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

Catalogue Number: BEK-2239-1P/2P

For the quantitative determination of human NGFR/p75^{ECD} in urine if used as directed.

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} in human urine only if used as directed.

This kit has not been tested for any other sample matrix. 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 nerve growth factor receptor (NGFR), also known as 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.

A potential pathological role for p75^{NTR} has become evident in recent years. Altered p75^{NTR} expression levels are implicated in degeneration of spinal motor neurons in human and mouse models of amyotrophic lateral sclerosis (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} could be an early biomarker for ALS in humans, as significantly elevated p75^{ECD} levels are found in urine of ALS patients as compared to healthy controls, and levels increase with progression of disease (Shepheard *et. al.* 2014 and 2017).

The Biosensis NGFR/p75^{ECD} *Rapid*™ enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the quantification of human p75^{ECD} in 4 hours. This kit consists of a pre-coated mouse monoclonal anti-p75^{ECD} capture antibody, a biotinylated mouse anti-p75^{ECD} 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 p75^{ECD} present in samples and protein standards.

The provided Quality Control (QC) sample serves as a positive control with a defined concentration range of p75^{ECD} protein, formulated in a stabilized buffer solution and designed to assure assay performance.

This NGFR/p75^{ECD} ELISA kit employs a recombinant human p75^{ECD}-Fc chimera as standard. While there is a current lack of a commercially available, true human p75^{ECD} standard, this ELISA kit allows quantification of human p75^{ECD} as p75^{ECD}–Fc equivalents. Please note that this ELISA assay does not cross-react with mouse p75^{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 human p75^{ECD} standard	2 x 8000 pg	4 x 8000 pg
Quality Control (QC) Sample	2 Vials (see vial label for amount)	4 Vials (see vial label for amount)
p75^{ECD} 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 protein 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
- For best assay reproducibility, a consistent environmental temperature is important. Bring the microplate and all reagents and solutions to room temperature before starting the assay. **Hint:** Reagents can be left at room temperature overnight for complete equilibration, reagents are stable under this condition
- 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.

While an inter-batch variability of < 10% can be achieved, we recommend running control urine samples (internal controls/standards) throughout a study for additional reproducibility.

6. Sample Preparation

The assay diluent provided in this kit is suitable for measuring p75^{ECD} in urine. Urine samples should be diluted in this Assay Diluent A at a minimum required dilution of 1:4. 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 if 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 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 p75^{ECD}. 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.

Human Urine

The preparation of urine samples for analysis has been adapted from the Human Kidney and Urine Proteome Project (HKUPP) workshop (Yamamoto 2010).

- Collect mid-stream urine, preferably of the 2nd morning urine
- Centrifuge for 10 min at 2-8°C at 1,000 x g within 30 minutes of collection
- Analyze 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
- Vortex well

- An additional centrifugation step (5 min at 2-8°C at 1,000 x g) is recommended to remove potential precipitate
- Dilute urine samples in Assay Diluent A with a minimum required dilution of 1:4

Note: Internal validation showed that p75^{ECD} can be quantified in urine from healthy subjects (subjects from ages 20 to 75 have been tested).

7. Preparation of p75^{ECD} Standard

- Reconstitute the lyophilized antigen standard with 1 mL of Assay Diluent A.
- Label the vial with the reconstituted p75^{ECD} standard as “8000 pg/mL”; vortex and let stand for 15 minutes
- Dilute the reconstituted 8000 pg/mL standard 2-fold (eg. 500 uL of 8000 pg/mL standard + 500 uL of diluent) and label the 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 sample 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

