biosensis® Human proNGF *Rapid*™ ELISA Kit

Catalogue Number: BEK-2226-1P/2P

For the quantitative determination of human proNGF in human serum, human heparin plasma, cell supernatants and cell lysates 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 human proNGF in human serum, human heparin plasma, cell supernatants and cell lysates only if used as directed. This kit has not been tested for other sample applications. In particular, human urine has not been validated by Biosensis for absence of immunoglobulin interference. 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.

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

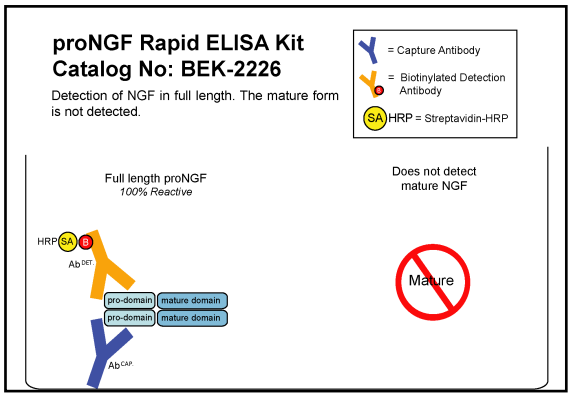
2. Introduction

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.

The Biosensis proNGF *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the quantification of full-length proNGF protein in less than 4 hours. This kit consists of a pre-coated anti-proNGF capture antibody, a biotinylated anti-proNGF 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 kit is designed NOT to detect the mature form of NGF.

This proNGF ELISA kit contains a recombinant human proNGF standard expressed in *E.coli*. 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 does not cross-react with the mouse form of proNGF, and due to sequence homology of mouse and rat proNGF is not expected to detect rat proNGF. The antibodies used in this ELISA kit bind epitopes within the pro-domain of the protein and therefore recognize proNGF and the pro-domain, but do not cross-react with mature NGF!



This kit has not been tested for other applications. Sufficient amount of proNGF standard is supplied to allow for spike- and recovery experiments in order to validate this ELISA assay for other sample matrices if required.

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 A (1x)	2 x 25 mL	4 x 25 mL
Heterophilic antibody Blocker BL-003-1000*	1 vial	2 vials
Recombinant human 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)
Human proNGF 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	Supplier	

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

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard and QC sample	Use within 1 hour of reconstitution; remaining standard may be aliquoted and reused if stored at -20°C for up to 1 week; vortex gently 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
Assay Diluent A, with BL-003-1000 as additive	2 months at 2-8°C

Note:

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

4. Equipment Required but Not Supplied

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

5. Before You Start….

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

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

6. Sample Preparation

The assay diluent provided in this kit is suitable for measuring human proNGF in serum, human heparin plasma, cell lysates and cell culture supernatants. See the following sample preparation guidelines for specific substrate preparation recommendations and sample dilution instructions, including instructions for use of a heterophilic antibody blocker for human serum and plasma samples. 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.

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 need to be diluted with plain culture medium and/or Assay Diluent A 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 may be required
- Quantification of low levels of proNGF in cell culture supernatants may require concentrating the supernatant in ultrafiltration devices with filters of 10 kDa molecular cut-off or lower.

Cell Lysates

Cell lysis and protein extraction can be accomplished by a variety of methods (eg., chemical or mechanical). This ELISA kit has been tested on DU 145 (prostate cancer) cells lysed with a modified RIPA buffer extraction process. The sample preparation procedure given here is only a guideline and is 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.

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

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 proNGF. Use **Assay Diluent A** as sample diluent for cell lysates prepared with RIPA buffer (minimum required sample dilution is 1/4)

General Notes on Cell Lysates:

- The addition of a protease inhibitor cocktail to buffers used in protein extraction just prior to cell lysis is highly recommended
- As an alternative, freeze whole cell pellets and store at -80°C for cell lysis at later time-point

Human Serum and Heparin Plasma

Internal validation has demonstrated that the quantification of proNGF in normal human serum and heparin plasma is difficult and typically below the detection level of this assay (see data in **Quantification of proNGF in Human Serum and Heparin Plasma**, Section 12)

It is strongly recommended to dilute all human serum and heparin plasma samples in a Sample Diluent specifically designed to reduce or eliminate heterophilic antibody (HA) interferences. The addition of HA blocker BL-003-1000 to Assay Diluent A is strongly recommended to avoid matrix interferences and reporting false positive or false negative results.

