biosensis® Neurotrophin 4/5 (NT4/5) *Rapid*™ ELISA Kit: Human, Monkey*, Rat* and Mouse*

Catalogue Number: BEK-2218-1P/2P

For the quantitative determination of NT4/5 in cell culture supernatants, human citrate plasma and brain extracts only if used as directed.

Please refer to the Sample Preparation Section for specific use instructions for each substrate application, in particular human plasma.

**See page 2 regarding the use of this kit for monkey, rat and mouse samples.*

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 NT4/5 cell culture supernatants, human citrate plasma 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.

The capture and detection antibodies will also detect NT4/5 from other species due to a high degree of NT4/5 amino acid sequence homology. Therefore, this ELISA kit can be used to quantify NT4/5 in many species including mouse, rat and monkey.

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

2. Introduction

Neurotrophin 4/5 (NT4/5) is a member of the neurotrophin family of growth factors that modulate neuronal functions within the central and peripheral nervous systems. While the (patho-) physiological roles of NT4/5 have not been studied as detailed as compared to the other neurotrophin family members, recent reports have assigned NT4/5 roles in neuronal development, axon regeneration after peripheral nerve injury, autism, Down's syndrome, progression of neurodegenerative diseases and cancer. Reliable methods for the detection and quantification of NT4/5 are thus required to further enhance the understanding of NT4/5 actions.

The Biosensis Neurotrophin 4/5 *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the specific, fast and reliable quantification of NT4/5 in less than 4 hours. This kit consists of a pre-coated polyclonal anti-NT4/5 capture antibody, a biotinylated anti-NT4/5 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 NT4/5 present in samples and protein standards.

This NT4/5 ELISA kit employs a recombinant human NT4/5 standard. The high degree of amino acid homology of NT4/5 among species allows quantification of NT4/5 in non-human samples, and results may be expressed as "human NT4/5 equivalents".

The provided Quality Control (QC) sample serves as a positive control with a defined concentration range of NT4/5 protein, formulated in a stabilized buffer solution and designed to assure assay performance.

The antibodies used in this kit bind to epitopes within the mature domain of NT4/5. While not tested, this ELISA kit may therefore detect the full-length pro- form of NT4/5.

3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
NT4/5 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
Recombinant human NT4/5 standard	2 x 2 ng	4 x 2 ng
Quality Control (QC) Sample	2 Vials (see vial label for amount)	4 Vials (see vial label for amount)
NT4/5 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
- Assaying NT4/5 in human citrate-plasma requires the use of a heterophilic antibody (HA) blocker cat# BL-003-1000 for accurate quantification. This sample diluent additive can be purchased as an add-on reagent, please refer to www.biosensis.com.

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 NT4/5 in cell culture supernatants, human citrate plasma (with addition of HA blocker BL-003-1000) and brain extracts. See the following sample preparation guidelines for specific substrate preparation recommendations and sample dilution instructions, including instructions for use of a heterophilic antibody blocker for human citrate plasma. For brain extracts, refer to Takahashi *et al.*, 2016 for use. 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 NT4/5 in samples, it is important to perform several dilutions of the sample to allow the NT4/5 concentration to fall within the range of the NT4/5 standard curve (15.6-1000 pg/mL). Also, with unknown samples of all types it is highly recommended to run some NT4/5 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 A for best results. Appropriate serum free, cell-free controls must be used for accurate detection.
- Quantification of low levels of NT4/5 in cell culture supernatants may require concentrating the supernatant in ultrafiltration devices with filters of 10 kDa molecular cut-off or lower

Human Citrate Plasma Samples

Internal validation has demonstrated that the quantification of NT4/5 in normal human citrate plasma is difficult and typically below the detection level of this assay (see data in **Quantification of NT4/5 in Human Citrate plasma**, Section 12).