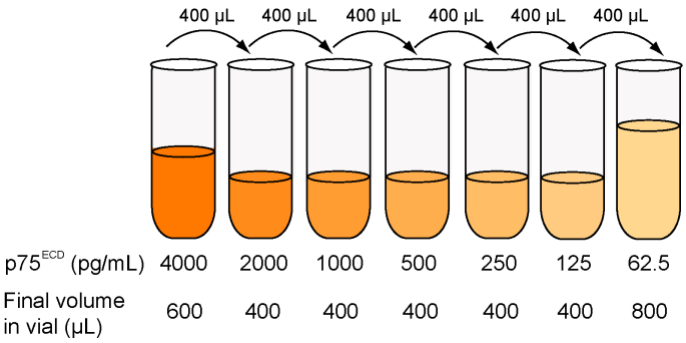

Figure 1: Schematic representation of p75^{ECD} standard preparation

8. Other Reagents and Buffer Preparation

- Quality Control (QC) sample: Reconstitute the lyophilized sample with 1 mL of Assay Diluent A and use without further dilution
- Detection antibody (100x) and Streptavidin-HRP conjugate (100x): Dilute each vial one hundred-fold with Assay Diluent A to yield a 1x working solution, 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

Maintaining a consistent environmental temperature (20-25°C, 70-75°F) is important for best inter-assay reproducibility. Bring all kit components including the required number of plate strips to room temperature before beginning the assay.

1. Add 100 µL of diluted p75^{ECD} 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, as well as an internal standard (urine sample)
3. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 150 minutes (2.5 hours)
4. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 µL per well). See the

technical hints section for a detailed description of the washing procedure

5. Add 100 µL of the detection antibody (1x) into each well
6. Seal the plate (eg., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 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 4
11. Add 100 µL of TMB into each well and incubate plate at room temperature for 10-25 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. Vortex urine samples after thawing
3. At least duplicate measurements for each standard and sample dilution is recommended; for highest accuracy of data we suggest triplicate measurements
4. Dilute samples to a p75^{ECD} concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
5. Avoid touching the inside surface of the wells with the pipette tip
6. 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
7. 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
8. Add TMB and the stop solution to the wells in the same order
9. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading
10. Stopping the TMB reaction after 10-25 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 (23-24˚C, 73-75˚F) in our laboratories.
11. TMB incubation times can vary between 10-25 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 should 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 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; do not use linear regression analysis!
5. Perform a 4-PL regression analysis to calculate the concentration of p75^{ECD} in the QC sample. An observed concentration within the range of 700 – 1300 pg/mL indicates acceptable assay performance
6. Perform a 4-PL regression analysis to calculate the concentration of p75^{ECD} in the samples. Multiply the result by the sample dilution factor
7. **Normalisation of p75^{ECD} concentrations using creatinine**. Normalisation of urinary p75^{ECD} concentrations with creatinine in each sample is required, as described in Shepheard *et al.* (2014, 2017). Appendix C explains how to normalise p75^{ECD} concentration using creatinine concentrations. Example data is also provided.

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 in Figure 2, a sample with OD_{450} = 0.50 reads as 319 pg/mL p75^{ECD}. 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 the example graph in Figure 2, recombinant human p75^{ECD} standards were run in triplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 15 minutes.

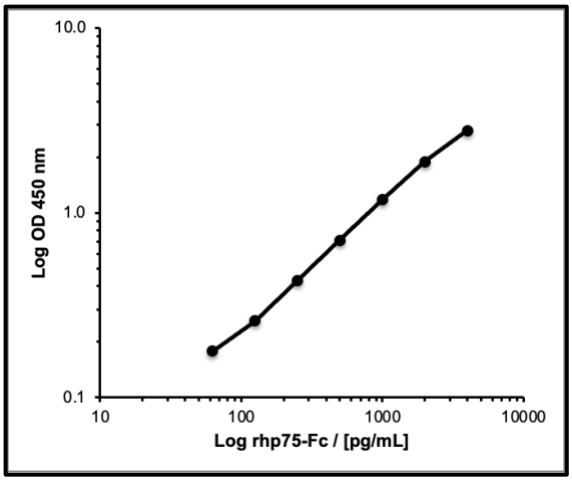

Figure 2: Human p75^{ECD} standard curve

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

	OD 450 nm	
p75^{ECD} / [pg/mL]	**Mean**	**CV**
4000	2.780	1.7%
2000	1.887	2.3%
1000	1.178	2.4%
500	0.708	2.7%
250	0.430	0.7%
125	0.260	1.3%
62.5	0.178	3.8%
Blank	0.099	7.7%

CV: coefficient of variation

Limit of Detection

This ELISA kit typically detects a minimum of 50 pg/mL of human p75^{ECD} (defined as the concentration at blank OD plus 3 x standard deviations of blank OD, n = 10).

