Phosphorylated NEFH (Human) ELISA Kit

Catalog Number KA1479

96 assays

Version: 07

Intended for research use only

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Introduction

Intended Use

Phosphorylated NEFH (Human) ELISA Kit is an assay intended to measure the concentration of phosphorylated forms of heavy neurofilaments in peripheral blood and cerebrospinal fluid. The kit is capable of detection of only human, porcine, bovine and rat neurofilaments, it is not capable of detection of mouse neurofilaments.

Background

Neurofilaments are cytoskeletal proteins that are found specifically in neuronal cells. They are composed of three subunits assigned according to their molecular weight as heavy (H), medium (M) and light (L). Phosphorylation of the C-terminal part of heavy and medium neurofilaments shows topological dependence in relation to neuron. Neurofilaments in axons are heavily phosphorylated, crosslinked and spatially organized, whereas neurofilaments found in the neuronal body and in dendrites have low level of phosphorylation, the crosslinking level is low and their orientation is random (Sternberger et al., 1983 and Hirokawa et al., 1984). Phosphorylated heavy neurofilaments were detected in higher concentrations in diseases that involve damage to the central nervous system (Petzold et al, 1993, Shaw et al. 2005).
The ELISA uses a combination of the mouse monoclonal antibody NF01 (Lukas et al., 1993) that binds to the phosphorylated epitopes on heavy neurofilaments and of the mouse monoclonal antibody NF05 that reacts independently upon the phosphorylation status of the neurofilament (Porchet et al., 2003).

Principle of the Assay

Phosphorylated NEFH (Human) ELISA Kit is a one-step sandwich immunoassay in which the surface of the microtitre plate is coated with the mouse monoclonal antibody NF01 that specifically binds the phosphorylated forms of NF-H. Neurofilaments from the tested sample bind to the NF01 antibody and simultaneously the neurofilaments react with the enzyme labelled monoclonal antibody NF05 which is added to the reaction together with the sample. All the molecules that are not captured in sandwich consisting of NF01-neurofilament protein-NF05 are washed away during the washing step of the assay. Then a chromogenic substrate for the enzyme is added. The reaction is stopped by adding an acidic solution. The colour intensity is directly proportional to the amount of phosphorylated neurofilaments in the sample.

General Information

Materials Supplied

List of component

Component	Amount
Microtitre plate with immobilised antibody NF01	96 (6x16) wells
pNF-H Standard (bovinne pNF-H, 1 µg/ml)	100 µl x 2 vials
NF05-HRP 100x, Detection antibody conjugated to horseradish peroxidase, 100x concentrated	120 µl
TBS, r.t.u.[1)]	30 ml
Wash buffer 10x concentrated	100 ml
Dilution buffer r.t.u.	15 ml
Chromogenic substrate (TMB) r.t.u.	13 ml
Stop solution r.t.u.	13 ml
A plastic bag with a press-to-seal zipper	1 bag
Adhesive membrane	1 slice

[1)]r.t.u., read to use

Storage Instruction

- ✓ Store the kit and the kit reagents at 2 to 10°C, in a dry place and protected from the light.
- ✓ Store unused strips in the sealable pouch and keep the desiccant inside.
- ✓ Kits are shipped in cooling bags.
- ✓ Expiration date is indicated at the ELISA kit label and at all reagent labels to the ELISA reagents.
- ✓ If you find any damage at any part of the kit, please inform the manufacturer.

Materials Required but Not Supplied

- ✓ Distilled/deionised water
- ✓ Precision micropipets 20, 200 and 1000 µl and suitable tips
- ✓ Graduated cylinders (1000 ml)
- ✓ Microplate washer or other device for microplate washing
- ✓ Absorbent papers
- ✓ ELISA reader
- ✓ Microplate shaker

  Note: It is recommended to use a precise dispenser e.g. Multipette Xtream Eppendorf for the dispensing the diluted solution of detection antibody.