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

Sample Diluent Preparation for Human Citrate Plasma with HA Blocker BL-003-1000 as follows:

- Reconstitute HA blocker BL-003-1000 in 1 mL of Assay Diluent A to give an IgG concentration of 1,000 µg/mL, mix gently by vortex
- Add the 1 mL of reconstituted BL-003-1000 to 24 mL of Assay Diluent A to a final volume of 25 mL. Use this Sample Diluent to prepare all human citrate plasma dilutions and NT4/5 protein standard dilutions; do not use Assay Diluent A enriched with HA blocker to dilute detection antibody or streptavidin-HRP conjugate

Plasma Collection and Sample Dilutions

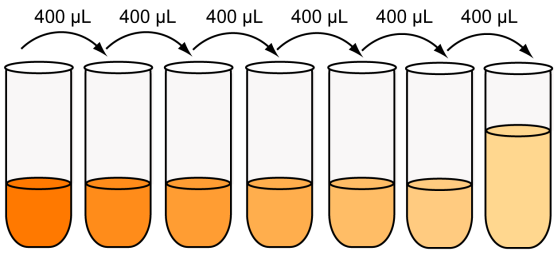
- Collect plasma using citrate as anticoagulant; EDTA and heparin treated plasma samples have not been tested
- Centrifuge for 15 min at 2-8°C at 1500 x g within 30 minutes of collection
- For eliminating the platelet effect we suggest further centrifugation for 10 min at 2-8°C at 10,000 x g
- It is recommend to analyze samples immediately or aliquot and store samples at -20°C to -80°C, thawing only once.
- Dilute human citrate plasma samples at least 1/8 – 1/10 with Sample Diluent containing HA blocker BL-003-1000 in order to measure NT4/5 concentrations

7. Preparation of NT4/5 Standard

- Reconstitute the lyophilized antigen standard with 1.0 mL of the **same assay diluent used for preparing sample dilutions**
- Label the vial with the reconstituted NT4/5 standard as 2000 pg/mL
- Vortex and let stand for 15 minutes

Perform a 1:2 serial dilution down to 15.6 pg/mL. The volumes used for the dilution series depends on the number of repeats per NT4/5 concentration. For triplicate measurement (100 µL per well) of each NT4/5 standard concentration, we recommend this procedure:

1. Label 6 tubes with “1000 pg/mL”, “500 pg/mL”, “250 pg/mL”, “125 pg/mL”, “62.5 pg/mL”, “31.3 pg/mL” and “15.6 pg/mL”, respectively
2. Aliquot 400 µL of the diluent into each tube
3. Take 400 µL from the Standard original tube “2000 pg/mL” and transfer to the “1000 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 “15.6 pg/mL” is prepared and mixed well



NT4/5 (pg/mL)	1000	500	250	125	62.5	31.3	15.6
Final volume in vial (µL)	400	400	400	400	400	400	800

8. Other Reagents and Buffer Preparation

- Quality Control (QC) sample: Reconstitute the lyophilized sample with 1 mL of the **same diluent used for preparing the NT4/5 standard curve**. This will provide a QC sample within 350 – 650 pg/mL
- Detection antibody (100x) and streptavidin-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent A; **do not use culture medium or assay diluent A enriched with HA blocker**; prepare enough volume to add 100 µL per well.

- Wash buffer (10x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly and dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

9. Assay Procedure

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

1. Add 100 µL of diluted NT4/5 standards, QC sample, samples and blank (assay 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 90 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 60 minutes
7. Discard the solution inside the wells and wash as described in step 4
8. Add 100 µL of the 1x streptavidin-HRP conjugate into each well
9. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
10. Discard the solution inside the wells and wash as described in step 3
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 5-8 minutes without shaking in the dark
12. Stop the reaction by adding 100 µL of the stop solution into each well. Visible blue color will change to yellow. Read the absorbance at 450 nm on a plate reader. **Note:** Color will fade over time; hence, we recommend plate to be read within 5 minutes after adding the stop solution or no longer than 30 minutes after addition
13. See Appendix B for a troubleshooting guide when unexpected difficulties are encountered

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

10. Technical Hints

1. Do not perform dilutions within the well
2. At least duplicate measurements for each standard and sample dilution is recommended
3. Dilute samples to a NT4/5 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 to reduce 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 5-8 minutes is usually sufficient to obtain a very sensitive standard curve. However, TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm and perform all incubations at room temperature (20-

25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.

