGF and does not cross-react with murine proNGF or the mature forms of NGF.

For preferential quantification of mature NGF independent of proNGF, we recommend the Biosensis NGF *Rapid*™ ELISA kits (Mouse: Cat# BEK-2213; Rat: Cat# BEK-2214).



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

Standard curve, blank and controls:

- Standard (10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.63 ng/mL, 0.31 ng/mL, 0.16 ng/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 for RIPA and TRIS Buffer Extraction of proNGF

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. Alternatively, neutral pH extraction buffers can be used which contain less detergents.

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. Complete proteinase inhibitor cocktails are added to this base buffer in all cases.

Biosensis In-house TRIS Buffer

50 mM Tris-HCL, 1 M sodium chloride; 0.1% Triton X-100; pH 7.2-8.0. Complete proteinase inhibitor cocktails are added to this base buffer in all cases. Tissue samples should be homogenized ice-cold.

Proper protein preparation procedures, including keeping homogenates on ice, are required for consistent and best results.

Note: Biosensis recommends evaluating the recovery of proNGF when preparing tissue extracts. In order to conduct a recovery experiment, known amounts of proNGF 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 and TRIS 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 7.0-8.0 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 and TRIS 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; other mechanical tissue homogenation methods can be used, but need to be optimized by the end-user
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 and TRIS Sample Dilution

Prepared, cleared, concentrated, homogenates are typically diluted at least 1:4 with Assay Diluent D 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, 1:4-1:8 (w/v) dilution for many, but some tissues can be greater 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.

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

Appendix C: Troubleshooting Guide

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