

# **biosensis<sup>®</sup> proNGF Rapid<sup>™</sup> ELISA** **Kit: Mouse, Rat**

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

**For the quantitative determination of mouse or rat proNGF in cell culture 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.**

## Table of Contents

1. Intended Use.....	2
2. Introduction .....	2
3. Materials Provided and Storage Conditions.....	2
4. Equipment Required but Not Supplied .....	3
5. Before You Start.....	3
6. Sample Preparation .....	3
7. Preparation of proNGF Standard.....	4
8. Other Reagents and Buffer Preparation .....	5
9. Assay Procedure .....	5
10. Technical Hints.....	5
11. Calculation of Results .....	6
12. Typical Data .....	6
13. Informational References .....	8
14. Other Information.....	8
Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments .....	9
Appendix B: Troubleshooting Guide .....	11

## 1. Intended Use

The purpose of this kit is the quantitative determination of mouse or rat proNGF in cell culture supernatants and cell lysates only if used as directed. 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.

This ELISA kit has been tested on mouse NGF samples as well as rat PC12 cell lysates. The antibodies used in the kit were raised against mouse NGF and are expected to detect rat proNGF equally well due to high degree of homology (96%) with mouse proNGF based on amino acid sequence, but this has not been exhaustively studied. Thus, in the absence of a true rat proNGF standard, however, results for rat may be expressed as “mouse proNGF equivalents”.

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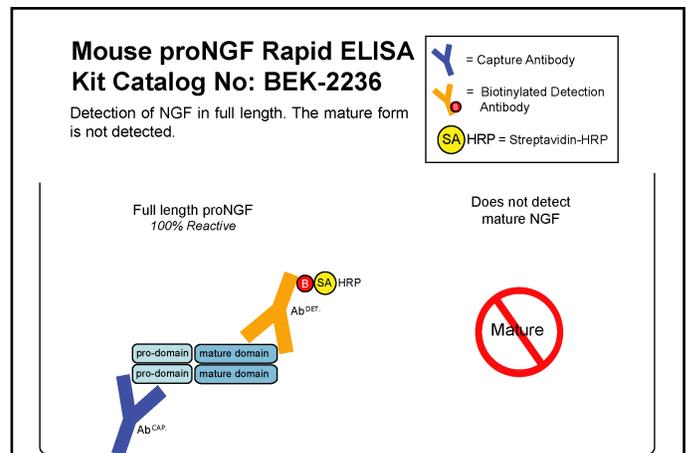
## 2. Introduction

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*<sup>TM</sup> 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-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.

This ELISA kit shows only 20% reactivity with the human form of 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. 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
Mouse 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	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard	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:**

- Pro-NGF 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*<sup>TM</sup> ELISA kits is available online at: <https://www.youtube.com/watch?v=7E0uc9qYL0E>
- 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 proNGF in cell lysates and cell culture supernatants. 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 6.8-7.5) 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 proNGF 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 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 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
- 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  $\beta$ -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 1/5 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.

**7. Preparation of proNGF Standard**

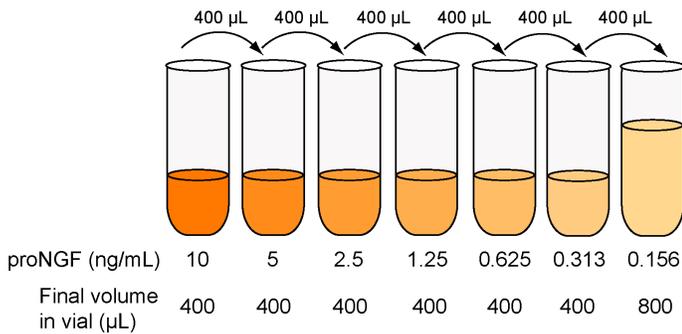
- Reconstitute the lyophilized antigen standard with 1 mL of the **same diluent used for preparing sample dilutions**
- Label the vial with the reconstituted proNGF standard as “20 ng/mL”; vortex and let stand for 15 minutes
- Dilute the 20 ng/mL proNGF standard 1:2 (eg., 400  $\mu$ L of 20 ng/mL standard + 400  $\mu$ L 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  $\mu$ L 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  $\mu$ L of the diluent into each tube
3. Take 400  $\mu$ L 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



- Repeat steps 3 and 4 for each consecutive concentration until the last tube “0.156 ng/mL” is prepared and mixed well



### 8. Other Reagents and Buffer Preparation

- 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).

