biosensis® Mouse/Rat proNGF *Rapid*™ ELISA Kit (1 Plate)

Catalogue Number: BEK-2236-1P

For the quantitative determination of mouse or rat proNGF expression in mouse or rat cell culture supernatants and/or mouse or rat cell lysate preparations. Assay has not been tested in mouse serum, plasma or other solutions.

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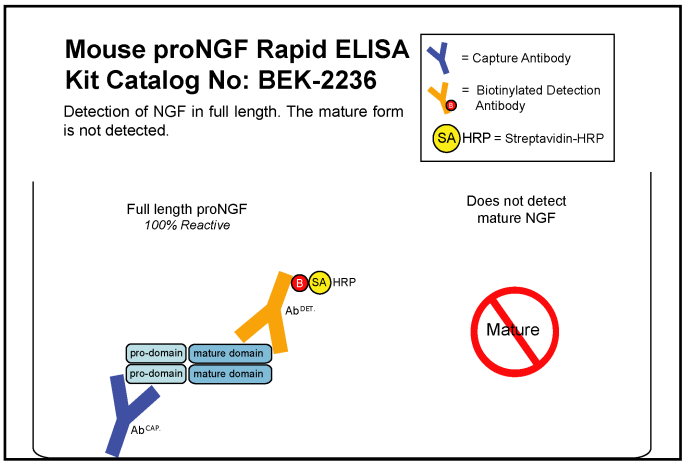
1. Introduction

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 mosue 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.

Note: *This ELISA kit has been extensively tested on mouse NGF samples as well as PC12 (rat) 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 be exhaustively studied. Thus, in the absence of a true rat proNGF standard however, results for rat may be expressed as mouse proNGF equivalents.*

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. 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.



The purpose of this kit is the quantitative determination of proNGF in cell culture supernatants and cell lysates. 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.

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

2. Materials Provided and Storage Conditions

Reagent	Quantity
proNGF antibody coated 96 well microplate	1 plate
Assay diluent D (1x)	2 x 25 mL
Recombinant mouse proNGF standard	2 x 20 ng
Mouse proNGF detection antibody (100x)	1 x 110 µL
Streptavidin-HRP (100x)	1 x 110 µL
Wash buffer (10x)	1 x 33 mL
TMB substrate (1x)	1 x 11 mL
TMB stop solution (1x)	1 x 11 mL
Plate sealer	1

Reagent	Storage and Stability
ELISA kit as supplied	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

3. 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

4. Before You Start….

- Read the entire protocol to familiarize yourself with the assay procedure
- 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 airtight
- 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

5. Sample Preparation

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).

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
- If required, dilute samples with cell culture medium in order to measure proNGF concentrations

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. Sample preparation procedures are given as guidelines.

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 1/5-1/10 with Assay Diluent D to quantify proNGF

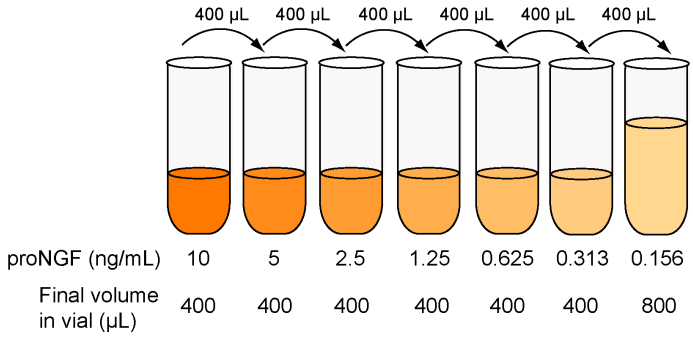
Note: As an alternative, freeze whole cell pellets and store at -80°C for cell lysis at later time-point.

6. 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

7. 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.

8. Assay Procedure

All steps are performed at room temperature (20-25°C, 70-75°F).

1. Add 100 µL of diluted proNGF standards, 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) 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) 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) for 30 minutes
10. Discard the solution inside the wells and wash as described in step 4
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 4-6 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 A for a troubleshooting guide when unexpected difficulties are encountered

9. 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 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.

