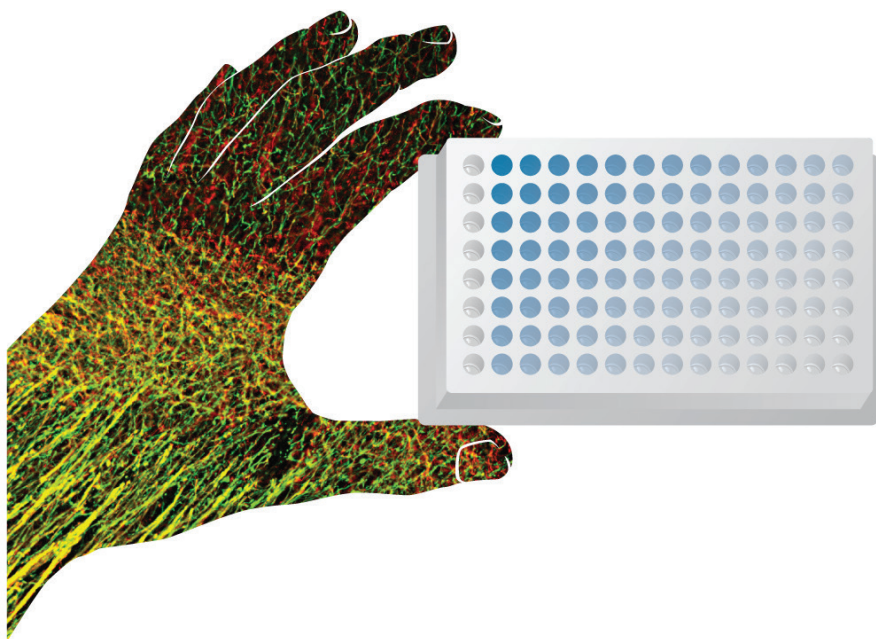# Colormetric Sandwich ELISA kit for the sensitive detection of Heavily Phosphorylated, Axonal Variants of the Major Neurofilament subunit NF-H (pNF-H), in plasma, serum, and CSF

Version 1 (Ch/Rb)

INTRODUCTION

Neurofilaments are the 10nm diameter or intermediate filament proteins found specifically in neurons are particularly concentrated in larger diameter

axons. They are generally composed of three major protein subunits referred to as NF-L, NF-M, and NF-H. This nomenclature is based on the apparent size of these proteins on SDS-PAGE gels, on which NF-L is typically ~68kDa, NF-M is ~150kDa, and NF-H is ~200kDa. The three proteins were discovered in the 1970s as a result of studies of axonal transported proteins (9).

The protein NF-H has some very unusual properties. It contains 50 back-to-back hexa-, hepta-, or octapeptide repeats with each containing the sequence Lysine-Serine-Proline (KSP). The serine residues in these peptide repeats are in vivo phosphorylation sites, which are heavily phosphorylated in axonal neurofilaments. The phosphorylated forms of NF-H are resistant to proteases. In being released from damaged and diseased axons and due to protease-resistance, this very abundant protein can be detected in cerebrospinal fluid (CSF) and blood. Detection level of phosphorylated forms of NF-H will provide information about the degree of axonal injury which has occurred (reviewed in reference 14).

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 (1).

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 (2). 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.

Other experiments have shown that pNF-H can be detected in the plasma of humans patients suffering from optic neuritis (15) and an elevated levels in the cerebrospinal fluid (CSF) of individuals suffering from brain tumors and stroke (16).

The original pNF-H ELISA kit (Version1, EnCor Biotechnology Inc.) was used to detect informative levels of pNF-H in the blood and CSF of patients suffering from aneurysmal subarachnoid hemorrhage (10), animal models of Multiple Sclerosis (7), animal models of traumatic brain injury (1), patients with Leber's hereditary optic neuropathy (8), and in both animal models and human patients with ALS (2).