- Add 100 µL of diluted proNGF standards, samples and blank (sample diluent only) to the pre-coated microplate wells
- If available, include a negative and positive control sample in the assay procedure
- Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G\*) for 2 hours
- 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
- Add 100 µL of the detection antibody (1x) into each well
- Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G\*) for 60 minutes

- Discard the solution inside the wells and wash as described in step 4
- Add 100 µL of the 1x streptavidin-HRP conjugate into each well
- Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G\*) for 30 minutes
- Discard the solution inside the wells and wash as described in step 4
- Add 100 µL of TMB into each well and incubate plate at room temperature for 4-6 minutes without shaking in the dark
- 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
- See Appendix B for a troubleshooting guide when unexpected difficulties are encountered

$$* RCF = 1.12 \times \text{Orbit Radius} \times (\text{rpm}/1000)^2$$

### 10. Technical Hints

- Do not perform dilutions within the well
- At least duplicate measurements for each standard and sample dilution is recommended
- Dilute samples to a proNGF concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
- Avoid touching the inside surface of the wells with the pipette tip
- Proper emptying and washing the plate is crucial for low background and non-specific binding. For manual plate washing, we recommend the following procedure:
  - 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
  - Blot and forcefully tap the microplate against clean paper towels for 3-5 times



- c. Wash the wells by pipetting 200  $\mu$ L 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 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<sub>450</sub> 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 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<sub>450</sub> = (the OD<sub>450</sub> of each well) – (the OD<sub>450</sub> of Zero well).

1. The **standard curve** can be plotted as the relative OD<sub>450</sub> 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

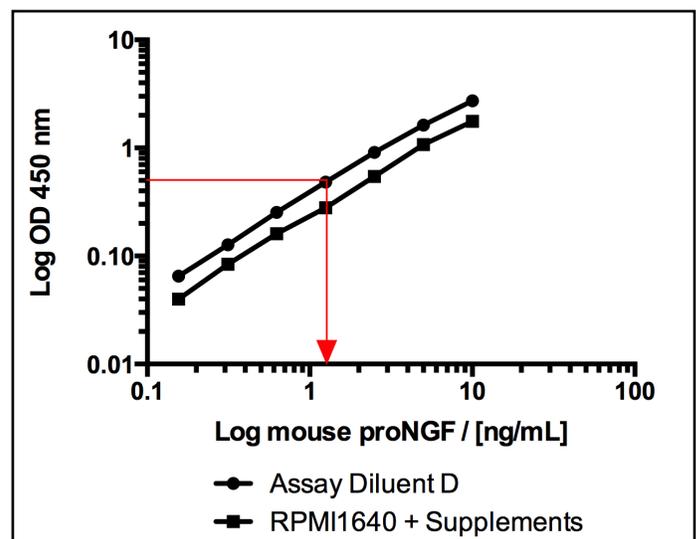
corresponding OD<sub>450</sub>. The greater the concentration of target protein in the solution, the higher the OD<sub>450</sub>

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<sub>450</sub> = 0.5 reads as 1.25 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, mouse proNGF was either reconstituted in Assay Diluent D or complete cell culture medium (RPMI1640 with 10% FBS and antibiotics) and serial dilutions performed using each diluent. The TMB reaction was stopped after 4.5 minutes. 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:

proNGF / [ng/mL]	OD 450 nm		
	Mean	SD	CV
10	2.854	0.084	2.9%
5	1.747	0.009	0.5%
2.5	1.025	0.032	3.1%
1.25	0.603	0.001	0.2%
0.625	0.372	0.005	1.3%
0.313	0.246	0.001	0.4%
0.156	0.184	0.001	0.5%
Blank	0.119	0.003	2.7%

