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biosensis® NGFR/p75^{ECD} *Rapid*™ ELISA Kit: Mouse

Catalogue Number: BEK-2220-1P/2P

For the quantitative determination of NGFR/p75^{ECD} cell culture supernatants and urine 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 NGFR/p75^{ECD} cell culture supernatants and urine 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.

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

2. Introduction

The low affinity nerve growth factor receptor (NGFR), also known as the p75 neurotrophin receptor (p75^{NTR}; TNFRS16; CD271) is a common receptor for the neurotrophins NGF, BDNF, NT-3 and NT-4/5. In neurons, p75^{NTR} mediates a variety of physiological functions including survival, apoptosis, neurite outgrowth and synaptic plasticity.

The importance of p75^{NTR} has grown with the demonstration that the pro-forms of the neurotrophins are potent agonists of p75^{NTR}, and that it is a biomarker for disease progression in Amyotrophic Lateral Sclerosis (ALS). Mouse models have helped to identify that altered p75^{NTR} expression levels are implicated in degeneration of spinal motor neurons ALS. Importantly, the extracellular domain of p75^{NTR} (herein referred to as p75^{ECD}) is shed from the cell membrane and excreted in urine (DiStefano & Johnson 1988). Recent findings further suggest that p75^{ECD} is an early biomarker for ALS in humans, as significantly elevated p75^{ECD} levels are found in urine of ALS patients compared to healthy controls (Shepheard *et al.* 2014).

The Biosensis NGFR/p75^{ECD} *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the quantification of mouse p75^{ECD} in less than 4 hours. This kit consists of a pre-coated mouse monoclonal anti-p75^{ECD} capture antibody, a goat anti-p75^{ECD} detection antibody and a horseradish peroxidase (HRP)-conjugated anti-goat antibody. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the concentration of p75^{ECD} present in samples and protein standards.

This NGFR/p75^{ECD} ELISA kit employs a recombinant mouse p75^{ECD}-Fc chimera protein as standard. While there is a current lack of a true mouse p75^{ECD} standard, this ELISA kit allows quantification of mouse p75^{ECD} as p75^{ECD}–Fc mouse equivalents. Please note that the antibodies used in this ELISA cross-react with human NGFR/75^{ECD}.

3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
p75^{ECD} 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 mouse p75^{ECD}-Fc standard	2 x 8 ng	4 x 8 ng
Lyophilized p75^{ECD} detection antibody	1 vial	2 vials
Anti-goat-HRP conjugate (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 on same day; aliquot unused standard to prevent multiple freeze-thaw cycles and store at -20°C for 2 weeks
Reconstituted detection antibody (100x)	6 months at 2-8°C
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 p75^{ECD} in cell culture supernatants and urine. 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 p75^{ECD} in samples, it is important to perform several dilutions of the sample to allow the p75^{ECD} concentration to fall within the range of the p75^{ECD} standard curve (62.5-4000 pg/mL). Also, with unknown samples of all types it is highly recommended to run some p75^{ECD} 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.

Urine

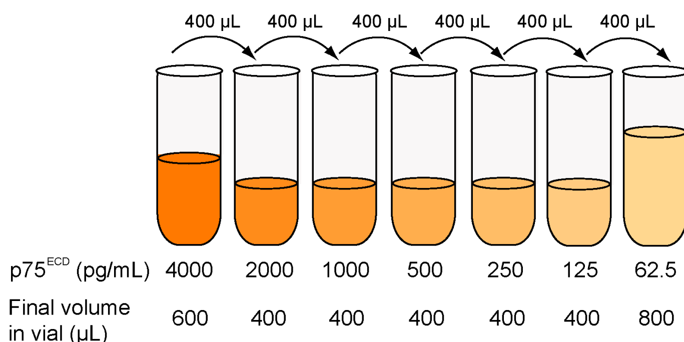
- Collect urine and centrifuge for 10 min at 2-8°C at 1,000 x g
- Analyse immediately or freeze sample aliquots at -20°C to -80°C within 4 hours of collection
- Thaw frozen urine samples at 37°C in a water bath

7. Preparation of p75^{ECD} Standard

- Reconstitute the lyophilized antigen standard with 1 mL of the **same diluent used for preparing sample dilutions**
- Label the vial with the reconstituted p75^{ECD} standard as “8000 pg/mL”; vortex and let stand for 15 minutes
- Dilute the 8000 pg/mL p75^{ECD} standard 1:2 (eg., 500 µL of 8000 pg/mL standard + 500 µL assay diluent); label this tube “4000 pg/mL”
- **Note:** 4000 pg/mL is the highest concentration of the p75^{ECD} standard curve

In order to generate a p75^{ECD} standard curve, perform a 1:2 serial dilution down to 62.5 pg/mL. The volumes used for the dilution series depends on the number of repeats per p75^{ECD} concentration. For triplicate measurement (100 µL per well) of each p75^{ECD} standard concentration, we recommend this procedure:

1. Label 6 tubes with “2000 pg/mL”, “1000 pg/mL”, “500 pg/mL”, “250 pg/mL”, “125 pg/mL” and “62.5 pg/mL”, respectively
2. Aliquot 400 µL of the assay diluent into each tube
3. Take 400 µL from the “4000 pg/mL” tube and transfer to the “2000 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 “62.5 pg/mL” is prepared and mixed well



8. Other Reagents and Buffer Preparation

- Detection antibody (lyophilized) and anti-goat-HRP conjugate (100x): reconstitute detection antibody vial with 110 µL Assay Diluent A to give a 100x concentrated solution. Dilute each vial one hundred-fold with Assay Diluent A to yield a 1x working solution; **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 p75^{ECD} standards, 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 anti-goat-HRP conjugate into each well
9. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
10. Discard the solution inside the wells and wash as described in step 4

11. Add 100 µL of TMB into each well and incubate plate at room temperature for 10-15 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 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 p75^{ECD} 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 10-15 minutes is usually sufficient to obtain a 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 p75^{ECD} standard concentration, blank and sample
2. Plot a standard curve with the p75^{ECD} standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the p75^{ECD} 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 regression analysis to calculate the concentration of p75^{ECD} in the samples. Multiply the result by the sample dilution factor
6. For urine studies, Biosensis recommends the use of creatinine as a way of reducing variability in assessment of dilute or concentrated samples, as described in Shepheard *et al.* (2014). Results are expressed as pg p75^{NTR}/ mg creatinine.

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 p75^{ECD} standard solution (Y-axis) vs. the respective known concentration of the p75^{ECD} 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}

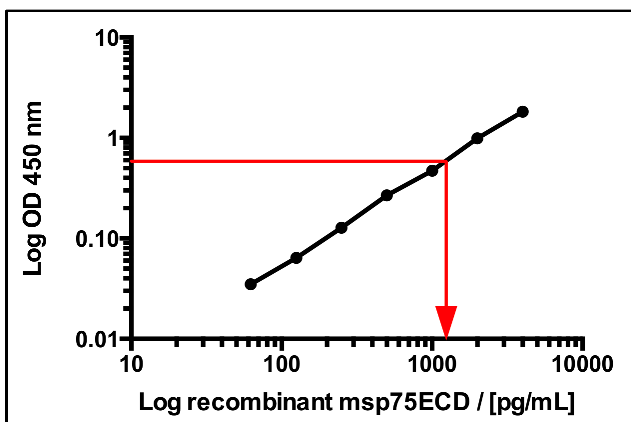
2. **Determine concentration of target protein in unknown sample.** The p75^{ECD} protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of p75^{ECD} in the unknown sample. In the exemplary standard curve on the right, a sample with OD_{450} = 0.5 reads as 1150 pg/mL p75^{ECD} (red line). If the samples measured were diluted, multiply the concentrations from interpolation (see step 1) with the dilution factor to obtain the actual p75^{ECD} 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 NGFR/p75^{ECD} 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, recombinant mouse p75^{ECD} standards were run in triplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 12 minutes.

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

	OD 450 nm			
p75^{ECD} / [pg/mL]	Mean	SD	SEM	CV
4000	1.823	0.074	0.043	3.55 %
2000	0.985	0.003	0.002	0.26 %
1000	0.467	0.002	0.001	0.31 %
500	0.265	0.002	0.001	0.38 %
250	0.124	0.002	0.001	0.52 %
125	0.060	0.011	0.006	3.46 %
62.5	0.028	0.006	0.004	2.25 %
Blank	0 (0.255)	0.005	0.003	1.92 %

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

Limit of Detection

This ELISA kit typically detects 20-40 pg/mL of mouse p75^{ECD} (defined as blank OD plus 3x the standard deviation of the blank OD, n=10).

Specificity

The antibodies used in this ELISA kit are known to cross-react with human p75^{ECD} protein.

13. Informational References

DiStefano PS, Johnson EM (1988) **Identification of a Truncated Form of the Nerve Growth Factor Receptor.** Proc Natl Acad Sci USA 85: 270-274.

Shepheard SR *et al.* (2014) **The Extracellular Domain of Neurotrophin Receptor p75 as a Candidate Biomarker for Amyotrophic Lateral Sclerosis.** PLoS ONE 9(1): e87398. doi:10.1371/journal.pone.0087398**.**

14. Other Information

For quantification of p75^{ECD} in human samples we recommend the Biosensis Human NGFR/p75^{ECD} *Rapid*^{TM} ELISA kit (BEK-2219) which utilizes a recombinant human p75^{ECD}-Fc protein as standard.

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

Standard curve, blank and controls:

- Standard (4000 pg/mL, 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 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 p75^{ECD} 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 > 0.35)	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 p75^{ECD} 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 p75^{ECD} into your sample or use a sample with known concentration of p75^{ECD} 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
	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	p75^{ECD} 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