Version 22-04/24

User's Manual

Phosphorylated Neurofilament NF-H ELISA Kit

REF **DEIA7367R**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

Phosphorylated Neurofilament NF-H ELISA Kit is an Enzyme Immunoassay kit for the quantification of Phosphorylated Neurofilament NF-H in plasma, serum, CSF and tissue extracts. For laboratory research only, not for drug, diagnostic or other use.

General Description

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. The pNF-H protein has been detected in large amounts following experimental spinal cord and brain injury in rats. 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.

Principles of Testing

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.

Reagents And Materials Provided

Store unopened kit at 2-8°C. HRP-Anti-pNF-H Antibody (100×)should be stored at -20°C upon receipt. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	8 strips X 12 wells	4°C
Standard	2 vial (lyophilized)	4°C
HRP-Anti-pNF-H Antibody (100X)	150 µl	Ship at 4°C, store at -20°C upon receipt. (Protect from light)
1X TBS	12 ml (Ready-to-use)	4°C
10X TBST	12 ml	4°C
Blocker	1 g	4°C
TMB substrate	12 ml (Ready-to-use)	4°C (Protect from light)

Materials Required But Not Supplied

1. Microplate reader capable of measuring absorbance at 450nm
2. 2N H_2SO_4 for stopping color development
3. 1× TBST for washing

   TBST buffer: 1× TBS buffer (0.01M Tris-HCl, 0.15M NaCl, pH 7.5) with 0.1% of Tween-20.
4. 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.
5. Pipettes and pipette tips
6. Deionized or distilled water
7. Automated microplate washer (optional)

Storage

Store components at 4°C or -20°C. Keep microplate wells sealed in a dry bag with desiccants. Do not expose test reagents to heat, sun or strong light during storage and usage. Please refer to the product user manual for detail temperatures of the components.

Specimen Collection And Preparation

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 plasma, serum, CSF and tissue extracts. 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×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 1000×g 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

Wash buffer (1× 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 7.5 using concentrated HCl (be careful!).
4. Add 1mL Tween-20 into 1× TBS buffer from step 3, mix well.
5. Bring final volume to 1L with deionized water.

Dilution Buffer: Add 0.5 g of Blocker into 5 ml of 10× TBST (supplied in the kit). Then add distilled water to adjust final volume to 50 ml, dissolve well. The reconstituted Dilution Buffer can be stored at 4°C for 2 days.

1× 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 **1:100** in **Dilution Buffer** to yield a 1× HRP-conjugated anti-pNF-H antibody, vortex to mix thoroughly. (E.g.: Dilute 10 µl of HRP-conjugated antibody in 1000 µl of Dilution Buffer.) Only prepare amount needed, and discard remainders after use.

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. 2.5× dilution: 40 µl sample + 60 µl Dilution Buffer; 5× 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: It is recommended to prepare this reagent immediately prior to use. Reconstitute the standard with **400 µl** of **Dilution Buffer (DB)** to yield a highest standard concentration of **10 ng/ml** (S1). Brief vortex the tube to make sure the standard is dissolved completely before making serial dilutions. The Dilution Buffer (DB) serves as zero standard (0 ng/ml), and the rest of the standard serial dilution can be diluted as according to the suggested concentration below: **10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.3125 ng/ml, 0.156 ng/ml.** DO NOT reuse the reconstituted standard.

Dilution table for pNF-H protein standard preparation:

Standard #	pNF-H protein Concentration	Volume of standard (µl)	Volume of DB (µl)
S1	10 ng/ml	400	-
S2	5 ng/ml	200 (S1)	200
S3	2.5 ng/ml	200 (S2)	200
S4	1.25 ng/ml	200 (S3)	200
S5	0.625 ng/ml	200 (S4)	200
S6	0.3125 ng/ml	200 (S5)	200
S7	0.156 ng/ml	200 (S6)	200
S0	0	0	200

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.

Assay Procedure

TMB substrate should be equilibrated to room temperature (RT) before use. Standards, samples and controls should be assayed in duplicates. A standard curve must be prepared for each plate!

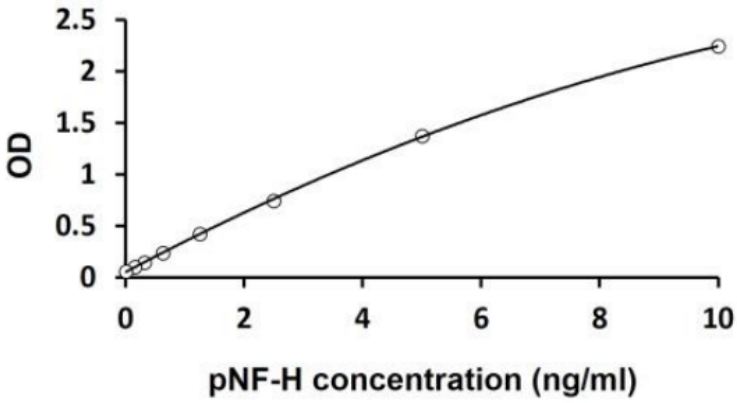
1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add **100 µl** of 1× TBS (provide in this kit) to rehydrate the plate, and then aspirate it.
3. Add **50 µl** per well of **standards and diluted samples** in duplicates into appropriate wells. Incubate the plate at **RT for 2 hours** on a microplate shaker at 250-300 rpm (alternately, brief mix the reagent by gently shaking/tapping the plate and cover the plate with a plate sealer and incubate the plate at **4°C for overnight**).
4. Aspirate each well and wash, repeating the process 2 times for a **total 3 washes.** Wash by filling each well with **1× TBST wash buffer (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.
5. **Rotate the plate 180°, and wash** again as according to **step 4**.
6. Add **100 µl** of working **1× HRP-conjugated pNF-H Antibody** (1:100 diluted) into each well. Incubate the plate for 2 hour at RT on a microplate shaker (250-300 rpm).
7. **Wash** as according to **step 4 and step 5**. (Before washing step, bring TMB substrate to room temperature.)
8. 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.
9. 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.
10. Read the OD with a microplate reader at **450 nm** immediately.

Calculation

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 for serum/plasma will be 2.5. So the measured concentration of samples calculated from the standard curve must then be multiplied by 2.5. E.g. If measured concentration from standard curve is 1 ng/ml, then the actual concentration should be 1 ng/ml (from standard curve) × 2.5 (dilution factor) = 2.5 ng/ml.

Typical Standard Curve



Precision

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

Detection Range

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

Sensitivity

70 pg/ml

Precautions

1. Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
2. This kit can be stored for up to 6 months. All components should be stored at 4°C except the HRP-Anti-pNF-H Antibody (100X), which should be stored frozen at -20°C.
3. Avoid using reagents from different batches.
4. Briefly mix and spin down the components before use.
5. If an incubation step needs to be extended, avoid letting the plate dry out by keeping sample or detection antibody solution in the wells.
6. 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.

7. Avoid bubblesin wells at all pipetting steps. Bubbles may lead to variable results, and are particularly a problem during optical density determination.
8. When using an automated plate washer, rotating the plate 180 degrees between washing steps may improve assay precision.
9. Gently tap the plate against paper towel to remove residual fluid after washing.
10. 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.
11. It is highly recommended that the standards, samples and controls be assayed in duplicates.
12. Change pipette tips between the addition of different reagent or samples.