10. 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
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_{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)
2. 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}
3. **Determine concentration of target protein in unknown sample.** The proNGF protein

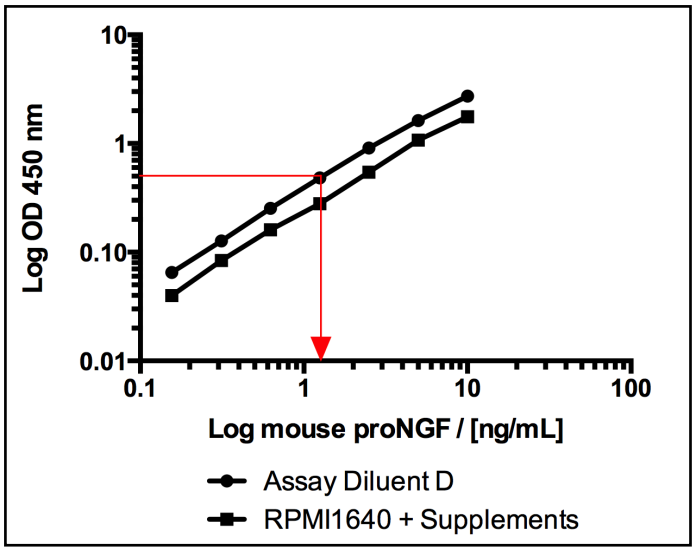
concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of proNGF in the unknown sample. In the exemplary standard curve on the right, a sample with OD_{450} = 0.5 reads as 1.25 ng/mL proNGF (red line)

4. If the samples measured were diluted, multiply the concentrations from interpolation (see step 3) with the dilution factor to obtain the actual proNGF concentration in the sample

11. 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. 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 are summarized in the following table:

	OD 450 nm		
proNGF / [ng/mL]	**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%

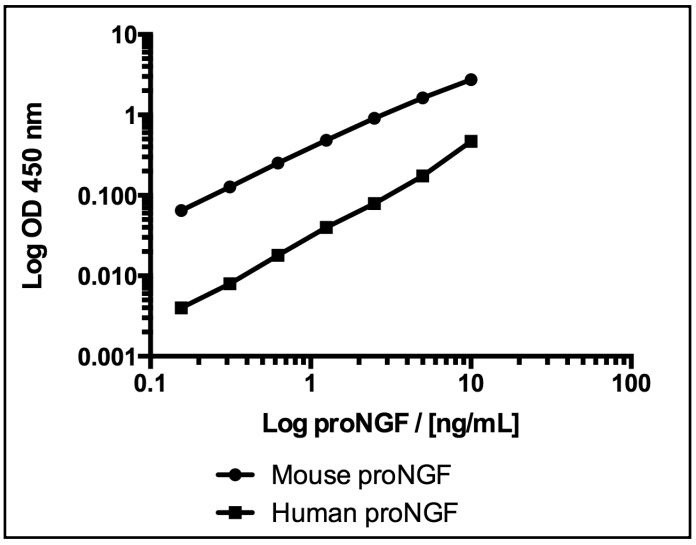
SD: standard deviation; CV: coefficient of variation

Limit of Detection

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

Specificity

This proNGF ELISA assay detects mouse proNGF and rat proNGF based on experiments completed with rat PC-12 cells though the precise differiental reactivity between these species has not been tested. The human form of proNGF shows a reactivity of only 20% in this ELISA assay.



No cross-reactivity was observed for mature mouse NGF and proBDNF tested at 25 ng/mL. 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 10^6 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 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 at least 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.

12. General 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.

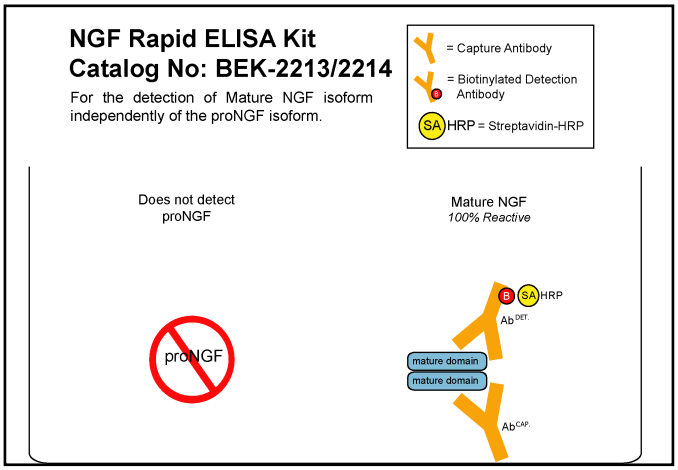
Pundavela J., Demont Y., *et al.*, **ProNGF correlates with Gleason Score and is a potential driver of nerve infiltration in prostate cancer.** Am J Pathol. 2014, Oct 3; pii: S0002-9440(14)00494-5. doi: 10.1016/j.ajpath.2014.08.009.

13. 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 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: 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 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
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