Sample Diluent Preparation for Human Serum and Heparin Plasma with HA Blocker BL-003-1000:

- Reconstitute HA blocker BL-003-1000 in 1 mL of Assay Diluent A to give an IgG concentration of 1,000 µg/mL, mix gently by vortex
- Add the 1 mL of reconstituted BL-003-1000 to 24 mL of Assay Diluent A to a final volume of 25 mL. Use this Sample Diluent to prepare all serum and heparin plasma dilutions

Serum Collection and Sample Dilutions:

- Allow the serum to clot in a serum separator tube (about 30 minutes to 4 hours) at room temperature
- Centrifuge at approximately 1,000 x g for 15 minutes
- Analyze the serum immediately or aliquot and store frozen at -20°C to -80°C
- Dilute serum samples in Sample Diluent (above) to measure proNGF concentrations in human serum
- Testing with a limited number of normal human serum samples indicate a minimum required sample dilution with Sample Diluent of 1/20 or higher to avoid matrix interferences and achieve acceptable recoveries of spiked proNGF

Plasma Collection and Sample Dilutions:

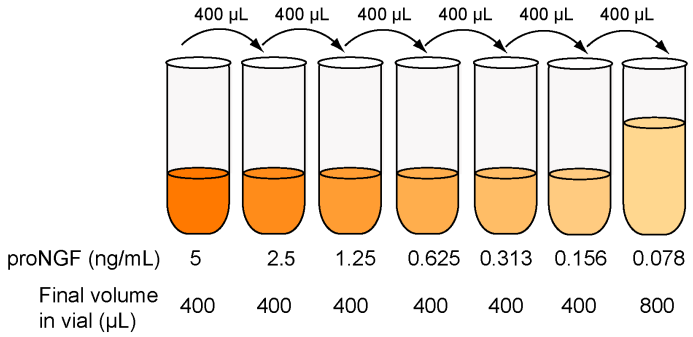
- Collect plasma using heparin as anticoagulant; EDTA and citrate treated plasma samples have not been tested
- Centrifuge for 15 min, 2-8°C at 1500 x g within 30 minutes of collection
- For eliminating the platelet effect we suggest further centrifugation for 10 min, 2-8°C at 10,000 x g
- It is recommend to analyze samples immediately or aliquot and store samples at -20°C to -80°C, thawing only once.
- Dilute human heparin plasma samples at least 1/10 with Sample Diluent containing HA blocker BL-003-1000 in order to measure proNGF concentrations

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 1:4 (eg., 200 µL of 20 ng/mL standard + 600 µL diluent); label this tube “5 ng/mL”
- **Note:** 5 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 0.078 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 “2.5 ng/mL”, “1.25 ng/mL”, “0.625 ng/mL”, “0.313 ng/mL”, “0.156 ng/mL” and “0.078 ng/mL”, respectively
2. Aliquot 400 µL of the diluent into each tube
3. Take 400 µL from the “5 ng/mL” tube and transfer to the “2.5 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 “0.078 ng/mL” is prepared and mixed well



proNGF (ng/mL)	5	2.5	1.25	0.625	0.313	0.156	0.078
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 proNGF Standard curve**.
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent A; **do not use cell culture medium or assay diluent A enriched with HA blocker;** 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 5-12 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 B 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 9 minutes is usually sufficient to obtain a very sensitive standard curve. However, TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm and perform all incubations at room temperature (20-25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.
10. TMB incubation times can vary between 6 – 12 minutes, and assay sensitivity can be affected if color development is stopped too early. In order to determine the optimal time to stop the TMB incubation, blue color development can be monitored at **650 nm**. Addition of Stop Solution will convert blue into yellow color, with an ~2.5-3X increase in OD at **450 nm**. Once the highest standard reaches an OD of ~1.0-1.2 at 650 nm, immediately stop the reaction by adding Stop Solution. Note that before taking a 650 nm measurement, the plate should be briefly and gently shaken by hand to homogenise the blue TMB reaction product within the wells for most accurate readings.

11. Calculation of Results

1. Average the readings for each 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 4-PL regression 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).