11. Calculation of Results

1. Average the readings for each NT4/5 standard concentration, blank and sample
2. Plot a standard curve with the NT4/5 standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the NT4/5 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
5. Perform a 4-PL regression analysis to calculate the concentration of NT4/5 in the QC sample. An observed concentration within the range of 350 – 650 pg/mL indicates acceptable assay performance
6. Perform a regression analysis to calculate the concentration of NT4/5 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).

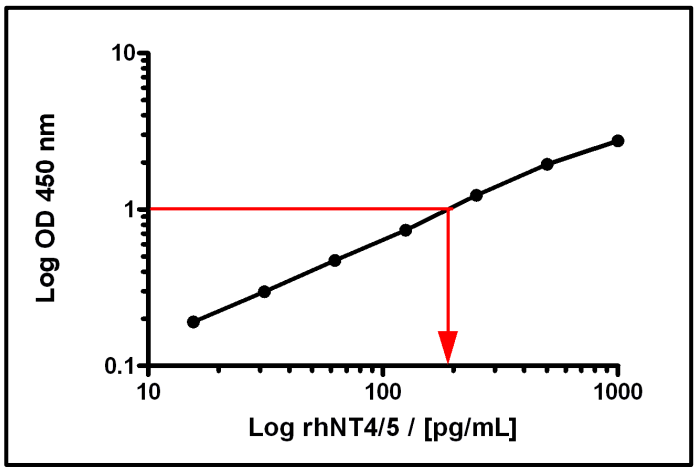
1. The **standard curve** can be plotted as the relative OD_{450} of each NT4/5 standard solution (Y-axis) vs. the respective known concentration of the NT4/5 standard solution (X-axis). The standard curve should result in a graph that shows a direct relationship between target protein concentrations 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 NT4/5 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 NT4/5 in the unknown sample. In the exemplary standard curve on the right, a sample with OD_{450} = 1 reads as 190 pg/mL NT4/5 (red line). If the samples measured were diluted, multiply the concentrations from interpolation (see step 1) with the dilution factor to obtain the actual NT4/5 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 NT4/5 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, NT4/5 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 NT4/5 standards are summarized in the following table:

	OD 450 nm			
NT4/5 / [pg/mL]	**Mean**	**SD**	**SEM**	**CV**
1000	2.748	0.038	0.027	0.027
500	1.948	0.004	0.003	0.003
250	1.231	0.003	0.002	0.002
125	0.737	0.014	0.010	0.010
62.5	0.473	0.001	0.000	0.000
31.3	0.298	0.003	0.002	0.002
15.6	0.191	0.008	0.006	0.006
Blank	0.088	0.000	0.000	0.000

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Limit of Detection

This NT4/5 ELISA kit typically detects a minimum of 10 pg/mL NT4/5 (defined as 150% of blank value).

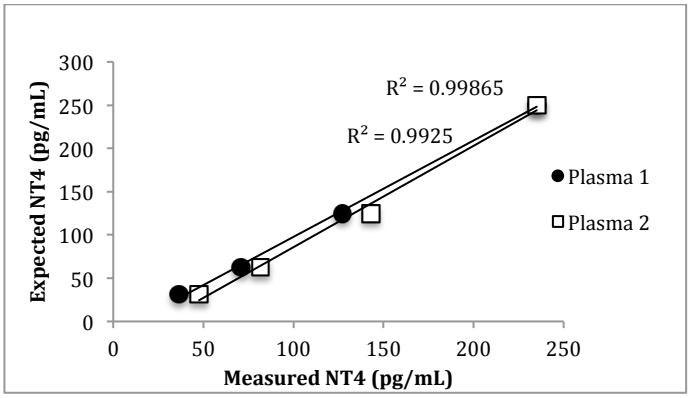
Recovery of NT4/5 Spike in Human Citrate Plasma

Two human citrate plasma samples were diluted in sample diluent containing the HA blocker # BL-003-1000. These samples were then spiked with exogenous NT4/5 protein (250 pg/mL) and measured in the NT4/5 *Rapid*™ ELISA as per the kit protocol (Section 9). The results in the table below show at ≥ 1:8 dilution of sample, between 97-138% of the spike was recovered.

