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biosensis® NGF *Rapid*™ ELISA Kit: Mouse

Catalogue Number: BEK-2213-1P/2P

For the quantitative determination of mouse NGF in cell culture supernatants and brain extracts only if used as directed.

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

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

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1. Intended Use

The purpose of this kit is the quantitative determination of mouse NGF in cell culture supernatants and brain extracts 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 is designed to measure the murine form of NGF. Note that NGF from other species will cross-react!

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

2. Introduction

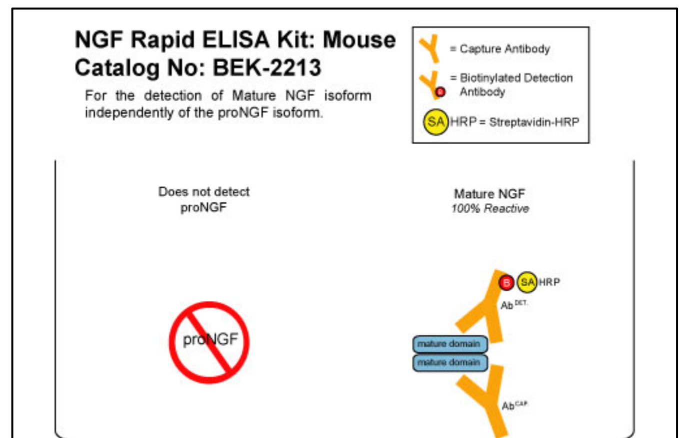
Nerve growth factor (NGF) is a secreted growth factor, vital for the survival, growth and maintenance of several populations of neurons in the central and peripheral nervous systems. Altered concentrations of NGF are also implicated in numerous neurodegenerative diseases. For instance, increased NGF concentrations in serum have been reported in patients suffering from Huntington's disease and there is a loss of NGF in brain in patients with diagnosed Alzheimer's disease. Further, administrating NGF in clinical trials has shown positive results in diabetic polyneuropathy, HIV associated peripheral neuropathy, Alzheimer's disease and skin ulcers.

The Biosensis NGF *Rapid* enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the quantification of mouse NGF in less than 3 hours. This kit consists of a pre-coated mouse monoclonal anti-NGF capture antibody, a biotinylated anti-NGF detection antibody and horseradish peroxidase (HRP)-conjugated streptavidin. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the concentration of NGF present in samples and protein standards.

This NGF ELISA kit is designed to measure mouse NGF and thus employs a mouse NGF standard. The mouse NGF standard supplied has been purified from mouse submaxillary glands according to published procedures. The calibrator standard reflects the native state of mouse NGF protein and has been chosen to give most accurate quantification of natural NGF protein levels in mouse samples.

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

Due to a high degree of NGF sequence homology, the antibodies used in this kit will also detect NGF from other species including human and rat! This ELISA kit preferentially detects the mature form of NGF. Cross-reaction of full-length mouse proNGF was < 0.1% when assayed at 25 ng/mL in assay buffer, and not detectable when assayed across the mature NGF calibration range.



3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
NGF 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
Native mouse NGF standard	2 x 500 pg	4 x 500 pg
Quality Control (QC) Sample	2 Vials (see vial label for amount)	4 Vials (see vial label for amount)
NGF detection antibody (100x)	1 x 110 µL	2 x 110 µL
Streptavidin-HRP (100x)	1 x 110 µL	2 x 110 µL
Wash buffer (10x)	1 x 33 mL	2 x 33 mL
TMB substrate (1x)	1 x 11 mL	2 x 11 mL
TMB stop solution (1x)	1 x 11 mL	2 x 11 mL
Plate sealer	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard and QC sample	Use on same day; aliquot unused standard to prevent multiple freeze-thaw cycles and store at -20°C for 2 weeks
Diluted detection antibody and HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer (1x)	2 weeks 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 NGF in cell culture supernatants and brain extracts. See the following sample preparation guidelines for specific substrate preparation recommendations and sample dilution instructions. For brain extracts, please also see information provided in Appendix B and C. Final working pH of any assay sample should be near neutral (pH 6.8-7.5) for best results, adjust with mild acid or base as needed.

