Abstract

This is an enzyme linked immunosorbent assay (ELISA) for the sensitive detection of pNF-H, one of the major proteins of the axon. When neurological disease or injury occurs, pNF-H leaks from damaged axons into the cerebrospinal fluid and blood, and can be detected by ELISAs such as this one. The level of pNF-H detected can give information about the degree of injury, the progression and recovery state and response of an individual to therapies. The kit can be used to quantify levels of this protein in tissue extracts, in cells in tissue culture in cerebrospinal fluid (CSF) and blood, both plasma and serum. It has been shown to detect pNF-H, released from damaged or degenerating axons in a variety of animal models of human disease and also in several human disease states. The kit has been shown to work in human, pig, rat, mouse, cat and dog, and is expected to work in many other species also.

Key words: neurofilament, NF-H, pNF-H, Biomarker, Axonal Injury
1 Introduction

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 superfamily 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, on which NF-L is typically ~68 kDa, NF-M is ~150 kDa and NF-H is ~200 kDa. The three proteins were discovered in the 1970s as a result of studies of axonally transported proteins (Hoffman and Lasek 1975). These three proteins are referred to as the neurofilament triplet. A fourth protein α-intermixin, a.k.a NF66, which was discovered a little later as a protein which copurifies 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 contain 50 back to back hexa, hepta or octapeptide repeats each containing the sequence Lysine-Serine-Proline (KSP). The serine residues in these peptide repeats are in vivo phosphorylation sites, and, in axonal neurofilaments, these sites are heavily phosphorylated. The phosphorylated forms of NF-H are also quite resistant to proteases, which suggests that on being released from damaged and diseased axons, this very abundant protein might be particularly resistant to proteases. This means that detection of this protein in cerebrospinal fluid (CSF) and blood would provide information about the degree of axonal injury which has occurred (reviewed in Petzold 2005).

The pNF-H 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, Boylan et al. 2008). 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 2007). More recent work has shown that the original pNF-H ELISA can detect informative levels of pNF-H in the blood and CSF.
Assay Procedure Summary

Antibody Coated Plate
- Inspect the plate and remove the liquid from the wells

Add 50 µL standards and diluted samples

Incubate at RT for 1 hr with gentle shaking

Wash plate 6x

Add 100 µL of HRP-conjugated detection antibody per well

Incubate at RT for 1 hr with gentle shaking

Wash plate 6x

Add 100 µL of TMB development reagent per well

Incubate at RT for 5-30 min with mild shaking

Add 50 µL of 2M H₂SO₄ stop solution

Read absorbance at 450nm and calculate results

of a variety of CNS damage and disease states, such patients suffering from aneurysmal subarachnoid hemorrhage (Lewis et al. 2008), animal models of Multiple Sclerosis (Gresle et al. 2008, 2012, 2014), animal models of traumatic brain injury (Anderson et al. 2008), patients with Leber's hereditary optic neuropathy (Guy et al. 2008) and both animal models and patients with ALS (Boylan et al. 2009). As part of the last study, Boylan et al. described a novel pNF-H ELISA that was developed making use of two monoclonal antibodies to pNF-H (Boylan et al. 2012). These were the clones MCA-AH1 (for pNF-H capture) and the clone MCA-NAP4 (for pNF-H detection). The MCA-NAP4 antibody is coupled directly to Horseradish peroxidase (HRP), resulting in a quicker assay with only two incubation steps. This kit is a commercial version of that assay.

2 Overview of how the kit works

The kit is an ELISA capture assay, in which a 96-well format ELISA plate is coated with a capture antibody, an affinity purified mouse monoclonal raised against the phosphorylated axonal form of NF-H (pNF-H). The plate has been blocked with a protein solution to remove any further non-specific binding, and was dried in the presence of a preservative. Samples plasma, serum, CSF, urine, tissue extracts or other liquid samples are incubated in the wells of this plate, and the pNF-H in the solution is bound by the capture antibody. Binding is detected with a second different monoclonal antibody to pNF-H labeled with horse radish peroxidase (HRP). Finally, the wells are incubated with 100 µL of TMB development solution for 5-30 minutes, producing a blue signal in positive wells. The reaction is stopped with 50 µL of 2N H₂SO₄, producing a yellow signal, which can quantified using a suitable ELISA plate reader reading at 450 nm.

3 Kit Contents

1. One 96-well plate coated with purified anti-pNF-H capture antibody and then blocked with an irrelevant protein to prevent non-specific binding. The plate is ready to use, and can be split into 8 rows of 12 wells each if required. The plate was dried in a solution containing sucrose to stabilize the antibody. Store at 4°C.

2. One vial of 6 µL affinity purified mouse detection antibody coupled to HRP at a concentration of about 1 mg/mL. Store at 4°C.

3. One vial of pNF-H protein standard. This is 15 µL at a concentration of 250 ng/mL and is derived from bovine spinal cord. (This is better stored at -20°C or lower. Shake on thawing out prior to use).