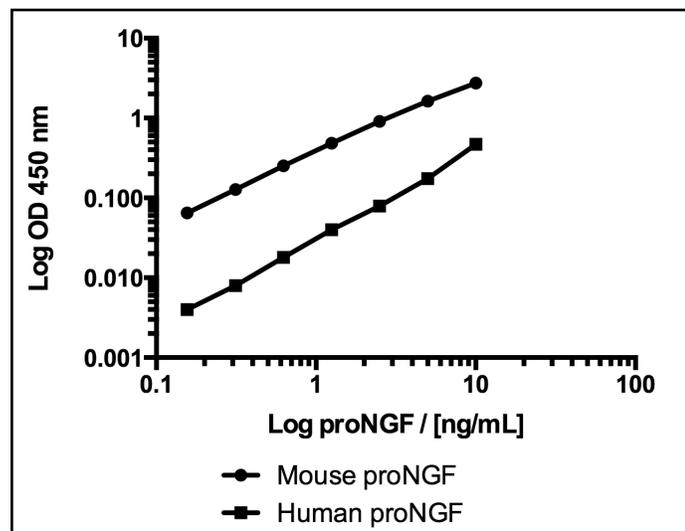
SD: standard deviation; CV: coefficient of variation

### Limit of Detection

This proNGF ELISA kit detects typically less than 50 pg/mL mouse proNGF (defined as Blank OD<sub>450</sub> 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, though the precise differential reactivity between these species has not been tested. 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. Due to the nature of capture and detection antibodies used, the pro-peptide is not detected.

### Intra- and Inter-Assay

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 Intra-Assay Coefficient of Variation (CV) was found to be ≤ 7.2%, the Inter-Assay CV was ≤ 6.7%.

### Spike- and Recovery in PC12 Cell Lysates

PC12 cells (5.2 x 10<sup>6</sup> 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 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 minimum required dilution for cell lysates is at least 1/10 with values improving at higher dilutions. We suggest to perform spike- and recovery for other sample types and sample preparation methods to assess accuracy of proNGF quantifications.

### 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. doi: 10.1074/jbc.M110.211714.

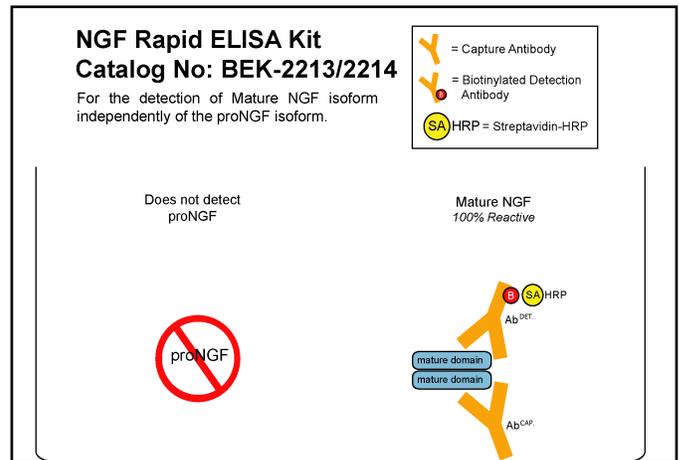
Pundavela J., Demont Y., *et al.*, **ProNGF correlates with Gleason Score and is a potential driver of nerve infiltration in prostate cancer.** Am J Pathol. 2014, Oct 3; pii: S0002-9440(14)00494-5. doi: 10.1016/j.ajpath.2014.08.009.

### 14. Other Information

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

The Biosensis Human proNGF *Rapid*<sup>TM</sup> 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*<sup>TM</sup> 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,  $9 \times 2 = 18$  **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.  $120 \times 2 @ 1:50 = 240$ , PLUS  $120 \times 2 @ 1:100 = 480$ ). Then the number of plates is determined by  $(480 \text{ wells required}) / (78 \text{ wells per plate available}) = 6.15$  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: 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
<b>High background (blank OD &gt; 0.30)</b>	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
<b>Low absorbance readings</b>	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 or use a sample with known concentration of proNGF as positive control
	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

Problem	Cause	Solution
<b>Low absorbance readings</b>	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
<b>Standard OD values above plate reader limit</b>	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
<b>Sample OD values above standard curve range</b>	proNGF concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
<b>High coefficient of variations (CV)</b>	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