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 NGF. Failure of

these essential validation experiments indicates that sample buffer, preparation and dilution and or blocking procedures will need to be optimized by the end user for consistent, accurate results with this ELISA assay. Please refer to our Technical Note #1 (ELISA Assay Validation) for further details.

Cell Culture Supernatants

- Remove particulates by centrifugation (10,000 x g for 5 minutes)
- Analyze immediately or store at -20°C to -80°C in aliquots to prevent multiple freeze thaw cycles
- Samples with high FBS or proteinaceous content will require to be diluted with plain culture medium and/or Assay Diluent A for best results. Also note, appropriate serum free, cell-free controls must be used for accurate detection
- Quantification of low levels of NGF in cell culture supernatants may require concentrating the supernatant in ultrafiltration devices with filters of appropriate molecular weight cut-off (MWCO)

Brain Tissue Extracts

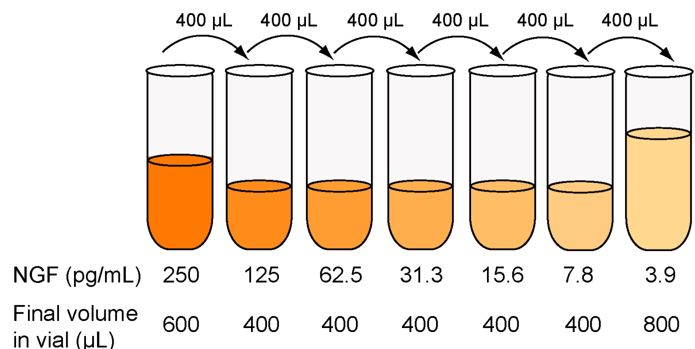
Several protocols have been published in the scientific literature using their respective diluent for NGF detection. This NGF ELISA kit is compatible with a range of sample diluents. However, based on our own experience we recommend following the protocol developed by Y-A. Barde's laboratory and published by Kolbeck *et al.* (1999). This sample preparation protocol allows measuring total NGF concentrations in acid-treated tissue homogenates by ensuring that NGF is released from binding proteins, particularly receptors prior to assay. See Appendix B for a detailed sample preparation protocol for acid-extraction. RIPA-based sample preparation guidelines are summarized in Appendix C.

7. Preparation of NGF Standard

- Reconstitute the lyophilized antigen standard with 1 mL of the **same diluent used for preparing sample dilutions**
- Label the vial with the reconstituted NGF standard as 500 pg/mL
- Vortex and let stand for 15 minutes
- Dilute the 500 pg/mL NGF standard 1:2 (eg., 500 µL of 500 pg/mL standard + 500 µL diluent); label this tube "250 pg/mL"

Perform a 1:2 serial dilution down to 3.9 pg/mL. The volumes used for the dilution series depends on the number of repeats per NGF concentration. For triplicate measurement (100 µL per well) of each NGF standard concentration, we recommend this procedure:

1. Label 6 tubes with 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 3.9 pg/mL, respectively
2. Aliquot 400 µL of the diluent into each tube except the tube labeled "250 pg/mL"
3. Take 400 µL from the "250 pg/mL" tube and transfer to the "125 pg/mL" tube.
4. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex.
5. Repeat steps 3 and 4 for each consecutive concentration until the last tube "3.9 pg/mL" is prepared and mixed well



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8. Other Reagents and Buffer Preparation

- Quality Control (QC) sample: Reconstitute the lyophilized sample with 1 mL of the **same diluent used for preparing the mouse NGF Standard curve**. This will provide a QC sample within 88 – 163 pg/mL.
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent A; **do not use** culture medium or other buffers and prepare enough volume to add 100 µL per well
- Wash buffer (10x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly and dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