Precautions for Use

✓ Safety Precautions

- All ingredients of the kit are intended for laboratory use only.
- The test procedure requires qualified laboratory personnel.
- Do not smoke, eat or drink during work.
- Do not pipette by mouth, use suitable pipetting device.
- Wear protective disposable gloves while handling reagents or samples and wash your hands thoroughly afterwards.
- Avoid spilling or producing aerosol.
- Autoclave all reusable materials that were in contact (spilled) with human samples, burn disposable ignitable materials, decontaminate liquid wastes and nonignitable materials with 3% chloramine. Liquid wastes containing acid (Stop solution) should be neutralized in 4% sodium bicarbonate solution.
- STOP solution contains 0.9 M sulphuric acid, handle with caution. Avoid contact with skin, eyes or mucous membranes. In case of contact with eyes, skin or mucous membranes rinse immediately with plenty of water and seek medical advice.

✓ Handling Precautions

- Manufacturer guarantees performance of the ELISA kit.
- Avoid microbial contamination and cross-contamination of samples and kit reagents.
- Avoid contact of the TMB substrate with oxidizing agents or metal surfaces.
- Follow the assay procedure indicated in the Instruction manual. Variations in the test results are usually due to:

  Insufficient mixing of reagents and samples.

  Inaccurate pipetting and inadequate incubation times.

  Poor washing technique or spilling the rim of well with sample or with solution of the detection antibody (NF05-HRP).

  Use of identical pipette tip for different solutions.

Assay Protocol

Reagent Preparation

Allow all the kit components to reach room temperature (~ 20 min). Mix all reagents well before use to ensure homogeneity.

✓ Wash buffer

Prepare Wash buffer by diluting the Wash buffer concentrate 10 times with an appropriate volume of distilled or deionised water (e.g. 100 ml of the concentrated Wash buffer + 900 ml of distilled water).
If there are crystals of salt present in the concentrated Wash buffer, warm up the vial to +32 to +37°C in a water bath. White turbidity in buffer may occur and is not a defect. Diluted Wash buffer is stable for one week if stored at +2 to +10°C.

✓ Detection antibody NF05 conjugated to HRP (NF05-HRP)

For one microplate prepare 7.5 ml of the NF05-HRP solution. Dilute the NF05-HRP concentrate 100x with the Dilution buffer.
If you intend to prepare a smaller amount of the NF05-HRP solution see the recommendations indicated in table 1.
Do not store the diluted NF05-HRP.

Table 1: Recommended volumes for preparation of the NF05-HRP solution

Number of 16-well strips	HRP conjugate concentrate (µl)	Dilution buffer (ml)
1	13.5	1.35
2	25.5	2.55
3	37.5	3.75
4	49.5	4.95
5	63	6.30
6	75	7.50