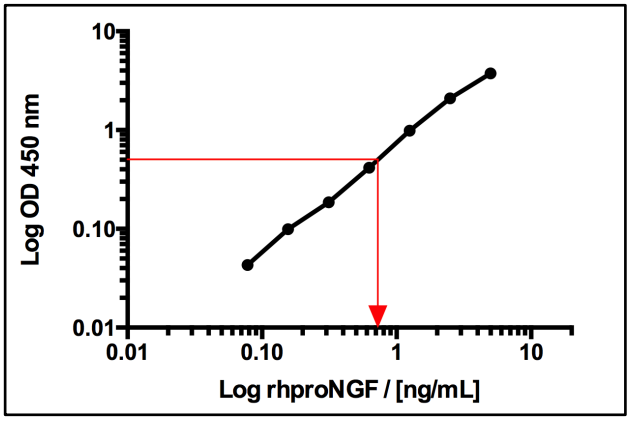
1. The **standard curve** can be plotted as the relative OD_{450} of each proNGF standard solution (Y-axis) vs. the respective known concentration of the proNGF standard solution (X-axis). The standard curve should result in a graph that shows a direct relationship between target protein concentrations 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. In the exemplary standard curve on the right, a sample with OD_{450} = 0.5 reads as 0.7 ng/mL proNGF (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, proNGF standards were run in triplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 5 minutes. Note that OD values and assay sensitivity may vary when using culture medium to prepare the calibration curve.

Typical optical densities and coefficient of variations for diluted proNGF standards are summarized in the following table using Assay Diluent A:

	OD 450 nm		
proNGF / [ng/mL]	**Mean**	**SD**	**CV**
5	3.857	0.071	1.8%
2.5	2.199	0.022	1.0%
1.25	1.087	0.013	1.2%
0.625	0.518	0.008	1.6%
0.313	0.287	0.002	0.7%
0.156	0.201	0.004	2.2%
0.078	0.145	0.001	0.9%
Blank	0.102	0.008	7.6%

SD: standard deviation; CV: coefficient of variation

Limit of Detection

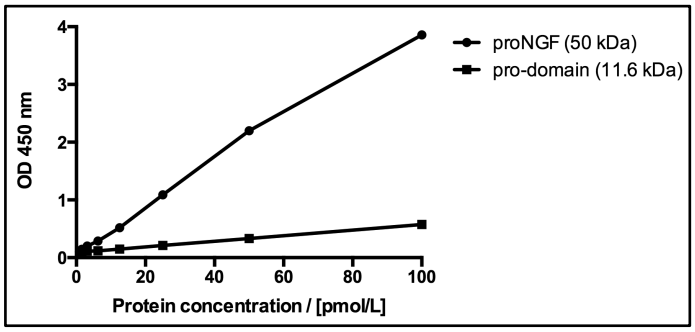
This proNGF ELISA kit detects a typically less than 50 pg/mL proNGF (defined as Blank OD_{450} plus 3x standard deviation, n=10).

Specificity

This proNGF ELISA assay detects the human form of proNGF and the pro-domain peptide. No cross-reactivity was observed for mature rhNGF and proBDNF tested at 25 ng/mL in assay buffer. The murine form of proNGF is not detected by this ELISA assay. Due to sequence homology of mouse and rat proNGF, this ELISA is expected to **not** cross-react with rat proNGF!

Detection of pro-Domain Peptide

The antibodies used in this ELISA kit detect the human pro-domain peptide, but less efficiently than the full-length protein.



Quantification of proNGF in Human Serum

Experiment A: Six Human serum samples were diluted 1/20 in Sample Diluent in the presence or absence of BL-003-1000 blocker and measured in duplicate.

	Sample Diluent + Blocker	*Sample Diluent Only*
Serum sample	**proNGF Concentration (ng/mL)**	
1	0	0
2	0	7
3	0	0
4	0	0
5	0	0
6	0	0

This data demonstrates that one out of six serum samples contained apparent endogenous proNGF in the absence of the blocker. However, in the presence of BL-003-1000 endogenous proNGF levels were reduced, indicating that these concentrations were false-positives.

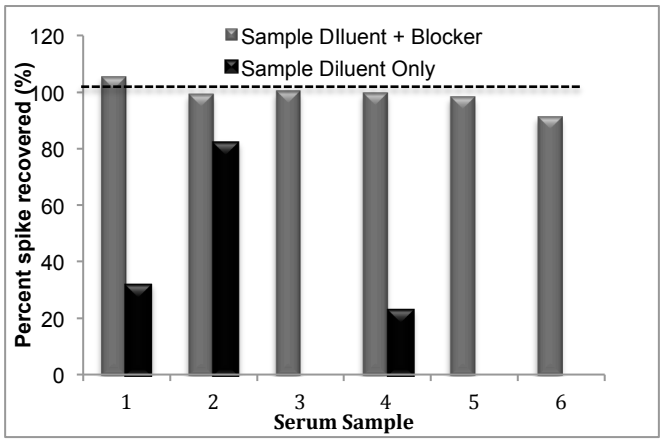
Experiment B: 60 Human Serum samples were diluted 1:20 with the sample diluent containing blocker BL-003-1000 and then tested in triplicate in the Biosensis human proNGF *Rapid*™ ELISA (BEK-2226).

