Creative Diagnostics®

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User's Manual

Human Phosphorylated Neurofilament H ELISA Kit

REF **DEIA4756**

Σ **96T**

RUO

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

The Human Phosphorylated Neurofilament H ELISA is a sandwich enzyme immunoassay for the quantitative measurement of human phosphorylated neurofilament H (pNF-H).

General Description

Neurofilaments are the 10nm diameter filaments which are the most abundant protein components of neurons and are particularly concentrated in axons. They belong to the intermediate or 10nm filament protein/gene superfamily which also includes keratins, the major structural proteins of skin. Neurofilaments (NF) consist predominantly of three subunits:, NF-L (low), NF-M (medium), and NF-H (heavy or high). NF-H protein is about 200 kDa and contains unusual multiple repeated sequence lysine-serine-proline (KSP), and in axonal neurofilaments essentially all serine residues are heavily phosphorylated. Because phosphorylated forms of NFH (pNF-H) are quite resistant to proteases, pNF-H released from damaged and diseased axons should remain in fluid undegraded. This means that detection of pNF-H in blood and CSF points unambiguously to neuronal damage due to the fact that pNF-H is found exclusively in neurons.

This protein can be detected in quite large amounts following experimental spinal cord and brain injury in rats. Levels of greater than 100 ng/ml of pNF-H were detectable in blood following serious cord injury and lower, but still easily detectable levels, were found in blood of animals given experimental brain injury. In recently studies with rats subjected to traumatic brain injury (TBI) using a controlled cortical impact (CCI) device, elevated blood pNF-H levels were found. Results show time-dependent changes in the detectable pNF-H levels and these levels correspond with the severity of the injury and the amount of cortical damage.

Studies with mice transgenic for mutations of human copper/zinc superoxide dismutase 1 which are associated with amyotrophic lateral sclerosis (ALS) have revealed corresponding increased amounts of pNF-H in blood of these animals. These mice develop axonal degeneration pathology similar to that seen in humans with ALS, and blood pNF-H levels can be used to monitor this degeneration. Interestingly, pNF-H is detectable before the onset of obvious disease symptoms.

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

Principles of Testing

In the Human Phosphorylated Neurofilament H ELISA, standards, quality controls and samples are incubated in microplate wells pre-coated with chicken polyclonal anti-pNF-H antibody. After 60 minutes incubation and washing, detection rabbit polyclonal anti-pNF-H antibody is added and incubated with captured pNF-H for 60 minutes. After another washing, HRP conjugated antibody against rabbit antibody is added. After 60 minutes incubation and the last washing step, the remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of pNF-H. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

Reagents And Materials Provided

Antibody Coated Microtiter Strips, ready to use, 96 wells

Detection Antibody Solution, ready to use, 13 ml

HRP Conjugate Solution, ready to use, 13 ml

Master Standard, lyophilized, 2 vials

Quality Control HIGH, lyophilized, 2 vials

Quality Control LOW, lyophilized, 2 vials

Dilution Buffer, ready to use, 13 ml

Wash Solution Conc. (10×), concentrated, 100 ml

Substrate Solution, ready to use, 13 ml

Stop Solution, ready to use, 13 ml

Materials Required But Not Supplied

1. Deionized (distilled) water
2. Test tubes for diluting samples
3. Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
4. Precision pipettes to deliver 10-1000 µl with disposable tips
5. Multichannel pipette to deliver 100 µl with disposable tips
6. Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
7. Vortex mixer
8. Orbital microplate shaker capable of approximately 300 rpm
9. Microplate washer (optional). [Manual washing is possible but not preferable]
10. Microplate reader with 450±10 nm filter, preferably with reference wavelength 630 nm (alternatively another one from the interval 550-650 nm)
11. Software package facilitating data generation and analysis (optional)

Storage

Store the complete kit at 2-8°C. Under these conditions, the kit is stable until the expiration date (see label on the box).

Specimen Collection And Preparation

The kit measures pNF-H in human serum, plasma and CSF.

Samples should be assayed immediately after collection or should be stored at -20°C. Mix thoroughly thawed samples just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. Avoid using hemolyzed or lipemic samples.

Dilute samples 3× with Dilution Buffer just prior to the assay, e.g. 50 µl of sample + 100 µl of Dilution Buffer for singlets, or preferably 80 µl of sample + 160 µl of Dilution Buffer for duplicates. **Mix well** (not to foam). Vortex is recommended.