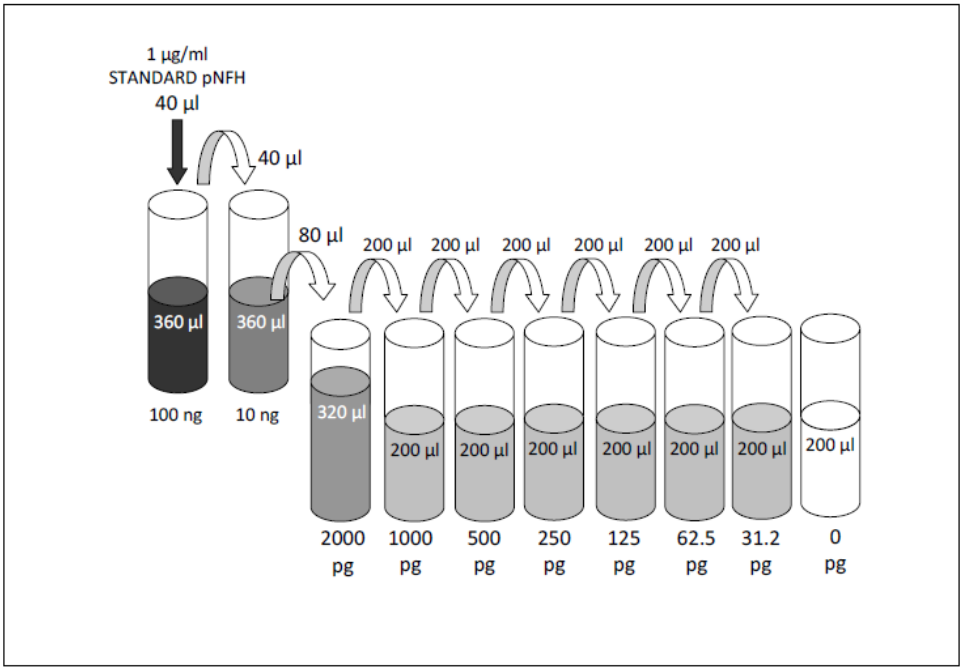
✓ Standard pNF-H

Standard is supplied as 1 µg/ml stock solution. Prepare the serial dilutions of the Standard as follows: Prepare ten (10) 1.5 ml PP microtubes and label them as 100 ng, 10 ng, 2000 pg, 1000 pg, 500 pg, 250 pg, 125 pg, 62 pg, 31 pg, 0 pg. Pipette sequentially 360 µl, 360 µl, 320 µl, 200 µl, 200 µl, 200 µ, 200 µl, 200 µl, 200 µl, 200 µl of the Dilution buffer to the microtubes. Mix well the Standard stock solution and pipette 40 µl of the stock solution to the first microtube (100 ng). Mix the content of the tube, remove 40 µl and add it to the second tube (10 ng). Mix the content, remove 80 µl and add it to the third tube (2000 pg). Then continue with dilutions as 200 + 200 µl to prepare the entire set of concentrations ranging from 2000 pg/ml to 31 pg/ml. Do not add any pNF-H into the tube labeled with 0, the zero standard is the Dilution buffer only.

Change the pipette tip after each dilution, always mix well the content.

For easier understanding of the Standard dilution procedure look at the scheme bellow (Dilution of the pNF-H Standard).

Figure 1 Dilution of the pNF-H Standard



Do not dilute Dilution buffer, TMB solution, STOP solution! They are ready to use.

Sample Preparation

- ✓ Store serum, plasma and cerebrospinal fluid samples frozen at –18 °C or lower.
- ✓ Thaw plasma samples quickly in a water bath at 37°C, the plasmatic proteins may precipitate if thawed slowly. Thaw serum and cerebrospinal fluid samples either in a water bath at 37°C or at the laboratory temperature.
- ✓ Prepare enough volume to measure each sample in replicates a 25 µl/well.
- ✓ Do not dilute the samples unless you expect pNF-H concentrations higher then 2000 pg/ml, in such case dilute the samples with the Dilution buffer to obtain the concentration that will fall within the Standard range (2000-31 pg/ml).

Assay Procedure

1. Allow all the kit components to reach temperature (~ 20 min).
2. Prepare the working concentrations (in the volume needed) of Wash buffer and of the NF05- HRP solution.
3. Dilute pNF-H Standard to concentrations 2000, 1000, 500, 250, 125, 63, 31 and 0 pg/ml.
4. Open the aluminium bag containing the microplate and remove the desired number of strips. Put the unused strips together with the desiccant to the provided plastic bag and seal it. Store the unused strips

at 4°C.

5. Pipette a 100 µl of TBS to the wells and incubate them for 10 min in laboratory temperature.
6. Remove the TBS from the wells and make the wells dry by tapping the inverted plate against a pile of absorbent papers.
7. Pipette a 25 µl of Standards (2000-0 pg/ml) and samples to the wells (see Plate Layout).
8. Continue by pipetting a 75 µl of NF05-HRP to the wells.
9. Cover the strips with the sealing membrane.
   The cover prevents evaporation from the wells during the incubation.
10. Place the ELISA plate into the shaker and mix the content within the wells with vigorous shaking at 1000 rpm for 10-20 sec.
11. Incubate 4 hours with continuous shaking (~600 rpm) at laboratory temperature.
12. Aspirate and wash 5 times with 250-400 µl of the Wash buffer. Invert and tap the plate against a pile of absorbent papers (see Safety precautions).
13. Pipette a 100 µl of TMB solution to the wells.
14. Incubate in dark place for 20 +/- 1 minutes.
15. Pipette a 100 µl of STOP solution to the wells.
16. Tap the microplate side gently to ensure complete mixing of the TMB with the STOP solution.
17. Read the absorbance at 450 nm, it is recommended to use a reference reading 620-690 nm.