Number of Serum samples	Total Mean proNGF Concentration (ng/mL)
56	0
4	1-9

This data shows proNGF was only detected in 4 out of the 60 human serum samples tested suggesting that serum only contains very low levels of proNGF.

Spike- and Recovery in Human Serum

Experiment A: The accuracy of results was assessed using a spike-and-recovery assay by spiking exogenous proNGF (2 ng/mL) into six serum samples diluted in Sample Diluent in the presence or absence of BL-003-1000 blocker



In the absence of the BL-003-1000 blocker only 1 spiked serum sample (serum 2) gave an acceptable recovery of the spike (80%). In comparison in the presence of the BL-003-1000 blocker spiked proNGF serum samples were all within the acceptable range (80-120%).

Experiment B: 60 Human serum samples were spiked with 2 ng/mL of exogenous proNGF and assayed for recovery of the exogenous proNGF in the presence of the BL-003-1000 blocker.

Number of Serum samples	Mean Percent of Spike Recovered (%)	+/- 1 Standard Deviation	Minimum Recovered (%)	Maximum Recovered (%)
60	100	12.57	57	122

Recovery levels of spiked proNGF in serum were only acceptable (80-120%) in the presence of the BL-003-1000 blocker.

Overall this data demonstrates accurate proNGF quantification in human serum diluted 1:20 in the presence of HA blocker BL-003-1000.

Quantification of proNGF in Human Platelets

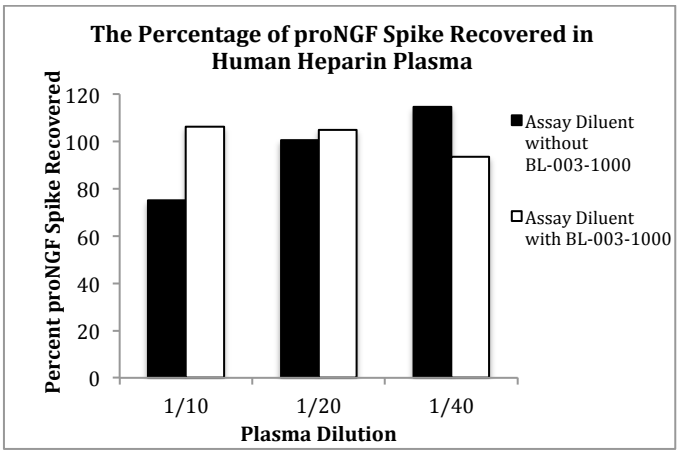
Platelets from normal human blood samples were pooled and lysed by repeated freeze-thaw cycles. The total protein amount was determined by Bradford Assay. Spike- and recovery experiments were performed with 1 ng/mL proNGF.

Dilution	pg proNGF/mg total protein	Recovery
1/5	21.4	96%
1/10	14.7	100%
1/20	Not detected	94%
Average	18.3	

Quantification of proNGF and Spike and Recovery of proNGF in Human Heparin Plasma

Human heparin plasma was diluted 1/10 in assay diluent in the presence and absence of BL-003-1000. The plasma was then spiked with proNGF protein (2 ng/mL) and further serially diluted to 1/20 and 1/40. The ELISA assay was repeated on 3 separate occasions (n=3).

The following figure shows that the recovery of spiked proNGF heparin plasma increases with increase in sample dilution (75-115%) when diluted in assay diluent without BL-003-1000, indicating assay interference. In comparison, samples diluted in assay diluent containing BL-003-1000 give consistent recoveries of the spike (94-106%) across the dilution range.



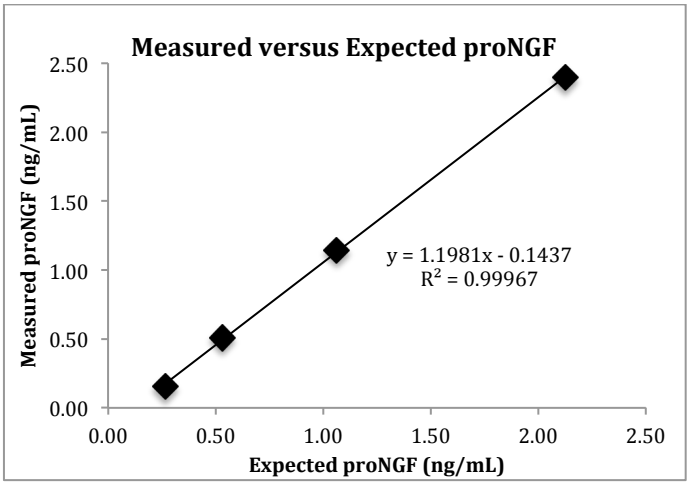
The following table demonstrates that human heparin plasma requires the BL-003-1000 blocker to prevent false-positive readings. ProNGF was not detectable at all dilutions.

