Phosphorylated Neurofilament H (pNF-H) Sandwich ELISA Kit

Cat. No. NS170

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
Australia +61 3 9839 2000
www.millipore.com

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Introduction

The Millipore Phosphorylated Neurofilament H (pNF-H) kit is a sandwich enzyme immunoassay (EIA), which measures pNF-H. The kit will measure pNF-H from a variety of mammalian species including human, rat, mouse, bovine, porcine, and feline.

Neurofilaments are the 10nm diameter filaments which make up the major part of the cytoskeleton of neurons and are particularly concentrated in larger diameter axons. They belong to the intermediate or 10nm filament protein/gene super family which also includes the keratins, the major structural proteins of skin. Neurofilaments are generally regarded as being composed of three major protein subunits referred to as NF-L, NF-M and NF-H. NF-L is the ”low” or ”light” subunit, NF-M is the ”medium” or ”middle” subunit and NF-H is the ”heavy” or ”high” subunit. This nomenclature is based on the apparent size of these proteins on SDS-PAGE gels; NF-L is typically about 68kDa, NF-M is about 150kDa and NF-H is about 200kDa. These three proteins, referred to as the neurofilament triplet, were discovered in the 1970s as a result of studies of axonally transported proteins (Hoffman and Lasek 1975). A fourth protein, α-internexin, a.k.a NF66, was later discovered as a protein co-purifiing with the neurofilament triplet proteins (Pachter and Liem 1985). A fifth protein, peripherin, is found in some neurofilaments along with the other four proteins, mostly in the peripheral nervous system (Portier et al. 1983). Neurofilaments are major proteins of neurons and are particularly concentrated in axons. The protein NF-H has some very unusual properties. Firstly it contains 50-60 back to back hexa, hepta or octapeptide repeats each containing the sequence Lysine- Serine-Proline (KSP). The serine residues in these peptide repeats are phosporylated in vivo, and in axonal neurofilaments, these sites are heavily phosphorylated. The phosphorylated forms of NF-H are also quite resistant to proteases, suggesting that this very abundant protein might be particularly resistant to proteotytic cleavage upon release from damaged and diseased axons. This means that detection of this protein in cerebro spinal fluid (CSF) and blood would provide information about the degree of axonal injury which has occurred (reviewed in Petzold 2005). As recently shown, this protein can be detected in quite large amounts following experimental spinal cord and brain injury in rats (Shaw et al. 2005). Levels of greater than 100 ng/ml of pNF-H were detectable in blood 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 mice transgenic for mutations of human copper/zinc superoxide dismutase 1 which are associated with amyotrophic lateral sclerosis (ALS). 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 this. Interestingly, pNF-H is detectable before the onset of obvious disease symptoms. Other studies show that pNF-H can be detected in tissue homogenates. Other experiments show that pNF-H can be detected in the plasma of humans suffering from optic neuritis (Petzold et al 2004) and in elevated levels in the cerebrospinal fluid (CSF) of individuals suffering from brain tumors and stroke (Petzold and Shaw, unpublished).

Test Principle

With the Millipore pNF-H assay system, chicken polyclonal antibodies generated against pNF-H, are pre-coated onto a 96-well plate and are used to capture pNF-H from a sample. Captured pNF-H is detected using pNF-H specific rabbit polyclonal antibodies and a goat anti-rabbit alkaline phosphatase conjugate. After addition of the substrate solution the amount of pNF-H is determined. The standard curve demonstrates a direct relationship between Optical Density (OD) and pNF-H concentration: i.e., the higher the OD the higher the pNF-H concentration in the sample.

Application

The Millipore pNF-H kit is designed to measure the amount of pNF-H in tissue homogenates and biological fluid (serum, plasma, and serum-free) samples from mouse and rat. There are enough reagents included in this kit for one 96-well immuno-assay plate. Running duplicate wells for samples and standards is recommended.