Stability and storage: Serum and plasma samples should be stored at -20°C, or preferably at -70°C for long-term storage. CSF samples should be stored at -70°C. **Do not store the diluted samples.**

Note: It is recommended to use a precision pipette and a careful technique to perform the dilution in order to get precise results.

Stability of samples stored at 2-8°C

Samples should be stored at –20°C. However, no decline in concentration of pNF-H was observed in CSF and serum samples after 7 days when stored at 2-8°C. To avoid microbial contamination, samples were treated with ε-aminocaproic acid and sodium azide, resulting in the final concentration of 0.03% and 0.1%, respectively.

CSF sample	Incubation Temp, Period	pNF-H (pg/ml)
1	-20°C	785
	2-8°C, 1 day	778
	2-8°C, 7 days	750
2	-20°C	2 633
	2-8°C, 1 day	2 684
	2-8°C, 7 days	2 605
3	-20°C	4 439
	2-8°C, 1 day	4 363
	2-8°C, 7 days	4 074

Serum sample	Incubation Temp, Period	pNF-H (pg/ml)
1	-20°C	370
	2-8°C, 1 day	434
	2-8°C, 7 days	385
2	-20°C	1 591
	2-8°C, 1 day	1 561
	2-8°C, 7 days	1 356
3	-20°C	3 333
	2-8°C, 1 day	2 901
	2-8°C, 7 days	3 003

Effect of Freezing/Thawing

No decline was observed in concentration of human pNF-H in CSF and serum samples after repeated (5×) freeze/thaw cycles. However it is recommended to avoid unnecessary repeated freezing/thawing of the samples.

CSF sample	Number of f/t cycles	pNF-H (pg/ml)
1	1x	778
	3x	726
	5x	779
2	1x	2 585
	3x	2 663
	5x	2 660
3	1x	4 235
	3x	4 265
	5x	4 048

Serum sample	Number of f/t cycles	pNF-H (pg/ml)
1	1x	440
	3x	329
	5x	329
2	1x	1 608
	3x	1 695
	5x	1 669
3	1x	3 124
	3x	3 017
	5x	2 956

Plate Preparation

Example of a work sheet.

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	Standard 4 000	QC HIGH	Sample 7	Sample 15	Sample 23	Sample 31
B	Standard 2 000	QC LOW	Sample 8	Sample 16	Sample 24	Sample 32
C	Standard 1 000	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
D	Standard 500	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
E	Standard 250	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
F	Standard 125	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
G	Standard 62.5	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
H	Blank	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38

Reagent Preparation

All reagents need to be brought to room temperature prior to use.

Always prepare only the appropriate quantity of reagents for your test.

Do not use components after the expiration date marked on their label.

1. **Assay reagents supplied ready to use:**

a. Antibody Coated Microtiter Strips

Stability and storage: Return the unused strips to the provided aluminium zip-sealed bag with desicant and seal carefully. Remaining Microtiter Strips are stable 3 months stored at 2-8°C and protected from the moisture.

b. Detection Antibody Solution, HRP Conjugate Solution, Dilution Buffer, Substrate Solution, Stop Solution

Stability and storage: Opened reagents are stable 3 months when stored at 2-8°C.

2. **Assay reagents supplied concentrated or lyophilized:**

a. pNF-H Master Standard

Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution of standard!

Reconstitute the lyophilized Master Standard with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam). The resulting concentration of the pNF-H in the stock solution is **4000 pg/ml**.

Prepare set of standards using Dilution Buffer as follows:

Volume of Standard	Dilution Buffer	Concentration
Stock	-	4 000 pg/ml
250 µl of stock	250 µl	2 000 pg/ml
250 µl of 2 000 pg/ml	250 µl	1 000 pg/ml
250 µl of 1 000 pg/ml	250 µl	500 pg/ml
250 µl of 500 pg/ml	250 µl	250 pg/ml
250 µl of 250 pg/ml	250 µl	125 pg/ml
250 µl of 125 pg/ml	250 µl	62.5 pg/ml

Prepared Standards are ready to use, do not dilute them.

Stability and storage: **Do not store the Standard stock solution and set of standards.**

b. Quality Controls HIGH, LOW

Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution and for current Quality Control concentration!

Reconstitute each Quality Control (HIGH and LOW) with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).

Reconstituted Quality Controls are ready to use, do not dilute them.