As part of the last study, Boylan et al. described a novel pNF-H ELISA platform, that was developed to use two different monoclonal antibodies to pNF-H (3). Detection mouse 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.

PRINCIPLE OF THE ASSAY
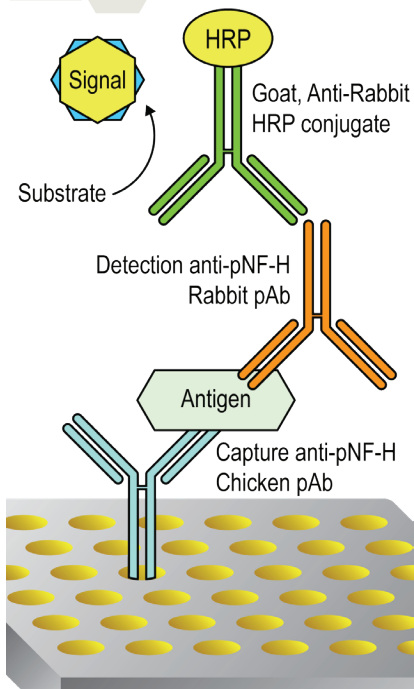
ELISA provides a convenient method for measuring the level of protein targets within a single, small-volume sample. ELISA-pNF-H-V1 is a 2-site sandwich enzyme linked immunoassay. EnCor provides a plate pre-coated with capture chicken polyclonal antibody against pNF-H. The user incubates the samples on the plate and any pNF-H protein in the sample binds to the capture antibody. The user then incubates the plate with a solution containing rabbit polyclonal pNF-H detection antibody, which binds to the complex of antigen and captured Ab. The detection antibody is then itself bound by goat anti-rabbit-Horse Radish Peroxidase (HRP) conjugated antibodies. The user adds a substrate TMB solution which produces a blue colored product in wells containing HRP. The intensity of the color corresponds to the amount of pNF-H in the sample. Finally the user stops the reaction by adding H2SO4. The color changes from blue to yellow and can be quantified by measurement on a suitable ELISA plate reader using an absorbance wave length of 450 nm.

REAGENTS SUPPLIED:

Kit Component	Kit Component	Quantity	Storage
Plate	Pre-coated anti-pNF-H strip plate	8 x 12 wells	4ºC
pNF Standard	Bovine pNF-H protein, lyophilized	2 vials	4ºC
RPCA-pNF-H	Detection, anti-pNF-H, rabbit polyclonal antibody (1,000X)	1 x 20µL	-20ºC
α-Rb-HRP	Goat anti-rabbit IgG, HRP conjugated antibody (100X)	1 x 200µL	-20ºC
Block	Blocking protein	1 x 1g	4ºC
TBS	1XTBS, ready to use rinsing plate solution	1 x 12 mL	4ºC
TBST	1XTBS - Tween concentrate	1 x 12 mL	4ºC
TMB	1XTMB (Tetramethylbenzidine) substrate ready to use solution	1 x 12 mL	4ºC

ADDITIONAL MATERIALS/EQUIPMENT:

- Microplate reader (450nm)
- Microplate shaker
- Automated plate washer
- Multichannel pipette
- Appropriately-sized tubes for reagent preparation
- Polypropylene microcentrifuge tubes for preparing dilutions
- Stop Solution (2N H_2SO_4)
- Wash buffer (1X TBST)

LIMITATIONS OF THE ASSAY

- For research use only. Not for use in diagnostic procedures.
- Absorbance values beyond the range of the standard curve are not valid.

Warning: Before starting the assay, read the instructions for use completely and carefully. Make sure that everything is understood!

BEST PRACTICES + TECHNICAL HINTS

1. Do not mix or substitute reagents from different sources or different kit lots.

2. Dilute samples in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution. Two common pipetting methods used for ELISA are standard (forward) and reverse. Use standard (forward) pipetting for the preparation of sample dilutions, and reverse pipetting for the addition of diluted samples, controls and reagents. Careful pipetting is crucial for accurate test results. Become familiar with the pipette and both methods before running actual tests. Be sure to use the correct pipette and tip (volume capacity) for the volume being transferred.