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Analytical Sensitivity and Detection Limits

Sensitivity: 0.0585 ng/mL
Range of Detection: 0.0293 ng/mL to 15 ng/mL

Kit Materials

1. Immunoplate Pre-coated with Chicken anti-pNF-H Polyclonal Antibody: (Part No. 2006263). One 96-well immunoplate pre-coated with chicken anti- pNF-H polyclonal antibody sealed in a foil pouch.
2. Rabbit anti- pNF-H Polyclonal Antibody: (Part No. 2006919). One vial (100 μL) of rabbit anti- pNF-H polyclonal antibody.
3. Goat anti-Rabbit, Alkaline Phosphatase Conjugated Antibody: (Part No. 2006917). One vial (10 μL) of goat anti-rabbit alkaline phosphatase conjugate.
4. Bovine pNF-H Standard: (Part No. 2006918). One vial (5 ng) of bovine pNF-H standard.
5. 20X TBS: (Part No. 2004913). One bottle (50 mL) of 20X TBS.
6. 20% Tween-20™ : (Part No. 2004915). One vial (3 mL) of 20% Tween-20™.
7. Elisa Assay Diluent: (Part No. 2006921) One bottle (30 mL) of Elisa assay diluent containing 0.05% Kathon as a preservative, provided in a ready to use format.
8. pNPP Buffer: (Part No. 2006261). One bottle (12 mL) of pNPP buffer for use with 50X p-NPP, provided in a ready to use format.
9. 50X pNPP: (Part No. 2006262). One vial (250 μL) of 50X pNPP (p-nitrophenyl phosphatase) alkaline phosphatase substrate.
10. Plate Covers - Two Plate Covers.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Pipettors & tips capable of accurately measuring 10-1000 μL
3. Graduated serological pipettes
4. 96-well microplate reader capable of measuring absorbance at 405nm
5. Graph paper for manual plotting of data
6. 1.5 mL tubes
7. Mechanical vortex
8. Two1 liter containers
9. Plate shaker (optional)
10. 3N NaOH (optional)
11. Distilled or deionized water.

Precautions

The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.

Storage

Prior to use maintain the kit components at 2°-8°C and -20°C, as indicated, until expiration date.

1. Wash Buffer – After dilution maintain the Wash Solution at 2°-8°C for up to one month or until expiration date.

2. Tween-20™ - Store at room temperature upon receipt of kit.

3. Elisa Assay Buffer – Aliquot as required to avoid repeated freeze-thaw cycles. After thawing, maintain the Elisa Assay Buffer at 2°-8°C for up to one month, or until expiration date.

4. Immunoplate Pre-coated with Chicken anti- pNF-H Polyclonal Antibody – Unused strip wells may be returned to the foil pouch and stored at 2°-8°C for up to one month, or until expiration date.

Technical Hints

- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values.
- **Recommended Method for Plate Washing:**
  1. Remove existing fluid from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels.
  2. Forcefully pipet 200 μL of diluted Wash Buffer into each well with a multi-channel pipet or manifold.
  3. Remove fluid from each well by flicking the plate over a sink. Subsequently blot the plate on clean paper towels.
  4. Repeat washing and flicking 6 times.
  5. Agitation of the wells during the ELISA wash steps is recommended to reduce non-specific background.

Preparation of Reagents

1. Wash Buffer

Warm the 20X TBS concentrate to room temperature and mix to ensure that any precipitated salts have re-dissolved. For 500 mL of Wash Buffer, combine 25 mL of 20X TBS, 1.25 mL of 20% Tween-20, and 473.75 mL distilled or deionized water. Stir to homogeneity.

2. TBS

Warm the 20X TBS concentrate to room temperature and mix to ensure that any precipitated salts have re-dissolved. Dilute 1 volume of the 20X TBS concentrate with 19 volumes of distilled or deionized water. Stir to homogeneity.

3. pNF-H Standard

Note: During shipment, small volumes of product may accumulate in the vial cap. For maximum recovery of product, centrifuge the vial prior to removing the cap.

The pNF-H standard is 250 ng/mL. A suggested scheme for a two-fold serial dilution, in duplicate, is as follows:

a) Label 12 microfuge tubes 0, 0.0146, 0.0293, 0.0586, 0.1172, 0.2344, 0.4688, 0.9375, 1.875, 3.75, 7.5, and 15 ng/mL. Add 120 µL of Elisa Assay Buffer to each of the tubes except the 15 ng/mL tube.

b) Prepare the 15 ng/mL pNF-H standard by adding 14.4 µL of the pNF-H Standard to 225.6 µL of Elisa Assay Buffer and vortex.

c) Prepare a two-fold serial dilution by transferring 120 µL of the preceding sample to 120 µL of Elisa Assay Buffer. For example, the 7.5 ng/mL standard is prepared by adding 120 µL of the 15 ng/mL pNF-H standard to 120 µL of Elisa Assay Buffer, mixing thoroughly before the next transfer. Repeat this process to make successive two-fold dilutions. Use 120 µL of Elisa Assay Buffer in the last tube for the zero standard.