Specificity

This ELISA kit does not cross-react with mouse p75^{ECD} protein.

Intra-Assay Precision (Within Assay Precision)

Three urine samples from healthy individuals with 3 different levels of p75 were diluted out with Assay diluent A. The validation samples were selected at high, medium and low concentrations to ensure that as far as possible all parts of the standard curve were controlled for. The plate layout was carefully selected to ensure that all sample concentrations were distributed across the plate and to test for the possible occurrence of geographical plate differences (for example, plate coating or edge or temperature effects). Each validation sample was tested 4 times on a single plate in a single run to test intra-assay precision. Three plates were run by three individual technicians on different days. The results summarized in the table below, show excellent intra-assay precision with CV (coefficient of variation) values ranging from 4.3 – 9.0%.

Intra-Assay Precision				
	Sample	**High**	**Medium**	**Low**
Plate 1 (Operator 1)	n	4	4	4
	Mean (pg/mL)	1483	1073	278
	Stdev.	91.9	77.2	24.4
	CV (%)	6.2	7.2	8.8
Plate 2 (Operator 2)	n	4	4	4
	Mean (pg/mL)	1266	954	246
	Stdev.	114	57	14
	CV (%)	9.0	6.0	5.7
Plate 3 (Operator 3)	n	4	4	4
	Mean (pg/mL)	1376	1070	303
	Stdev.	58.9	54.5	17.6
	CV (%)	4.3	5.1	5.8
Range CV (%)		**4.3 – 9.0**		

Inter-Assay Precision (Precision Between Plates)

Inter-assay precision was calculated on twelve values from three runs for each of the low, medium and high validation samples. Each concentration of validation sample was tested 4 times per run. Three assays were performed by three individual technicians on two different days. The results summarized in the table below, show excellent precision with CV values < 11%.

Inter-Assay Precision			
Sample	High	Medium	Low
n	12	12	12
Mean (pg/mL)	1375	1033	276
Stdev.	124	82	30
CV (%)	9.0	7.9	10.9
Range CV(%)	**7.9-10.9**		

Quantification of p75^{ECD} and Parallelism in Human Urine

Parallelism between the calibration standard curve (in Assay Diluent A) and serially diluted urine samples was assessed to detect possible matrix effects. Each urine sample was diluted to three to four concentrations with Assay Diluent A. The precision between diluted samples in a dilution series ranged from 1.2 to 13.9%, which meets the European Medicines Agency (EMA), Bioanalytical Method Validation Guideline requirements for a CV of less than 20%.

All samples showed detectable levels of p75^{ECD} demonstrating that p75^{ECD} levels can be detected in normal human urine.