Stability and storage: **Do not store the reconstituted Quality Controls.**

Note: Concentration of analyte in Quality Controls need not be anyhow associated with normal and/or pathological concentrations in serum or another body fluid. Quality Controls serve just for control

that the kit works in accordance with PDS and CoA and that ELISA test was carried out properly.

c. Wash Solution Conc. (10×)

Dilute Wash Solution Concentrate (10×) ten-fold in distilled water to prepare a 1x working solution. Example: 100 ml of Wash Solution Concentrate (10×) + 900 ml of distilled water for use of all 96-wells.

Stability and storage: The diluted Wash Solution is stable 1 month when stored at 2-8°C. Opened Wash Solution Concentrate (10×) is stable 3 months when stored at 2-8°C.

Assay Procedure

1. Pipet **100 µl** of diluted Standards, Quality Controls, Dilution Buffer (=Blank) and samples, preferably in duplicates, into the appropriate wells. See Figure for example of work sheet.
2. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.
3. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
4. Add **100 µl** of Detection Antibody Solution into each well.
5. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.
6. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
7. Add **100 µl** of HRP Conjugate Solution into each well.
8. Incubate the plate at room temperature (ca. 25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
9. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
10. Add **100 µl** of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.
11. Incubate the plate for **15 minutes** at room temperature. The incubation time may be extended [up to 25 minutes] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.
12. Stop the colour development by adding **100 µl** of Stop Solution.
13. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550-650 nm). Subtract readings at 630 nm (550-650 nm) from the readings at 450 nm. The absorbance should be read within 5 minutes following step 12.

    Note: If some samples and standard/s have absorbances above the upper limit of your microplate reader, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine pNF-H concentration of off-scale standards and samples. The readings at 405 nm should not replace the readings for samples that were "in range" at 450 nm.

    Note: Manual washing: Aspirate wells and pipet 0.35 ml Wash Solution into each well. Aspirate wells and repeat twice. After final wash, invert and tap the plate strongly against paper towel. Make certain that Wash Solution has been removed entirely.

Calculation

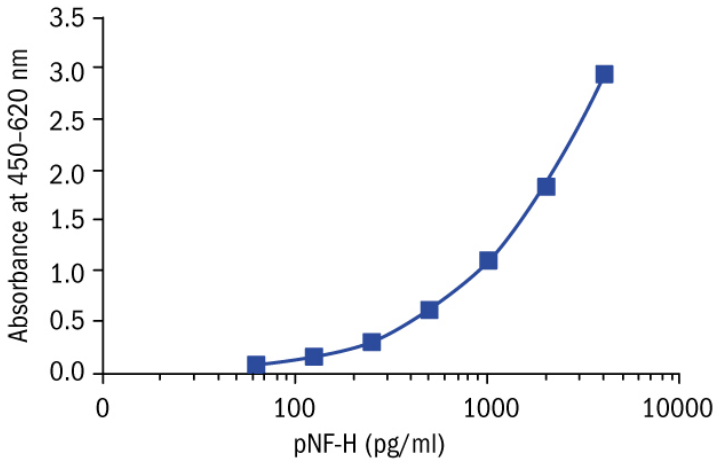
Most microplate readers perform automatic calculations of analyte concentration. The standard curve is constructed by plotting the mean absorbance (Y) of Standards against the known concentration (X) of Standards in logarithmic scale, using the four-parameter algorithm. Results are reported as concentration of pNF-H pg/ml in samples.

Alternatively, the logit log function can be used to linearize the standard curve, i.e. logit of the mean absorbance (Y) is plotted against log of the known concentration (X) of Standards).

The measured concentration of samples calculated from the standard curve must be multiplied by their respective dilution factor, because samples have been diluted prior to the assay, e.g. 400 pg/ml (from standard curve) × 3 (dilution factor) = 1200 pg/ml = 1.2 ng/ml.

Standard in this assay is human brain extract based. Native pNF-H is about 200 kDa protein.

Typical Standard Curve



Performance Characteristics

It is intended for research use only.

The total assay time is less than 4 hours.

The kit measures pNF-H in serum, plasma, cerebrospinal fluid (CSF) and tissue samples

Assay format is 96 wells.

Standard and Quality Controls are human brain extract based. No animal sera are used.

Components of the kit are provided ready to use, concentrated or lyophilized.