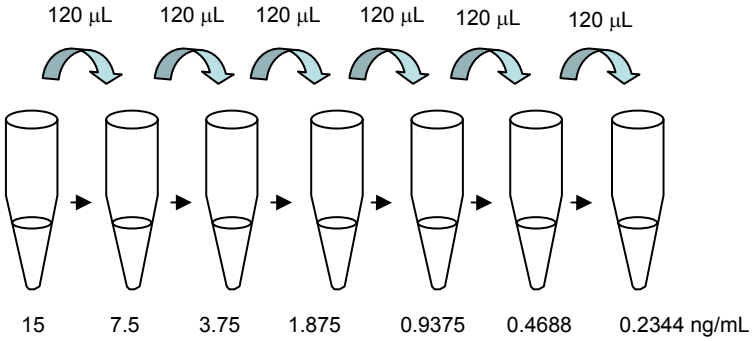


Figure 1: Serial Dilution of pNF-H Standard

Note: *The pNF-H Standard curve can be set up with a different serial dilution scheme by making appropriate adjustments to the dilution pattern*.

4. Rabbit Anti- pNF-H Polyclonal Antibody

Immediately before use dilute the anti-pNF-H Antibody 1:100 with Elisa Assay Diluent as follows: For each 12 well strip prepare 1.4 mL of diluted anti-pNF-H Antibody by adding 14 µL of anti-pNF-H Antibody to 1386 µL of Elisa Assay Diluent.

5. Goat anti-Rabbit Polyclonal Antibody, Alkaline Phosphatase Conjugated

Immediately before use dilute the goat anti-rabbit alkaline phosphatase conjugate 1:2,000 with Elisa Assay Diluent as follows: For each 12 well strip prepare 1.4 mL of diluted goat anti-rabbit alkaline phosphatase conjugate by adding 0.7 µL of goat anti-rabbit alkaline phosphatase conjugate anti-pNF-H Antibody to 1400 µL of Elisa Assay Diluent.

6. 1X pNPP Alkaline Substrate Solution

Immediately before use dilute the 50X pNPP to 1X pNPP with the pNPP buffer as follows: For each 12 well strip prepare 1.4 mL of 1X pNPP alkaline phosphatase substrate solution by adding 28 µL of 50X pNPP to 1372 µL of pNPP buffer.

Preparation of Samples

It is recommended that you test each sample in duplicate.

Tissue Samples: Tissue samples should be rapidly excised, weighed and homogenized in 4M urea, 1 mM EDTA, 1mM EGTA, 0.2 mM PMSF in 10 mM Tris-HCl at a pH of 7.2. It is recommended to use 10 mgs of wet weight of tissue per mL of buffer, but the most appropriate ratio needs to be determined by the user for each tissue. After homogenization the material should be centrifuged in microfuge tubes for 5 minutes at top speed in an Eppendorf type centrifuge. 1 µL or less of CNS tissue will give a strong signal in the ELISA.

CSF Samples: Samples of CSF from control rodents have undetectable levels of pNF-H, while those with CNS injuries frequently have levels in the range of 5 ng/mL. A suggested sample dilution for CSF samples from injured animals is in the range of 1:6 (for example dilute 10 µL of the CSF sample with 50 µL of sample diluent). Optimal dilutions will need to be determined by the end user based on the concentration of pNF-H in the sample.

Blood Samples: Blood can be assayed directly, but for most assay purposes is either allowed to clot or is centrifuged down to pellet out red and white cells. Clotting requires several hours at room temperature, and the clear yellow liquid, the serum, can be used for pNF-H analysis. More rapid and convenient is to spin the blood down at top speed in an Eppendorf centrifuge and take the clear yellow liquid, the plasma, for analysis (serum is therefore basically the same as plasma, except that the blood clotting factors are absent). Workable results have been obtained with blood, serum and plasma, but the kit has been standardized on plasma for reproducibility. A series of blood samples can be taken and frozen at -20°C or lower. Then, when a complete series of samples have been collected they can be thawed out and the red and white cells can be pelleted out in microfuge tubes at top speed for 5 minutes in a microfuge centrifuge. The plasma is then run in the ELISA. It has been found that rats with serious spinal cord injuries will give strong signals with as little as 1 µL of plasma. Rats with brain injuries needed 10 µL or more. Mice generally appear to give stronger signals, and very strong signals have been seen in the plasma for mice transgenic for mutant forms of human copper/zinc superoxide dismutase 1 (SOD1). Humans with these mutations develop a serious form of amyotrophic lateral sclerosis (ALS) and the transgenic mice develop a progressive ALS-like phenotype. The pNF-H ELISA can therefore be used to measure axonal degeneration in these and presumably many other transgenic models of human disease.

