Phosphorylated Neurofilament NF-H ELISA Kit

Enzyme Immunoassay kit for the quantification of Phosphorylated Neurofilament NF-H (pNF-H) in plasma, serum, CSF and tissue extracts.

Catalog number: ARG81165

distributed in the US/Canada by:
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INTRODUCTION

Neurofilaments are type IV intermediate filament heteropolymers composed of light, medium, and heavy chains. Neurofilaments comprise the axoskeleton and functionally maintain neuronal caliber. They may also play a role in intracellular transport to axons and dendrites. This gene encodes the heavy neurofilament protein. This protein is commonly used as a biomarker of neuronal damage and susceptibility to amyotrophic lateral sclerosis (ALS) has been associated with mutations in this gene. [provided by RefSeq, Oct 2008]

The pNF-H protein has been detected in large amounts following experimental spinal cord and brain injury in rats (18). Levels of greater than 100ng/mL of pNF-H were detectable in blood samples following serious spinal cord injury and lower but still easily detectable levels were seen in blood of animals given experimental brain injury.

More recent studies have revealed considerable amounts of this protein in the blood of transgenic mice carrying mutations of human copper/zinc superoxide dismutase-1 which are associated with amyotrophic lateral sclerosis. These mice develop an axonal degeneration pathology similar to that seen in humans with ALS, and blood pNF-H levels can be used to monitor progression of the disease. Interestingly, pNF-H was detectable before the onset of obvious disease symptoms.

PRINCIPLE OF THE ASSAY

This assay employs the sandwich enzyme immunoassay technique for the detection of pNF-H protein in plasma, serum, CSF and tissue extracts samples. pNF-H protein in samples and standards will bind to the capture mouse monoclonal antibody coated on the microtiter plate. After appropriate washing steps, HRP-conjugated anti-pNF-H monoclonal antibody binds to the captured protein. After washing away any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and color develops in proportion to the amount of pNF-H protein bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450nm ±2nm. The concentration of pNF-H protein in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	8 strips X 12 wells	4°C
Standard (5 µg/ml)	10 µl	Ship at 4°C, store at -20°C upon receipt
HRP-Anti-pNF-H Antibody (4000X)	6 µl	Ship at 4°C, store at -20°C upon receipt
10X TBST	5 ml	4°C
Blocker	0.5 g	4°C
TMB substrate	12 ml (ready-to-use)	4°C (Protect from light)

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- 2N H_2SO_4 for stopping color development
- 1X TBST for washing

  TBST buffer: 1X TBS buffer (0.01M Tris-HCl, 0.15M NaCl, pH 7.4-7.5) with 0.1% of Tween-20)
- Microplate shaker (shaking amplitude 3 mm; approx. 300 rpm), or an orbital shaker. If other kind of shaker is used, the rotating speed may need to be optimized by user.
- Pipettes and pipette tips
- Deionized or distilled water
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- This kit can be stored for up to 6 months. All components should be stored at 4°C except the pNF-H protein standard, which should be stored frozen at -20°C.
- If crystals are observed in the 20X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Avoid using reagents from different batches.
- Briefly spin down the Primary, Secondary Antibody and standard before

use.

- If an incubation step needs to be extended, avoid letting the plate dry out by keeping sample or detection antibody solution in the wells.
- Avoid prolonged exposure of HRP-conjugated antibody (stock or diluted) to light. During the antibody incubation step, plates do not need to be shielded from light except for direct sunlight.
- Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results, and are particularly a problem during optical density determination.
- The TMB developer solution should be at room temperature when added to the plate. Keeping time intervals consistent between adding developing buffer and reading the plate should improve inter-plate precision.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

The kit measures PNF-H PROTEIN in mammalian CSF, serum, plasma and urine. Mix thawed samples thoroughly just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot and store samples at-20°C or store at -70°C for long-term storage. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using heparin as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay immediately or aliquot and store at -20°C or store at -70°C for long-term storage. Avoid repeated freeze-thaw cycles.

CSF: Centrifuge to remove particles in the samples. CSF samples should be aliquoted and stored at-70°C. Avoid repeated freeze-thaw cycles.

REAGENT PREPARATION

- Dilution Buffer:
  - **1X TBST:** Dilute 5 ml of 10X TBST buffer (supplied in the kit) into 45 ml of distilled water to yield 50 ml of 1X TBST buffer.
  - **Dilution Buffer**: Add 0.5 g of Blocker into 50 ml of 1X TBST, diluted from 10X TBST as above step, mix well. The reconstituted Dilution Buffer can be stored at 4°C for 2 days.
- **TMB substrate:** The solution should be stored at 4°C. For best results, bring the reagent to room temperature prior to use.
- **Samples** If the measuring absorbance of samples is higher than the highest standard, dilute the samples with Dilution Buffer before assay and assay again. A 1:2.5 dilution for serum/plasma samples and 1:5 dilution for CSF are suggested for the starting dilution. (E.g. 2.5X dilution: 40 µl sample + 60 µl Dilution Buffer; 5X dilution: 20 µl sample + 80 µl Dilution Buffer). For the calculation of the concentrations this dilution factor has to be taken into account.
  (It is recommended to do pre-test to determine the suitable dilution factor).
- **Standard**: The concentration of pNF-H protein Standard (**Stock 0**) is **5 µg/ml (5000 ng/ml).** Dilute the pNF-H protein Standard (Stock 0) at 1:500 dilution with Dilution Buffer to yield a **Stock 1** with **10 ng/ml** of pNF-H protein, gently vortex and mix well. Dilute the standard as the table at below with Dilution Buffer (DB) and the Dilution Buffer (DB) serves as zero standard (0 ng/ml).
  Dilution table for pNF-H protein standard preparation:

Standard #	PNF-H PROTEIN Concentration	Volume of standard (µl)	Volume of DB (µl)
Stock 1	10 ng/ml	2 (Stock 0)	998
Standard 1	5 ng/ml	100 (Stock 1)	100
Standard 2	2.5 ng/ml	100 (Standard 1)	100
Standard 3	1.25 ng/ml	100 (Standard 2)	100
Standard 4	0.625 ng/ml	100 (Standard 3)	100
Standard 5	0.3125 ng/ml	100 (Standard 4)	100
Standard 6	0.156 ng/ml	100 (Standard 5)	100
Blank	0	0	200

1X TBST for washing: 0.01M Tris-HCl, 0.15M NaCl, pH 7.5, 0.1% Tween-20

1. Tris base: 1.21g, NaCl 8.77g
2. Add deionized water to a final volume of 900mL.
3. Adjust pH to 7.4-7.5 using concentrated HCl (be careful!),
4. Add 1mL Tween-20 into 1x TBS buffer from step 3, mix well.
5. Bring final volume to 1L with deionized water.

- **Stop solution:** recommended stop solution is **2N H_2SO_4** solution

  E.g. 26.7mL concentrated H_2SO_4 + 223.3mL H_2O

  Stop solution can be stored at room temperature for up to 3 months.

- **HRP-conjugated anti-pNF-H antibody:** Prepare the HRP-conjugated antibody solution immediately prior to use. Briefly centrifuge vial before opening. Dilute HRP-conjugated anti-pNF-H antibody at **1:4000** in Dilution Buffer to yield a 1X working HRP-conjugated secondary antibody, mix thoroughly. (E.g.: Dilute 2 µl of HRP-conjugated antibody in 8 ml of Dilution Buffer (1:4000 dilution)) Only prepare amount needed, and discard remainders after use.

ASSAY PROCEDURE

TMB substrate should be equilibrated to room temperature (RT) before use. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **50 µl** per well of standards and diluted samples in duplicates into appropriate wells. Incubate the plate at **RT for 2 hours** on a microplate shaker at 250-300 rpm (alternately, cover the plate with a plate sealer and incubate the plate at **4°C for overnight** on a microplate shaker at 250-300 rpm)
3. Aspirate each well and wash, repeating the process 2 times for a **total 3 washes**. Wash by filling each well with **1XTBST (300 µl)** using a squirt bottle, manifold dispenser, or autowasher. Gently shake the plate for few seconds before removal of liquid, and complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining buffer by aspirating, decanting or blotting against clean paper towels.
4. **Turn the plate 180°, wash** as according to step 3.
5. Add **100 µl** of working **1X HRP-conjugated pNF-H Antibody** (1:4000 diluted) into each well. Incubate the plate for **2 hour at RT** on a microplate shaker (250-300 rpm).
6. **Wash** as according to step 3 and step 4. (Before washing step, bring TMB substrate to room temperature.)
7. Add **100 µl of TMB substrate** to each well. Incubate and shake plate on a shaker with gentle shaking for **5-20 minutes at RT in dark** (average time might be 7-10 min). Substrate will change from colorless to different

strengths of blue.

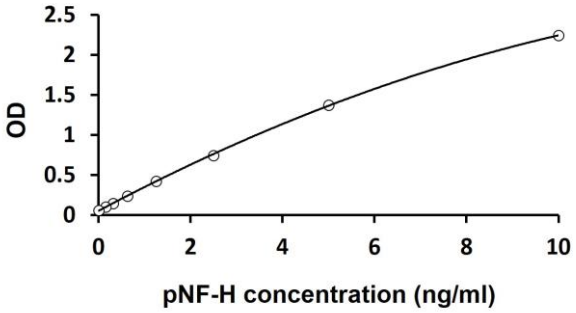
8. Add 50 µl of 2N H_2SO_4 to each well. The color of the solution should change from blue to yellow. Mix thoroughly by gently shaking/tapping the plate. Take care to avoid creating bubbles which will create a strong interfering absorbance signal.
9. Read the OD with a microplate reader at 450 nm immediately.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Use the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
5. The diluted samples must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above. If following the dilution suggestion as the protocol as above the dilution factor will be 2.5. So the measured concentration of samples calculated from the standard curve must then be multiplied by 2. E.g. 400 pg/ml (from standard curve) x 2.5 (dilution factor) = 1000 pg/ml.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Standard Range

The standard range can cover form 0.156 – 10 ng/ml.

Sensitivity

70 pg/ml

Intra-assay and Inter-assay precision

The CV value of intra-assay precision was <6% and inter-assay precision was < 10%.

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For further information about this kit, its application or the procedures in this kit insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.

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