9. Assay Procedure

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

1. Add 100 µL of diluted NGF standards, QC samples, 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 45 minutes
4. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 µL per well). See the technical hints section for a detailed description of the washing procedure
5. Add 100 µL of the detection antibody (1x) into each well
6. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
7. Discard the solution inside the wells and wash as described in step 3
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 3
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 5-15 minutes without shaking in the dark.
12. Stop the reaction by adding 100 µL of the stop solution into each well. Visible blue color will change to yellow. Read the absorbance at 450 nm on a plate reader. **Note:** Color will fade over time; hence, we recommend plate to be read within 5 minutes after adding the stop solution or no longer than 30 minutes after addition
13. See Appendix D for a troubleshooting guide when unexpected difficulties are encountered

* RCF= 1.12 x Orbit Radius x $(rpm/1000)^2$

10. Technical Hints

1. Do not perform dilutions within the well
2. At least duplicate measurements for each standard and sample dilution is recommended
3. Dilute samples to a NGF concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
4. Avoid touching the inside surface of the wells with the pipette tip
5. Proper emptying and washing the plate is crucial for low background and non-specific binding. For manual plate washing, we recommend the following procedure:
    a. To remove liquid from the wells, place the plate on the palm of the hand and quickly invert the plate over the sink. Forcefully move the arm downwards and stop abruptly to force the liquid out of the wells. When done correctly, this technique should prevent liquid from getting onto the fingers or onto the outside of the microplate wells
    b. Blot and forcefully tap the microplate against clean paper towels for 3-5 times
    c. Wash the wells by pipetting 200 µL of wash buffer into each well and empty the wells as described in step a-b)
    d. Repeat this procedure for a total of 5 times
6. Complete removal of liquid from the wells at each step is essential for reliable results. However, avoid letting the wells dry out at any time

7. Add TMB and the stop solution to the wells in the same order
8. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading
9. Stopping the TMB reaction after 5 minutes is usually sufficient to obtain a very sensitive standard curve. However, TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm (0.351 G) and perform all incubations at room temperature (20-25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.
10. TMB incubation times can vary between 5 – 15 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 NGF standard concentration, blank and sample
2. Plot a standard curve with the NGF standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the NGF 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 mouse NGF in the QC sample. An observed concentration within the range of 88 – 163 pg/mL indicates acceptable assay performance
6. Perform a regression analysis to calculate the concentration of NGF 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).

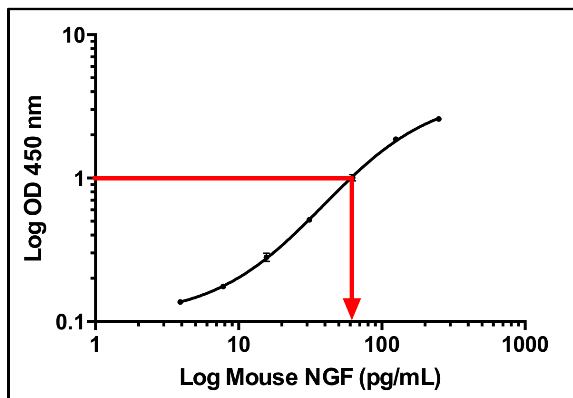
1. The **standard curve** can be plotted as the relative OD_{450} of each NGF standard solution (Y-axis) vs. the respective known concentration of the NGF standard solution (X-axis). The standard curve should result in a graph that shows a direct relationship between target protein concentrations and 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 NGF 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 NGF in the unknown sample. In the exemplary standard curve on the right, a sample with OD_{450} = 1 reads as 64 pg/mL NGF (red line). If the samples measured were diluted, multiply the concentrations from interpolation (see step 1) with the dilution factor to obtain the actual NGF 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 NGF ELISA assay.

In addition, we 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, mNGF standards were run in triplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 5 minutes.