3. Standard (Forward) Pipetting:
   d. Put a new tip on a single-channel pipette and make sure that it is on tight.
   e. Press the plunger to the first stop.
   f. Draw the calibrated volume of sample into the tip and pause for one second with the tip still in the sample. Be careful not to place the tip too deeply into the sample.
   g. Touch the tip to the side of the sample container to remove any excess liquid on the outside of the tip.
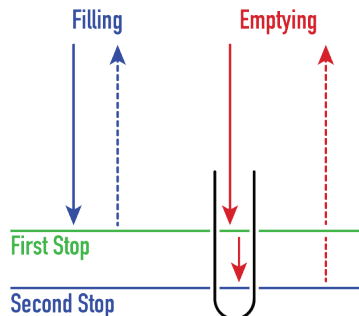
a. Dispense the sample into the measured diluent by depressing the plunger past the first stop to the second stop. Be careful not to place the tip too deeply into the sample diluent.
b. After dispensing the sample into the diluent, rinse the pipette tip in the diluent by pushing the plunger up and down 2–3 times before ejecting the tip.
c. Mix samples using vortex if they are in the tube, and tapping the plate if the samples added directly to the well.
d. Eject the tip into a waste container.

1. Reverse pipetting using single or multichannel pipette:
   a. Put new tips on the pipette. Make sure they are on tight and straight.
   b. Press the plunger past the first stop and halfway to the second stop.
   c. Draw the liquid in a slow motion, being careful not to draw any air bubbles into the tips, check for consistency of volume in the tips.
   d. Touch the tips to the edge of the reagent reservoir to remove excess liquid on the outside of the tips. If the wells on your plate are empty, position the tips into the lower corner of each well, making contact with the plastic. If the wells on your plate contain liquid, position the tips above the liquid, making contact with the plastic.
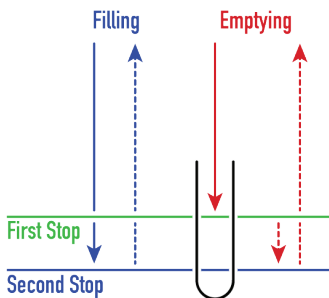
   e. Slowly dispense the liquid into the wells by depressing the plunger to the first stop. Be careful not to splash liquid out of the wells, and make sure there are no drops left on the tips.
   f. To repeat, hold the plunger at the first stop and continue with step "c".
   g. Eject the tips into a waste container.

   NOTE: Reverse pipetting uses more reagent/ volume (= "dead volume")

1. Measure desire amounts of reagents by weighing or pipetting them from original container.
2. 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.
3. Avoid bubbles in wells at all pipetting steps. Bubbles may lead to variable results, and are particularly a problem during optical density determination.
4. When using an automated plate washer, rotating the plate 180 degrees between washing steps may improve assay precision.
5. Gently tap the plate against paper towel to remove residual fluid after washing.
6. **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.
7. If an incubation step needs to be extended, avoid letting the plate dry out by keeping sample or detection antibody solution in the wells. If assay results are above the top of the calibration curve, dilute samples, and repeat the assay.

REAGENT PREPARATION

Wash solution –TBST:

Wash solution is 1X Tris buffered saline - tween (TBST) (10 mM Tris base, 150mM NaCl, 0.1% Tween-20), for 1L
- 1.21g - Tris base
- 8.77g - NaCl

Add deionized water to a final volume of 900mL.
Adjust pH to 7.5 using concentrated HCl (careful!), then add 1mL - Tween 20, mix well.
Bring final volume to 1L with dH_2O.