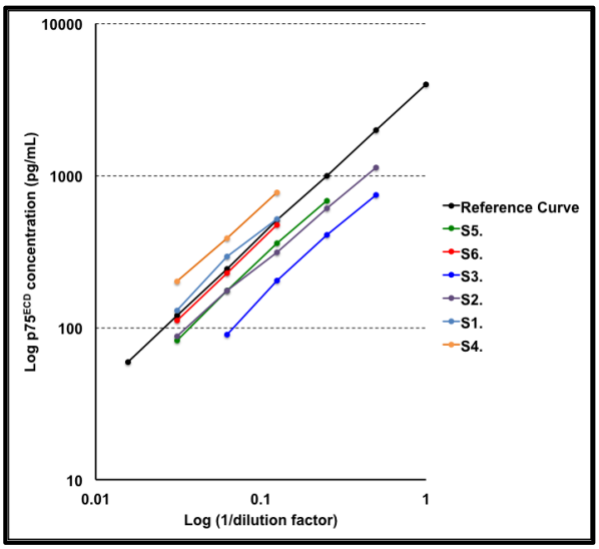

Figure 3: Parallelism in Human Urine

Parallelism in Human Urine			
Sample	**Dilution**	**p75^{ECD} (pg/mL)**	**p75^{ECD} (pg/mL) Dilution Corrected**
S1	1/4	824	3297
	1/8	512	4094
	1/16	290	4643
	1/32	133	4268
	Mean		**4075**
	%CV		**13.9**
S2	1/4	607	2426
	1/8	311	2486
	1/16	176	2823
	1/32	93	2988
	Mean		**2681**
	%CV		**10.0**
S3	1/4	401	1605
	1/8	204	1635
	1/16	95	1512
	Mean		**1584**
	%CV		**4.0**
S4	1/4	1345	5380
	1/8	779	6232
	1/16	383	6128
	1/32	202	6464
	Mean		**6051**
	%CV		**7.7**
S5	1/4	663	2653
	1/8	372	2975
	1/16	194	3104
	1/32	79	2515
	Mean		**2812**
	%CV		**9.7**
S6	1/4	508	2032
	1/8	260	2077
	1/16	127	2038
	Mean		**2049**
	%CV		**1.2**

Dilutional Linearity

To confirm the accuracy of p75^{ECD} levels in normal human urine, a spike of p75^{ECD} standard (3000 pg/mL) was added to 25% normal human urine sample (i.e. neat normal human urine diluted 1:4 in assay diluent). These samples were then serially diluted (1:2 – 1:16) in 25% normal human urine. The measured p75^{ECD} values were plotted against the expected p75^{ECD} values.

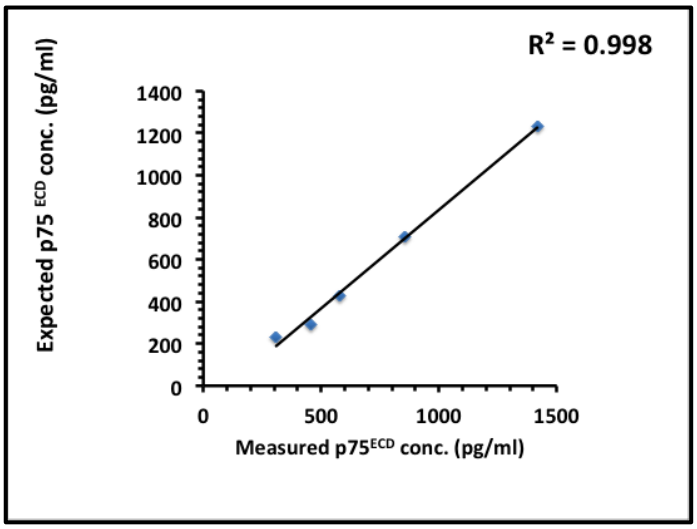

Figure 4: Linearity of Dilution in Human Urine

This data confirms that this ELISA kit is compatible with urine matrix and that a minimum required dilution of 1:4 is compatible with the assay.

Intra-Batch Variation

The variation between different plates from the same batch was determined by running samples at different dilutions across randomly sampled plates from a single manufactured batch of pre-coated plates. The CV ranged from 3.2 to 6.9%

Inter-Batch Variation

The variation between plates from different batches was tested by running samples at different dilutions on plates from 3 batches that were manufactured across a 14-day period. The precision ranged from 7.5 to 9.9%.

13. Other Information

For quantification of p75^{ECD} in murine samples we recommend the Biosensis Mouse NGFR/p75^{ECD} *Rapid*™ ELISA kit (BEK-2220-1P/2P).

14. Informational References

DiStefano and Johnson 1988, *Identification of a truncated form of the nerve growth factor receptor*. Proc Natl Acad Sci U.S.A.Jan;85(1):270-4

European Medicines Agency 2011, *Guidelines on Bioanalytical Method Validation.* 21July 2011

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Shepheard *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.