Typical optical densities and coefficient of variations for diluted NGF standards are summarized in the following table:

	OD 450 nm		
NGF / [pg/mL]	Mean	SD	CV
250	2.581	0.082	3.2%
125	1.867	0.096	5.1%
62.5	1.008	0.072	7.1%
31.3	0.511	0.002	0.4%
15.6	0.281	0.026	9.2%
7.8	0.175	0.003	1.5%
3.9	0.136	0.004	2.7%
Blank	0.119	0.004	3.4%

SD: standard deviation; CV: coefficient of variation

Limit of Detection

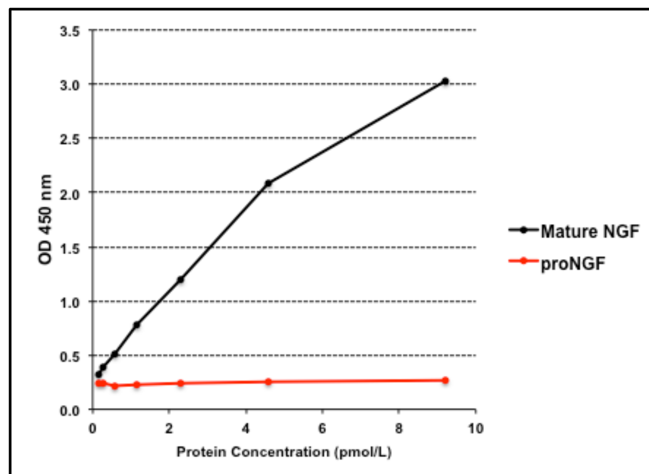
This NGF ELISA kit detects a minimum of 8 pg/mL mouse NGF (defined as 150% of blank value).

Specificity

The antibodies used in this ELISA kit bind epitopes within the mature domain of the protein. No cross-reactivity was observed with brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5 tested at 25 ng/mL in assay buffer. Due to a high degree of NGF sequence homology, the antibodies used in this kit will also detect NGF from other species including human and rat!

Cross-Reactivity with Mouse proNGF

Mature mouse NGF (27 kDa) and full-length mouse proNGF (50 kDa) were assayed in parallel at equimolar protein concentrations across the Mouse NGF ELISA calibration range (3.9-250 pg/mL; 0.14-9.2 pmol/L). OD readings for mouse proNGF were indistinguishable from the assay's blank OD readings.



In addition, mouse proNGF was assayed at 25 ng/mL (500 pmol/L) and cross-reactivity was found to be < 0.1%. Thus, this Mouse NGF *Rapid*™ ELISA preferentially detects mature NGF protein.

13. Informational References

Kolbeck R *et al.*, *Brain-derived neurotrophic factor levels in the nervous system of wild-type and neurotrophin gene mutant mice.* J Neurochem. 1999 May;72(5):1930-8.

14. Specific References

Mossa AH *et al.* (2020). *Antagonism of proNGF or its receptor p75 NTR reverses remodelling and improves bladder function in a mouse model of diabetic voiding dysfunction.* Diabetologia. [Epub ahead of print]. **Application: Mouse bladder extracts (RIPA).**

Roy S *et al.* (2020). *Neurogenic Tissue Nanotransfection in the Management of Cutaneous Diabetic Polyneuropathy.* Nanomedicine. [Epub ahead of print]. **Application: Mouse lysate.**

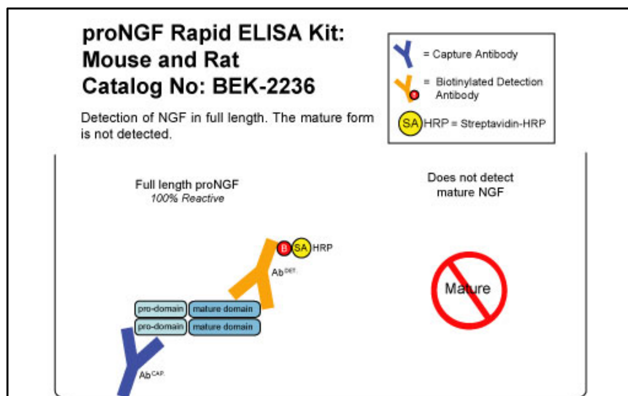
Vigli D *et al.* (2018). *Chronic treatment with the phytocannabinoid Cannabidivarin (CBDV) rescues behavioural alterations and brain atrophy in a mouse model of Rett syndrome.* Neuropharmacology. [Epub ahead of print]. **Application: Mouse hippocampus homogenate.**

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

15. Other Information

For accurate quantification of human and rat NGF, Biosensis offers ELISA kits for human NGF (cat# BEK-2211) and rat NGF (cat# BEK-2214).