Stop solution:

Stop solution is 2N H_2SO_4 solution
to make 250mL
- 26.7mL concentrated H_2SO_4
- 223.3mL H_2O

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

Dilution buffer (DB):

Weigh out 500mg of blocker and combine it with 5mL of 10X TBST (provided by EnCor), add dH_2O to have final volume of 50mL, dissolve well. The buffer can be stored at 4°C for 2 days.

Dilute detection antibody:

Prepare the detection antibody solution immediately prior to use.

Prepare the detection antibody solution immediately prior to use.
EnCor provides detection, rabbit polyclonal pNF-H antibody, (RPCA-pNF-H) as 1,000X stock solution. The working solution is 1X. For 1 plate combine:
- 12mL of dilution buffer (DB)
- 12µL of Detection antibody (RPCA-pNF-H), vortex well

Make enough solution for the experiment, and discard remainders after use.

Dilute α–Rb–HRP antibody:

Prepare the α–Rb–HRP antibody solution immediately prior to use.

EnCor provides Goat anti-rabbit IgG, HRP conjugated antibody (α-Rb-HRP) as 100X stock solution. The working solution is 1X. For 1 plate combine:
- 12mL of dilution buffer (DB)
- 120µL of α-Rb-HRP, vortex well

Make enough solution for an experiment and discard remainders after use.

PROTEIN STANDARDS PREPARATION

EnCor supplies pNF-H standard: lyophilized bovine pNF-H protein. The highest standard point (10ng/mL) is obtained by reconstituting one vial of lyophilized standard with 400uL of the dilution buffer. We recommend 7-point calibration curve in duplicates with 2-fold serial dilution steps and a zero standard blank.

Serial dilution of standards in the micro-tubes:

1. Label 7 micro-tubes, one for each standard point: 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156ng/mL.
2. The first tube (10) is original vial with reconstituted protein.
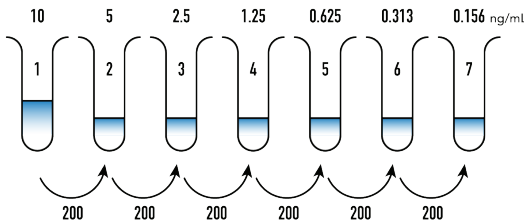
3. Add 200μL of dilution buffer (DB) into each tube starting from the second one
4. Perform serial 1:2 dilutions starting from the standard - 10ng/mL by transferring 200μL of the preceding sample to 200μL of DB in the next tube as shown on the picture.

5. Vortex well before the next transfer, and use a new pipette tip during each transfer. The last tube will contain 400μL.
6. Add 50μL of each standards to the desire wells.
7. Add 50μL of Dilution buffer along to the wells, designated for the Blank.

Reconstituted and diluted pNF-H standards should be used immediately and cannot be stored and reused.

Serial dilution of the standards directly in the plate:

Designate two columns of the plate (2X8 wells) for the standard curve. Add 50μL/well of the DB to the wells "B" through "H" in two columns. Add 100μL/well of the reconstituted ST (10ng/mL) to the wells "A". Perform serial 1:2

Column	Dilution Buffer	Standard	C (ng/mL)
A	0	100 μL	10.0
B	50	50 (A)	5.0
C	50	50 (B)	2.5
D	50	50 (C)	1.25
E	50	50 (D)	0.625
F	50	50 (E)	0.3125
G	50	50 (F)	0.156
H	50	0	0

dilutions (50μL/well) down the plate in the columns designated for the standard curve using multichannel pipette by transferring 50μL of the preceding sample to 50μL of DB in the next well as shown on the table. Do not add any standards solution to the last "H" wells, these are BLANK. Discard 50μL of the solution from wells "G".