	Concentration of proNGF (ng/mL)		
	Plasma Dilution		
	1/10	1/20	1/40
Assay Diluent A only	43	29	30
Assay Diluent A + BL-003-1000	<LOD	<LOD	<LOD

<LOD = Below limit of detection

Linearity-of Dilution in Human Heparin Plasma

A spiked heparin plasma sample was diluted 1/10 in assay diluent in the presence of BL-003-1000. The plasma was then further diluted 2-fold to 1/80. The ELISA was repeated on 3 separate occasions with 1 sample (n=3). Results show linearity-of dilution across the dilution range.



Quantification of proNGF in Cell Lysates and Cell Supernatant

DU145 (human prostate cancer) cells (9.5 x 10^6 cells) and PC3 cells (10 x 10^6) cells were lysed in RIPA buffer containing protease inhibitors as per Sample Preparation Section. Lysates and cell supernatants were then diluted with Assay Diluent A and assayed.

Sample (Lysate)	Dilution	ng proNGF/ mg total protein	Mean ng proNGF/ mg total protein
DU145	1/4	0.737	0.721
	1/8	0.705	
PC3	1/8	0.281	0.288
	1/16	0.294	

Sample (Supernatant)	Dilution	proNGF Concentration
PC3 (16X concentrated)	1/4	700 pg/mL
DU145 (not concentrated)	All dilutions	< LOD

13. Informational References

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

Pundavela J. *et al.* (2014). *ProNGF correlates with Gleason Score and is a potential driver of nerve infiltration in prostate cancer***.** Am J Pathol. 184(12):3156-62.

14. Specific References

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Rowe CW *et al.* (2019). *The precursor for nerve growth factor (proNGF) is not a serum or biopsy-rinse biomarker for thyroid cancer diagnosis*. BMC Endocr Disord. 19(1):128. **Application: Human serum.**

Stapledon CJM *et al.* (2019). *Human osteocyte expression of Nerve Growth Factor: The effect of Pentosan Polysulphate Sodium (PPS) and implications for pain associated with knee osteoarthritis*. PLoS One. 14(9):e0222602. **Application: Human primary culture supernatant.**

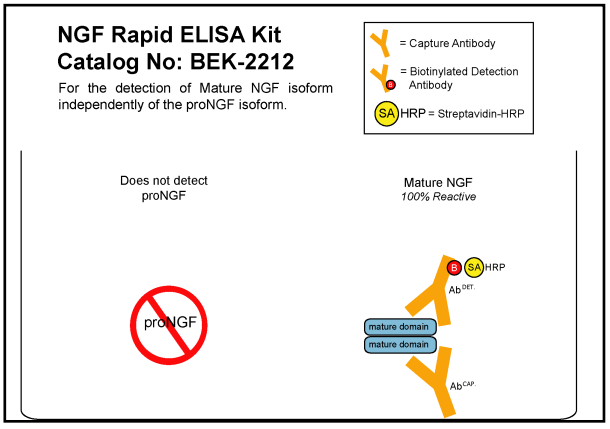
Ryu JC *et al.* (2018). Role of proNGF/p75 signaling in bladder dysfunction after spinal cord injury. J Clin Invest. [Epub ahead of print]. **Application: Human urine.**

Sherif IO & Al-Gayyar MMH (2018). Oleuropein potentiates anti-tumor activity of cisplatin against HepG2 through affecting proNGF/NGF balance. Life Sci. [Epub ahead of print]. **Application: Human cell culture.**

15. Related Products

Heterophilic antibody (HA) blocker cat# BL-003-1000 has been specifically developed to reduce or eliminate HA interference in human serum and heparin-plasma samples when measuring proNGF in serum with the Human proNGF *Rapid*™ ELISA and is available for purchase separately.

Biosensis offers a proNGF *Rapid*™ ELISA kit for quantification of mouse and rat proNGF protein (Cat# BEK-2236). For preferential quantification of human mature NGF independent of human proNGF, we recommend the Biosensis NGF *Rapid*™ ELISA kit (Cat# BEK-2212).



Please refer to our website (www.biosensis.com) for the full range of NGF/proNGF-related products.

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

Standard curve, blank and controls:

- Standard (5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.313 ng/mL, 0.156 ng/mL, 0.078 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 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: 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 either 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