Biosensis offers a proNGF *Rapid*™ ELISA kit for quantification of mouse and rat protein (cat# BEK-2236) independent of mature NGF.



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 (250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL, 3.9 pg/mL): 7 wells
- Blank (0 pg/mL): 1 well
- Control vial (either provided in the ELISA kit or the researcher): 1 well

Gives a total of 9 wells used per Standard/Control set.

We recommend that the standard curve, blanks, and controls be run on each separate plate for the most accurate calculations. Note that if two (or more) independent assays are run on one plate, each assay requires its own Standard/Control set. We also recommend that ALL samples (i.e. standards, controls, blanks and test samples) be performed in duplicate at least.

Thus, for standards, blanks and controls, 9 x 2 = **18 standard wells are required per assay.** This leaves 96 wells – 18 wells = **78 sample wells per plate** for test samples for EACH of our standard 1P ELISA kits run with a standard curve and controls.

1P kit: 78 experimental wells per 96 well ELISA plate experiment
2P kit: 156 experimental wells per 192 well ELISA plate experiment

The 2-Plate Optional Single Control Set:
Some researchers using our 2P kits run a single, duplicate standard curve, blank and control sample set (18 wells) for the entire 2-plate experiment. This allows 78 test samples on the "standard curve plate" (the plate containing the standard curve, controls and blanks) and a full 96 test samples on the "test only plate" for a total available number of 174 tests per 2 plate kit. (78 wells +96 wells = 174).

2P kit, single set of controls (in duplicate): 174 experimental wells per 192 well ELISA plate experiment

Example: 60 test samples, duplicate measurement at one single dilution
How many wells and plates do I need for sixty test samples at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 samples, x 2 draws each (e.g. before and after) = 120 stock samples
2. Factor in the number of repeat tests desired, in order to calculate the number of wells required. Biosensis recommends that ALL samples be run in duplicate at least.
3. Multiply the number of stock samples by the number of dilutions per sample. In this example, we will be testing EACH stock sample at a single dilution (i.e. 1:100) in duplicate, so 120 stock samples x 2 wells = 240 microplate wells required for sixty test samples. In summary: 60 samples, 4 tests per sample, equals 240 total number of wells required.
4. Decide on the number of standards, blanks and controls run on each plate. We recommend standard, control and blank duplicates for each 96-well plate. Alternatively, one standard, blank and control duplicate set can be run on every 2 plates (2-Plate Optional Single Control Set option discussed above).

Running the Test:
Running the 60 test sample experiment with a standard curve, blanks and controls on *each* test plate, in duplicate (our recommended option):

Single Dilution per Test Sample:

(240 wells required) / (78 wells per plate available) = 3.077 plates required, or just over 3 plates, thus researcher will need to order **4 x 1-plate kits or 2 x 2-plate kits** (there will be unused wells) to ensure enough wells for the entire sixty test samples, tested in duplicate (two draws per sample, 1 dilution, 4 wells per test, total of 240 wells). The unused 8-well strips can be used for other assays later.

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Two Dilutions per Test Sample:

If testing at two dilutions, (e.g. 1:50 & 1:100 per sample) and doing duplicates, then the number of wells/tests is doubled to 480 (e.g. 120 x 2 @ 1:50 = 240, PLUS 120 x 2 @1:100 = 480). Then the number of plates is determined by (480 wells required) / (78 wells per plate available) = 6.15 plates required, or just over 6 plates, thus the researcher will need to order **3 x 2-plate kits and 1 x 1-plate kit** to ensure the minimum number of wells for sixty test samples tested in duplicate at two dilutions (two draws per test, 2 dilutions, 8 tests/wells per test, total of 480 tests/wells).

2-Plate Optional Single Control Set Option:

If running 60 test samples with a single dilution, and a single duplicate standard curve, blank, and control sample for every two plates, then one has **174 available test wells per 2P kit**.

For the single dilution, sixty test samples, 2 draws per sample experiment (240 tests), one would need (240/174) = 1.38 2-plate kits, or **2 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty test samples.