SAMPLE PREPARATION

Serum and Plasma:

Plasma samples prepared in heparin tubes commonly display additional clotting following thawing. Remove clots and all solid material by centrifugation. Similarly serum may sometimes contain small amounts of particulate material which can be removed by centrifugation. For those systems and assays using neat samples or lower dilution factors, the sample can be put directly into the wells of the coated plates. Follow the sequence below:

1. Add the dilution buffer (DB) to the plate.
2. Add the sample into the DB.
3. Mix by tapping the plate or repeating pipetting.

We recommend starting with diluted plasma or serum samples 1:2.5 times with DB (or 30µL of dilution buffer plus 20µL of serum or plasma sample) at a single concentration for pilot experiments. If strong pNF-H signals are detected samples may be run at higher dilutions. Always run samples in duplicate or triplicate. This will provide enough data for statistical validation of the results.

CSF Samples:

Pathological human CSF samples are generally higher in pNF-H concentration. Start pilot experiments with 1:5 dilution of CSF in DB (In order to dilute samples directly in the plate, add 40µL of dilution buffer to the well then add 10µL of CSF sample, mix by tapping the plate). If strong signals are detected samples may be diluted more (dilute CSF samples in the tube, then add 50µL to the well.) Always run samples in duplicate or triplicate. This will provide enough data for statistical validation of the results.
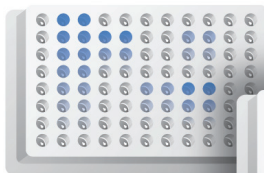
PROTOCOL

1. Remove the plate from a plastic bag. It is ready to use.
2. Rehydrate the plate with 1XTBS (provided). Add 100uL of the solution and aspirate it.
3. Designate two or more columns of the plate for the standard curve.   Perform standard curve (see "Protein Standards Preparation").
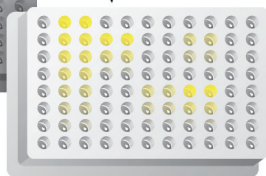
A standard curve must be prepared for each plate!

4. Add 50µL of diluted samples per well, or dilute them directly in the wells (see "Sample preparation"). Incubate the plate for at least 2 hours at room temperature with gentle shaking, for better performance overnight at 4˚C (Seal the plate).
5. Wash the plate 2X3 times with 300µL of TBST using an automated plate washer, rotate the plate 180 degrees between wash steps.
6. Add 100µL of detection antibody solution (RPCA-pNF-H, 1:1,000 dilution, or 12mL of DB + 12µL of RPCA-pNF-H) to each well. Incubate the plate for at least 2 hours at room temperature, or overnight at 4˚C with gentle shaking (Seal the plate).
7. Wash the plate 2X3 times with 300µL of TBST using an automated plate washer, rotate the plate 180 degrees between wash steps.
8. Add 100µL of α-Rb-HRP (1:100 dilution, or 12mL of DB + 120µL of α-Rb-HRP), to each well. Incubate the plate for 1-1.5 hours at room temperature with gentle shaking.
9. Bring TMB solution to room temperature.
10. Wash the plate 2X3 times with 300µL of TBST using an automated plate washer, rotate the plate 180 degrees between wash steps.

Before "STOP" solution

After "STOP" solution

11. Add 100µL of TMB (the solution should be at Room Temperature) devel-oping solution. Incubate the plate until you will reach a desire color change; the highest standard should be dark blue, while Blank should stay colorless. Time of incubation varies from 5 to 20 minutes (AVG time is 10min).
12. Stop reaction with 50µL of stop solution; 2N H_2SO_4. Note, that the color changes from blue to yellow. No incubation in stop solution is required be-fore reading the plate.
13. Read the plate on the plate reader -spectrophotometer at 450nm. There are many models and manufacturers of plate readers; refer to the manu-facturer's instructions for details of operation.