Tang *et al*. 2015, *Normalization of urinary biomarkers to creatinine for clinical practice and research – when and why.* SMJ Jan;56(1):7-10

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

Standard curve, blank and controls:

- Standard (500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 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 subjects) 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/patient 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/patient samples on the "standard curve plate" (the plate containing the standard curve, controls and blanks) and a full 96 test/patient samples on the "test only plate" for a total available number of 174 test/patients 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 patient samples, duplicate measurement at one single dilution
How many wells and plates do I need for sixty patients at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 patients, 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 patients. In summary: 60 patients, 4 tests per patient, 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 patients 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 patient sample, tested in duplicate (two draws per patient, 1 dilution, 4 wells per patient, 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 patient samples tested in duplicate at two dilutions (two draws per patient, 2 dilutions, 8 tests/wells per patient, total of 480 patient tests/wells).

2-Plate Optional Single Control Set Option:

If running 60 patients 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 patients, 2 draws per patient 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 patient samples.

For the two dilutions per sample, sixty patients, 2 draws per patient 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 patient samples.

Appendix B: Troubleshooting Guide

This NGFR/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 > 0.30)	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 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 and/or check that the QC sample value falls within the expected p75^{ECD} 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	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

Appendix C: Normalisation of p75^{ECD} Concentration with Creatinine

Urinary biomarker concentrations are frequently reported as a ratio to urinary creatinine, because water reabsorption in kidneys affects urinary solute concentrations. Creatinine is a breakdown product of creatine phosphate during muscle metabolism and is filtered out of the blood into the urine by the kidney. Creatinine production is usually at a fairly constant rate when renal function, metabolism and muscle mass are stable, but can be dependent on age, sex, and race (Tang *et al.*, 2015). A highly concentrated sample from a dehydrated individual will yield higher concentrations of p75^{ECD} compared to a sample from the same individual when they are well hydrated. Therefore, urine p75^{ECD} readings should be normalized against Creatinine levels from the same urine sample.

Measuring Creatinine Levels

Normalisation of urinary p75^{ECD} concentrations with creatinine in each sample is required, as described in Shepheard *et al.* (2014, 2017). See section **14 Informational References.**

Calculation of Normalised p75^{ECD} Concentration

1) Adjust p75^{ECD} and creatinine levels for sample dilution factors
2) Creatinine values are generally reported as mg/dL. Convert the results to mg/mL by dividing each value by 100. For example: 60.9 mg/dL = 0.61 mg/mL
3) p75^{ECD} values obtained in this kit are expressed as pg/mL. Convert each value to ng/mL by dividing each value by 1000. For example: 2027.9 pg/mL = 2.03 ng/mL
4) To express p75 values in ng/mg of Creatinine, divide the p75 value (ng/mL) obtained in step 3 by the creatinine value (mg/m) obtained in Step 2:

$$\frac{p75ECD\ (\frac{ng}{mL})}{Creatinine\ (\frac{mg}{mL})} = \text{p75}^{\text{ECD}} \text{ concentration in ng/mg creatinine}$$

For example: $\frac{2.03\ \frac{ng}{mL}}{0.61\ \frac{mg}{mL}} = 3.33$ ng/mg creatinine

Example: Normalised p75^{ECD} values from 9 healthy urine samples.

Sample	Creatinine concentration (mg/dL)	Creatinine concentration (mg/mL)	p75^{ECD} concentration (pg/mL)	p75^{ECD} concentration (ng/mL)	Normalised p75^{ECD} concentration (ng/mg Creatinine)
U1	60.9	0.61	2027.9	2.03	3.33
U2	27.8	0.28	712.1	0.71	2.54
U3	124.3	1.24	3368.4	3.37	2.72
U4	88.7	0.89	2708.0	2.71	3.04
U5	44.7	0.44	1545.7	1.54	3.50
U6	71.4	0.71	2600.9	2.60	3.66
U7	73.2	0.73	1164.9	1.16	1.59
U8	53.5	0.54	1235.2	1.24	2.30
9	176.3	1.76	4335.1	4.34	2.47