For the two dilutions per sample, sixty test samples, 2 draws per sample experiment (480 tests), one would need (480/174) = 2.76 2-plate kits, or **3 x 2-plate kits** would need to be ordered to ensure enough wells for all sixty test samples.

Appendix B: Tissue Lysate Preparation Procedures Acid-Extraction of NGF

Like other neurotrophins such as BDNF, NGF is bound to its receptors within many tissues, which prevents its detection in any ELISA. Acid extraction protocols have therefore been developed to release bound neurotrophins, which not only release the bound neurotrophins, but also precipitates the receptors. We recommend the following sample preparation protocol for measuring total NGF concentrations in acid-treated tissue samples. This protocol is based on Kolbeck *et al.* (1999).

Extraction Buffer

50 mmol/L sodium acetate (820 mg / 200 mL), 1 mol/L NaCl (11.7 g / 200 mL), 0.1% Triton X100 (200 µL / 200 mL), add glacial acetic acid until pH 4.0 is reached. Before use add one “Complete” or “Complete Mini” protease inhibitors cocktail tablet (Roche, cat. no. 11697498001 or 11836153001), to be used as recommended by the manufacturer.

Incubation/Neutralization Buffer

0.2 mol/L phosphate buffer, pH 7.6. For instance, weigh in the following amounts per 200 mL total buffer volume and adjust pH with concentrated NaOH solution (≥ 5 mol/L):

KH_2PO_4 (MW 136.09 g/mol): 2.72 g

Na_2HPO_4 (MW 141.96 g/mol): 2.84 g

Acid-Extraction Sample Diluent

Mix acid-extraction buffer and incubation/neutralization buffer at a ratio of 1 to 3 (eg., 1 mL of extraction buffer and 3 mL of incubation/neutralization buffer). Check pH of solution is approximately pH 6.6 to 7.0.

Protocol

1. Dissect brain structures, weigh tissue fragments and then freeze them rapidly in liquid nitrogen
2. For long term storage transfer the frozen tissue samples to -80°C
3. Re-suspend brain tissues in approximately 10 weight/volume-ratio of extraction buffer (for example, 100 µL extraction buffer for 10 mg tissue)
4. Sonicate the suspension to homogeneity on ice with a probe sonicator (e.g. Misonix Ultrasonic Liquid Processor)
5. Sonicate in short bursts (5-7 sec) to avoid excessive sample heating
6. Keep homogenates on ice for 30 min, then repeat sonication as well as incubation on ice
7. Centrifuge homogenates for 30 minutes at 10,000-20,000 x g and 4°C, then transfer clear supernatants into clean tubes and discard pellets
8. Measure total protein concentration (eg., BCA or Bradford protein assay)
9. These supernatants may be stored at -80°C and must be centrifuged again for 30 min at 10,000-20,000 x g and 4°C immediately after thawing and before being loaded into the wells of an ELISA plate
10. Reconstitute the supplied NGF standard with 1 mL of Acid-Extraction Sample Diluent; perform a 1:2 serial dilution as outlined in Section 6 (“Preparation of NGF Standard”).
11. Neutralization and preparation for ELISA: Prepare a sample dilution with 1 part tissue extract and 3 parts of Incubation/Neutralization Buffer (eg., 50 µL tissue extract and 150 µL Incubation/Neutralization buffer); check that pH of sample is near neutral. Note that the neutralization step equals a sample dilution factor of 4 which needs to be considered when calculating NGF concentrations in samples. The prepared, neutralized samples are now ready for the ELISA assay. Note: Freezing of prepared, neutralized, samples is not recommended. Typically, freezing of samples is best when protein concentrations are more concentrated, as in step 9.
12. If the NGF concentration in the sample is out of the assay range (3.9-250 pg/mL), after performing the initial tests, dilute the solution prepared in step 11 further with Acid-Extraction Sample Diluent for best results. Do not use Assay Diluent A for acid-treated/neutralized samples.
13. Results can then be reported as ng NGF/mg total soluble protein or g wet weight if tissue sample is large enough

Note: Biosensis recommends evaluating the recovery of NGF when preparing tissue extracts. In order to conduct a recovery experiment, known amounts of NGF standard are added to an aliquot of the brain tissue homogenates and assayed. Spike-recovery experiments allow comparison of technique and methods. Recoveries of 80-120% of spiked values are acceptable.