Assay Instructions

1. Place the desired number of Phosphorylated Neurofilament H (pNF-H) strips in the strip well plate holder. Return unused strips to the foil pouch. Tape may assist in holding the wells in place during the assay.
2. Hydrate the wells with 100 µL of TBS and incubate the plate at room temperature for 10 minutes.
3. Remove the TBS from the wells and blot dry.
4. Add 50 µL of standards or samples to wells (see reagent preparation section). It is recommended that standards and samples be run in duplicate.

   Note: *A standard curve must be run at each setting.*
5. Seal the plate with a plate sealer. Incubate the plate for 1 for hour at room temperature with gentle shaking.
6. **IMPORTANT WASH STEP:**

   Gently remove the plate sealer and wash the plate at least 6 times. A thorough washing of the plate is extremely important to reduce background. We recommend using a multi-channel pipette to fill each well with 200 µL of diluted Wash Buffer. Fluid removal from the wells is best accomplished by inverting the plate over a sink and flicking the fluid out of the wells and then blotting the plate on clean paper towels. Repeat this procedure for a total of 6 times.

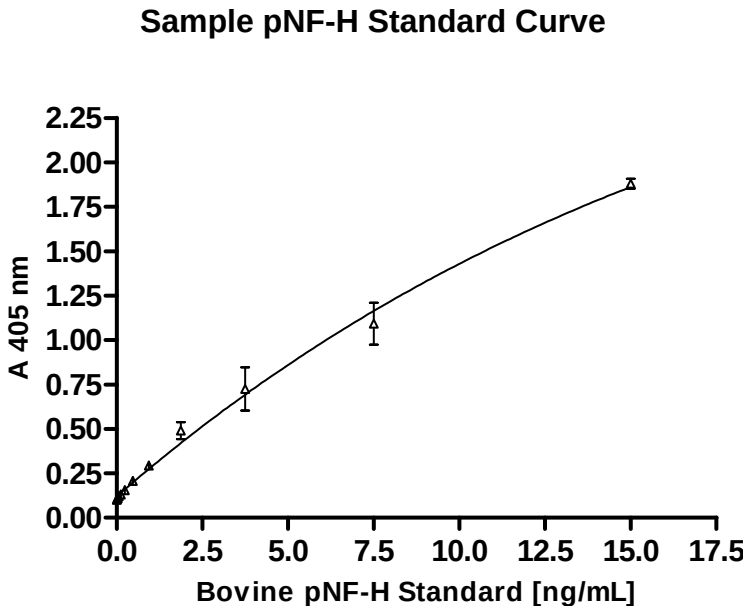
   For users of automatic plate washers: It is important to ensure that the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. The result may be poor precision and an unsuitable standard curve. For best results, we recommend at least 4 wash cycles.
7. Add 100 µL of the diluted anti- pNF-H antibody (see reagent preparation section) to each well. Cover the plate and incubate the plate for 1 hour at room temperature with gentle shaking.
8. Wash as described in Step 6.
9. Add 100 µL of the diluted alkaline phosphatase conjugated goat anti-rabbit polyclonal antibody (see reagent preparation section) to each well. Cover the plate and incubate the plate for 1 hour at room temperature with gentle shaking.
10. Wash as described in Step 6.
11. Rinse the wells with 200 µL of 1X TBS. Remove the fluid and blot the plate dry.
12. Add 100 µL of freshly diluted 1X pNPP alkaline phosphatase substrate (see reagent preparation section) to each well. Incubate the plate in the dark at room temperature for 30-60 minutes. Immediately read the plate at 405 nm. *The reaction may be stopped by the addition of 50 µL per well of 3N NaOH (not provided).*

CAUTION: *Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.*

Calculation of Results

Manual Plotting: Plot the standard curve on graph paper. Known concentrations of pNF-H are plotted on the X-axis and the corresponding OD on the Y-axis. The standard curve should result in a graph that shows a direct relationship between pNF-H concentrations and the corresponding ODs (absorbances). In other words, the greater the concentration of pNF-H in the sample, the higher the OD. The concentration of pNF-H in unknown samples may be determined by plotting the sample OD on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of pNF-H in the unknown sample.

Plate Reader/PC Interface: An alternative approach is to enter the data into a computer program curve fitting software. A good fit can be obtained with a linear regression analysis. Some data points at the top or bottom of the range tested may need to be dropped to get a good fit. Currently existing spreadsheet software can perform such plotting.



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