✓ Flow Chart

Prepare the working concentrations of reagents and Standards

↓

Pipette 100 µl of TBS into each well

↓

Incubate 10 min

↓

Remove TBS from wells

↓

Pipette 2 x 25 µl of Standards and samples

Add 75 µl of NF05-HRP solution into each well

↓

Incubate 4 hours / 600rpm

↓

Wash 5x (250-400 µl/well)

↓

Pipette 100 µl of TMB into each well

↓

Incubate 20 minutes

↓

Pipette 100 µl of STOP solution into each well

↓

Read the absorbance at 450 nm with a reference reading at 620-690 nm

Data Analysis

Calculation of Results

✓ Processing of Results

- Subtract the absorbance at the reference wavelength from the absorbance at 450 nm (usually performed automatically by the ELISA reader).
- Compute means in duplicates.
- Subtract the Standard 0 mean from all of the other mean values (Blank Difference data).
- Construct the standard curve by plotting the mean absorbance for each Standard (Blank Difference data) on the y-axis versus the concentration of pNF-H (pg/ml) on the x-axis. Draw the best fit curve through the standard points.
- Compute the concentrations of pNF-H in samples according to the standard curve formula.

✓ Interpretation of Results

Samples with concentrations lower then 31.25 pg/ml (the lowest standard) can not be assigned differently than that they contain pNF-H at concentration lower than 31.25 pg/ml pNF-H.

Samples with concentration higher than 2000 pg/ml consider as either with concentrations higher than 2000 pg/ml or dilute them and repeat the test with the diluted samples, e.g. 2x a 4x diluted (afterwards multiply the results by the dilution factor).

Test Characteristics

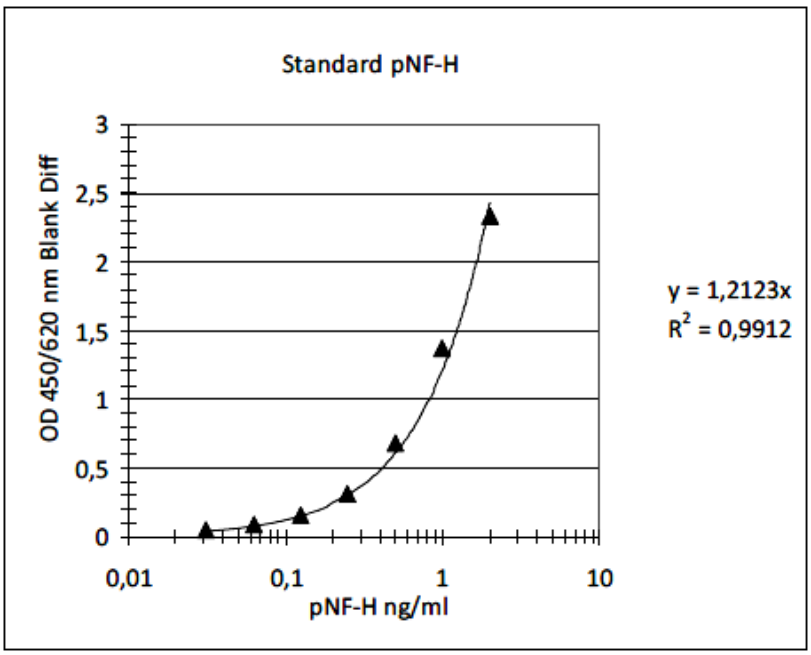


Figure 2: Example of standard curve

Performance Characteristics

✓ Validity criteria

- Absorbance 450/620 nm of the Standard 0 should be lower than 0.250.
- Absorbance of the Standard 2000 pg/ml should be higher than 1.400.
- The difference between the Standard 31.25 pg/ml absorbance and the Standard 0 absorbance should be higher or equal to 0.010 (450/620 nm data).

✓ Intraassay variability

(N = number of tests within the plate):

N	sample	AVG A450/620	SD	CV (%)
32	Standard 2000	2.348	0.088	3.7

✓ Interassay variability

(N= number of test repetitions)

N	sample	AVG pg/ml	SD	CV (%)
5	serum A	53	6	13
5	serum B	88	11	20

✓ Limit of detection

Limit of detection was calculated as the average interassay Standard 0 absorbance calculated from 10 test repetitions plus 3x SD of the mean.

Limit of detection of the test is 24 pg/ml.

Resources

References

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Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 0	Standard 0	Sample 1	Sample 1								
B	Standard 31	Standard 31	Sample 2	Sample 2								
C	Standard 62	Standard 62	Sample 3	Sample 3								
D	Standard 125	Standard 125	Sample 4	Sample 4								
E	Standard 250	Standard 250	…	…								
F	Standard 500	Standard 500										
G	Standard 1000	Standard 1000										
H	Standard 2000	Standard 2000										