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Appendix C: Tissue Lysate Preparation Procedures RIPA Buffer

The key with any RIPA extraction buffer is to provide enough detergent to solubilize the proteins, but not so much that it will interfere with subsequent uses, particularly native ELISAs where antibody binding is critical. We recommend the following RIPA extraction buffer, which has lower detergent amounts and has been successfully used in brain tissue extracts.

Biosensis In-house RIPA
50 mM Tris-HCL, 150 mM sodium chloride; 1.0% NP-40 or Triton X-100; 0.5% sodium deoxycholate; pH 7.5 to 8.0. (Note: 0.1% SDS is left out for ELISA samples, but can be added back for western or IP samples, which will be diluted before use). Complete proteinase inhibitor cocktails are added to this base buffer in all cases. Tissue samples should be homogenized in ice-cold RIPA buffer, and proper protein preparation procedures including lysates being kept on ice, etc. are required for consistent and best results.

Note: Biosensis recommends evaluating the recovery of NGF when preparing tissue extracts. In order to conduct a recovery experiment, known amounts of NGF standard are added to an aliquot of the brain tissue homogenates and assayed. Spike recovery experiments allow comparison of technique and methods. Recoveries of 80-120% of spiked values are acceptable.

It is recommended that tissue samples should be rapidly excised, weighed, and snap frozen in liquid nitrogen prior to storage at -80°C. We recommended using frozen samples within two weeks of freezing.

RIPA homogenates should be prepared in approximately 10 to 100 volumes of the homogenization buffer to tissue wet weight, but the most appropriate ratio needs to be determined by the user for each tissue. Typical ratios are 10-20 mg wet weight/100 µL of lysate, or 1 g tissue per 10 mL of lysis buffer. The tissue can be homogenized either via sonication or mechanical shearing or both (polytron). The homogenates are centrifuged at ~ 14,000 x g for 30 minutes. The resulting supernatants can be used for ELISAs. Check that sample pH is near 7.5-8.0 for best results.

Concentrated stock lysates should be divided into aliquots and frozen at -80°C and thawed and used only once. Lysate stability is fragile and should be used within two weeks for best results.

RIPA Buffer Tissue Lysis and Preparation

1. Sonicate the suspension to homogeneity on ice with a probe sonicator (e.g. Misonix Ultrasonic Liquid Processor). Sonicate in short bursts (5-7 sec) to avoid excessive sample heating
2. Keep homogenates on ice for 30 min, then repeat sonication as well as incubation on ice
3. Centrifuge homogenates for 30 minutes at 10,000 – 20,000 x g and 4°C, then transfer clear supernatants into clean tubes and discard pellets
4. Measure total protein concentration (e.g., BCA or Bradford protein assay)
5. These supernatants may be stored at -80°C and must be centrifuged again for 30 min at 10,000-20,000 x g and 4°C immediately after thawing and before being used in the ELISA

RIPA Sample Dilution
Prepared, cleared, concentrated, lysates are typically diluted at least 1:5 with Biosensis Assay Diluent A before use in the ELISA. After dilution, check that sample pH is within 7.0 – 8.0 with pH paper for best results.

Note: The final sample dilution will vary depending upon the tissue and exact extraction method. Typically, 1:5-1:20 (w/v) dilution for many, but some tissues such as hippocampus can be greater (e.g. 1:300) in order for the assay values to be consistent and fall within the linear range of the assay. Thus, the optimal dilution needs to be determined for each experimental set and laboratory. Spike recovery experiments are highly recommended, see note above.

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

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Appendix D: Troubleshooting Guide

This NGF 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 NGF 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 NGF into your sample and/or check that the QC sample value falls within the expected NGF 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

Problem	Cause	Solution
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	NGF concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
Low 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