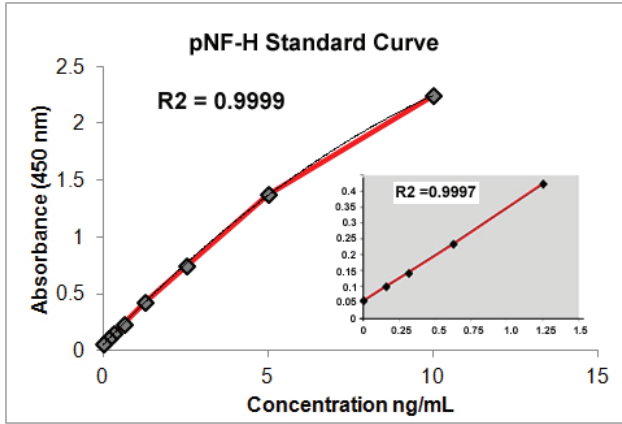# PERFORMANCE CHARACTERISTICS OF THE pNF-H ELISA

Fig.1

Representative plot of the full range pNF-H standard curve (bovine protein) obtained using the pNF-H-ELISA-V1 kit. The inset is an enlarged portion of the lower range (0-1.25ng/mL) of the standard curve. Trendline for the full range plot is polynomial, for inset is linear. Both trendlines are shown in black color. R-squared values displayed on the charts.

Measuring Range:

The standard curve covers the interval 0.156-10ng/mL pNF-H (see Fig.1). Extrapolation beyond the curve will not provide accurate results. Samples displaying signal outside the curve should be further diluted and remeasured.

Detection limit:

The least detectable concentration is 70pg/mL, defined as the mean of blank samples + 2 STDEV.

Sensitivity:

The intra-assay variability %CV < 6
The inter-assay variability %CV < 10

Shelf life:

When stored at an appropriate temperature, the product can be used within 6 months from date of purchasing. Detection rabbit pAb-pNF-H, and goat anti-rabbit HRP antibodies should always be stored at -20˚C.

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ELISA Kit
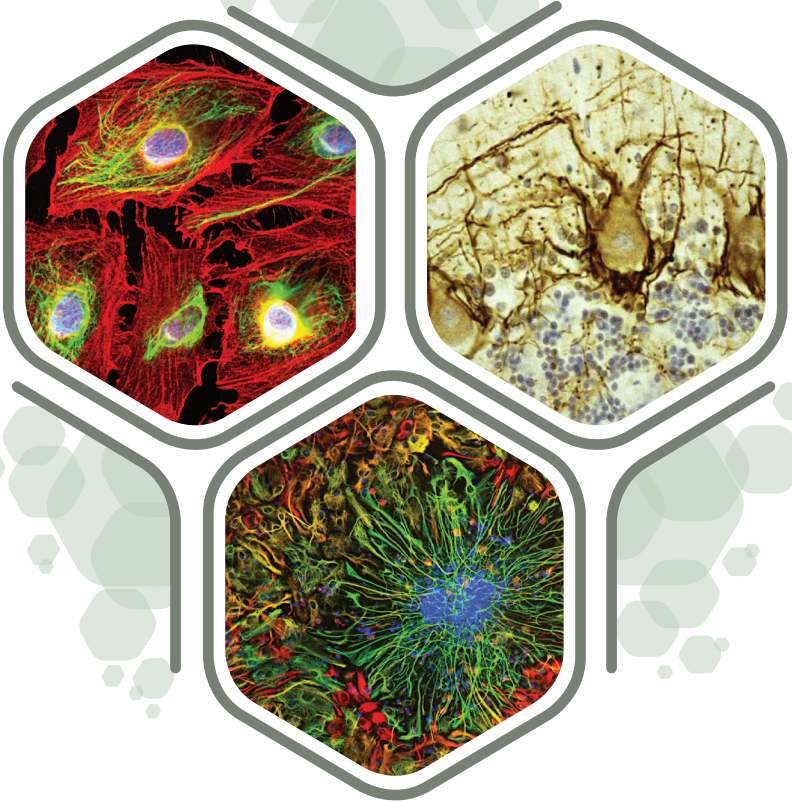
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