Detection Range

62.5-4000 pg/ml

Detection Limit

23.5 pg/ml

Sensitivity

Limit of Detection (LOD) (defined as concentration of analyte giving absorbance higher than mean absorbance of blank plus three standard deviations of the absorbance of blank: Ablank + 3xSDblank) is calculated from the real pNF-H values in wells and is 23.5 pg/ml.

Dilution Buffer is pipetted into blank wells.

Specificity

The antibodies used in this ELISA are specific for pNF-H from human and from mammalian species such as mouse, rat, rabbit, dog, pig, horse and bovine.

Linearity

CSF and serum samples were serially diluted with Dilution Buffer and assayed.

CSF sample	Dilution	Observed (pg/ml)	Expected (pg/ml)	Recovery O/E (%)
1	-	3 576	-	-
	2x	1 804	1 788	100.9
	4x	933	894	104.3
	8x	440	447	98.4
2	-	13 791	-	-
	2x	7 664	6 894	111.1
	4x	3 822	3 448	110.9
	8x	1 918	1 724	111.2

Serum sample	Dilution	Observed (pg/ml)	Expected (pg/ml)	Recovery O/E (%)
1	-	3 333	-	-
	2x	1 629	1 667	97.7
	4x	859	833	103.0
	8x	390	417	93.7
2	-	16 499	-	-
	2x	9 209	8 249	111.6
	4x	4 773	4 125	115.7
	8x	2 293	2 062	111.2

Recovery

CSF and serum samples were spiked with different amounts of pNF-H and assayed.

CSF sample	Observed (pg/ml)	Expected (pg/ml)	Recovery O/E (%)
1	985	-	-
	3 672	3 985	92.1
	2 713	2 485	109.2
	1 608	1 735	92.7
2	2 046	-	-
	2 572	2 796	92.0
	3 519	3 546	99.2
	5 419	5 046	107.4

Serum sample	Observed (pg/ml)	Expected (pg/ml)	Recovery O/E (%)
1	1 648	-	-
	2 180	2 398	90.2
	3 028	3 148	96.2
	4 863	4 648	104.6
2	2 128	-	-
	2 618	2 878	99.3
	3 399	3 628	93.7
	5 090	5 128	91.0

Reproducibility

1. Intra-assay (Within-Run) (n=8)

CSF sample	Mean (pg/ml)	SD (pg/ml)	CV (%)
1	963	48.2	5.0
2	1 829	73.2	4.0

2. Inter-assay (Run-to-Run) (n=8)

CSF sample	Mean (pg/ml)	SD (pg/ml)	CV (%)
1	1 410	64.1	4.5
2	3 212	76.9	2.4

Precautions

1. **For professional use only**
2. Wear gloves and laboratory coats when handling immunodiagnostic materials
3. Do not drink, eat or smoke in the areas where immunodiagnostic materials are being handled
4. This kit contains components of human origin. These materials were found non-reactive for HBsAg, HCV antibody and for HIV 1/2 antigen and antibody. However, these materials should be handled as potentially infectious, as no test can guarantee the complete absence of infectious agents
5. This kit contains components of animal origin. These materials should be handled as potentially infectious
6. Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents. Stop and/or Substrate Solutions may cause skin/eyes irritation. In case of contact with the Stop Solution and the Substrate Solution wash skin/eyes thoroughly with water and seek medical attention, when necessary

7. The materials must not be pipetted by mouth

Trouble Shooting

1. **Weak signal in all wells**

Possible explanations:

Omission of a reagent or a step

Improper preparation or storage of a reagent

Assay performed before reagents were allowed to come to room temperature

Improper wavelength when reading absorbance

2. **High signal and background in all wells**

Possible explanations:

Improper or inadequate washing

Overdeveloping; incubation time with Substrate Solution should be decreased before addition of Stop Solution

Incubation temperature over 30°C

3. **High coefficient of variation (CV)**

Possible explanation:

Improper or inadequate washing

Improper mixing Standards, Quality Controls or samples

Limitations

1. Reagents with different lot numbers should not be mixed
2. Use thoroughly clean glassware
3. Use deionized (distilled) water, stored in clean containers
4. Avoid any contamination among samples and reagents. For this purpose, disposable tips should be used for each sample and reagent
5. Substrate Solution should remain colourless until added to the plate. Keep Substrate Solution protected from light
6. Stop Solution should remain colourless until added to the plate. The colour developed in the wells will turn from blue to yellow immediately after the addition of the Stop Solution. Wells that are green in colour indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution
7. Dispose of consumable materials and unused contents in accordance with applicable national regulatory requirements