4. One vial of blocking protein. This is 300 mg and should be added to the TBS-Tween to make the dilution buffer.

5. One bottle containing 3 mL of 10X concentrated TBS-Tween (TBST, see above) - make up to 30 mL with distilled water and add the blocking protein. This solution can be used to dilute the samples, the detection antibody and the HRP-conjugated antibody.

6. One bottle containing 12 mL of development buffer.
4 Not included in the Kit
TBST wash solution, 2 N H₂SO₄, HRP reaction stopping solution

5 Storage of Reagents
The ELISA plate and most of the reagents can be stored for up to 3 months at 4°C. The dilution buffer and pNF-H standard are more labile and should be stored frozen at -20°C. Shake these up prior to use after thawing.

6 Equipment
We recommend three items of equipment: an ELISA plate reader, an ELISA plate washer and a rotary shaker. The plate reader should be equipped to measure optical density at a wavelength of 450 nm, the absorbance maximum for the HRP reaction product.

7 Standard ELISA Assay
We have standardized on 1-hour incubation with the coated plate, 1-hour incubation with detection antibody and 5-30 minute development, all performed at room temperature with mild shaking. Since each washing step typically takes 5-10 minutes, the total run time is about 2.5 hours. No doubt the assay will work with shorter incubation times, especially if incubation is performed at 37°C, but we have not examined this in detail.

8 Procedure

1). Unpack the ELISA plate and make sure there are no problems with it. The plate can be broken down to 8 strips of 12 wells per strip, and one or more strips can be run at a time if desired. In this case you will either need a plate washer which can be programmed to wash one row only, or else a set of used or blank strips if you are using a washer which does not have this facility (the plates are Immunon 4HBX plates manufactured by Thermo Labsystems, Franklin, MA). We routinely wash the plate once in an ELISA plate washer prior to adding the samples.

2). Apply the samples. We typically apply a total sample size of 50 µL per well, making up the total volume with an appropriate amount of dilution buffer. You can store the dilution buffer for a few hours at 4°C, for storage of more than one day we recommend freezing to avoid bacterial contamination.

**Standard Curve:** To generate a standard curve, we add 95 µL of dilution buffer to one well, and put 50 µL of dilution buffer in each subsequent well. We add 5 µL of 250 ng/mL sample to the first well, and serially dilute this down the series. Leave one well with just 50 µL of the dilution buffer as a background control. Following serial dilution, the first well therefore, has 12.5 ng/mL of pNF-H, the next has 6.25 ng/mL and so on. Depending on the sensitivity of your ELISA plate reader, 12.5 ng/mL will either saturate the detector or be close to saturation. The most useful range of the standard curve is likely to be from 3.125 ng/mL and lower.


**Reaction stopping solution**

2N H₂SO₄ • make up with care!

**11 Warranty**

Data presented here were obtained using the protocol outlined here. Any modification or change in the protocol may impact the results, in which event EnCor Biotechnology Inc, disclaims all warranties.

**12 Intended Use**

This ELISA capture assay is intended for research use only. The total assay time is less than 3 hours. The kit measures the levels of phosphorylated NF-H in blood, cerebrospinal fluid, urine, tissue extracts, and in cells in tissue culture. No commercial use of this product is allowed.

**13 References**


180° between washes. We routinely program the washer to perform 3 cycles of washing, giving 6 washes in total. **In our experience, careful and thorough washing is the single most important step in getting good quality data from ELISAs.**

**V.** Add 100 µL of HRP-conjugated detection antibody per well. This should be made by dilution of 6 µL antibody in the appropriate vial 1:2,000 in dilution buffer, a total volume of 12 mLs. Incubate for 1 hour at room temperature with gentle shaking.

**VI.** Wash plates extensively, as step iv) above.

**VII.** Add 100 µL TMB development reagent per well. A blue color should be visible in wells with the most concentrated standards after only a few minutes of incubation. Incubate with mild shaking for 5-30 minutes until your samples show a reasonable signal density.

**VIII.** Reaction is then stopped by addition of 50 µL per well of 2N H₂SO₄ which inhibits the HRP reaction and changes the reaction color to yellow.

**IX.** Measure the signals at 450nm on a suitable ELISA plate reader.

**X.** Plot out the data using software in your plate reader, or using Microsoft Excel or similar program (see below). Think about them!

### 9 Interpretation of results

The level of pNF-H detected in a plasma, serum or CSF sample provides information on the degree of ongoing axonal injury or degeneration. Levels detected in a tissue homogenate should give information about the axonal content. We typically use the top row of the plate for a standard curve which we generate by serially diluting standards 1:1 down one lane. We fill the last well with 50 µL of H₂SO₄, which 5 µL per well of 2N H₂SO₄ inhibits the HRP reaction and changes the reaction color to yellow.

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