Percentage (%) of Exogenous NT4/5 Spike Recovered				
Dilution	**1:8**	**1:16**	**1:32**	**1:64**
Plasma 1	98	106	119	122
Plasma 2	97	117	129	138

Linearity of Dilution of Human Citrate Plasma

A spike of NT4/5 (250 pg/mL) was added to two separate human citrate plasma samples at a 1:8 dilution in sample diluent containing the Biosensis heterophilic antibody blocker # BL-003-1000. These samples were then serially diluted (1:8 – 1:64) in sample diluent. The diluted samples were run on the NT4/5 ELISA as per protocol (Section 9). The observered or measured NT4/5 values were plotted against the expected NT4/5 values.



The above graph demonstrates linearity of dilution across the tested dilution range in citrate-plasma samples

Quantification of NT4/5 in Human Citrate Plasma

Two human citrate plasma samples were diluted 1/10 to 1/80 in Sample Diluent in the presence and absence of the BL-003-1000 blocker. The samples were spiked with exogenous NT4 (500 pg/mL) and measured in triplicate in the NT4/5 *Rapid*™ ELISA as per kit protocol (Section 9). The ELISA was repeated on two independent days (n=2)

	Without BL-003-1000		*With BL-003-1000*	
Plasma Dilution	% spike recovered	NT4/5 (pg/mL)	% spike recovered	NT4/5 (pg/mL)
10	66	4855	93	0
20	87	4746	97	0
40	74	5578	106	0
80	94	5235	109	0

The above table shows that when human citrate plasma is diluted in sample diluent in the presence of BL-003-100, endogenous NT4/5 levels are below the detection limit. In comparison, in th absence of BL-003-1000, false-positive NT4/5 levels are detected. Furthermore, the spiked exogenous NT4/5 in the plasma sample diluted in BL-003-100 are within an acceptable range (80-120%) at all plasma dilutions. In comparison, in the absence of BL-003-1000 in the sample diluent, there is matrix interference observed causing lower recovery levels of spiked NT4/5.

Specificity

No cross-reactivity is observed with NGF, BDNF or NT3 at concentrations of 25 ng/mL in assay buffer.

13. Specific References

Allen RS *et al.*, 2018. *TrkB signaling pathway mediates the protective effects of exercise in the diabetic rat retina.* Eur J Neurosci. [Epub ahead of print]. **Application: Rat retina homogenates.**

Maejima H *et al.*, 2017. *Exercise enhances cognitive function and neurotrophin expression in the hippocampus accompanied by changes in epigenetic programming in senescence-accelerated mice.* Neurosci Lett. [Epub ahead of print]. **Application: Mouse hippocampus homogenates.**

Takahashi K et al., 2016. *Exercise combined with low-level GABAA receptor inhibition up-regulates the expression of neurotrophins in the motor cortex.* Neurosci Lett. [Epub ahead of print]. **Application: Mouse cortex homogenates, prepared in native cell lysis buffer.**

14. Other Information

The NT4/5 capture antibody can be purchased from Biosensis (Product# S-058-100) and is useful for a variety of *in vitro* and *in vivo* applications, including western blotting and immunohistochemistry.

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

Standard curve, blank and controls:

- Standard (1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 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.

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: Troubleshooting Guide

This NT4/5 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 NT4/5 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 NT4/5 into your sample and/or check that the QC sample value falls within the expected NT4/5 concentration range
	Insufficient antibody or insufficient HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume
	Reagents expired	Ensure correct storage of the kit and do not use the kit beyond its expiry date
Low absorbance readings	Decreased incubation times and temperature	Please follow incubation times as stated in the protocol and warm reagents to room temperature before use
	Microplate wells dried out	Do not leave microplate wells without solution for an extended time
	Wash buffer not diluted	Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution
	Stop solution not added	Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm
Standard OD values above plate reader limit	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
Sample OD values above standard curve range	NT4/5 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