研究用試薬

Neurofilament (pNf-H)-high sensitive ELISA

Instruction for use

For in vitro diagnostic use IVD

ORDER NO.	ANTIGEN	SUBSTRAT	FORMAT
EQ 6562-9601	Phosphorylated neurofilament heavy chain	Ab-coated microplate wells	96 x 01 (96)

CE

Intended use

The ELISA test kit provides quantitative in vitro determination of the heavy chain of phosphorylated neurofilament (pNf-H) in human serum or plasma to support the diagnosis of amyotrophic lateral sclerosis (ALS). The product is designed for use as IVD.

Clinical significance

Motor neuron diseases (MND) are a group of neurodegenerative disorders characterised by degeneration of upper and lower motoneurons [1]. Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, has a prevalence of 2 per 100,000 persons and is the most common MND [2]. Primary lateral sclerosis, progressive muscular atrophy, progressive bulbar palsy and pseudobulbar palsy are other types of MND. Despite extensive research the cause of onset of ALS is not yet understood [1, 3]. The most common genetic cause of ALS is the hexanucleotide repeat expansion of the C9ORF72 gene, which may represent approximately 30% of familial ALS [2]. In 90% of cases, ALS occurs sporadically, and therefore early and accurate diagnosis of ALS requires tests that are not dependent upon genetic testing [2, 4].

The first signs of the disease are often very subtle, for example, muscle weakness in arms or legs or cramping, while others experience difficulty in swallowing or speaking, depending on the site of onset [5]. During the course of the disease these symptoms become increasingly severe and spread to other regions of the body. ALS patients eventually experience a loss of independence that includes the inability to communicate, swallow or walk [3-7].

ALS diagnosis is based on clinical symptoms and electromyography (EMG) [1, 3, 5-7]. Standardised criteria for the clinical diagnosis of ALS are the El Escorial criteria. Fulfilment of El Escorial criteria is typically used for inclusion in ALS clinical trials, though by this time in the disease process a significant number of motor neurons have already been lost [5, 6]. Commonly, there is a gap of more than 12 months [7, 8] between the first symptoms and the diagnosis. Therefore, methods to more rapidly diagnose ALS are needed and will likely include imaging or biochemical lab testing [3]. Diagnostic tests are needed to discriminate ALS from MND mimics, including polyneuropathy, myopathy and inclusion body myositis [1, 4]. Detection of increased values of the heavy chain of phosphorylated neurofilament (pNf-H) in CSF or serum is suited for the diagnosis, differential diagnosis and prognosis in MND [1, 3, 4, 8, 9]. Recent studies have suggested including pNf-H in routine diagnostic work-up for ALS [1].

Antibody

The microplate wells are coated with a polyclonal anti-pNf-H antibody.

Test principle

The test kit contains microplate strips each with 8 reagent wells coated with purified polyclonal anti-pNf-H antibody. The reagent wells are incubated in the first analysis step with monoclonal biotin-labelled anti-pNf-H antibodies (biotin) and patient samples. pNf-H contained in the samples is bound in a complex by the antibodies. In a second incubation step, this complex is labelled with peroxidase-labelled streptavidin (enzyme conjugate). In a third incubation step, the bound peroxidase catalyses a colour reaction with the substrate tetramethylbenzidine (TMB). The colour intensity is proportional to the pNf-H concentration in the sample.

Contents of the test kit

Component	Colour	Format	Symbol
1. Antibody-coated microplate wells 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrator 1, ready for use	light red to dark red	1 x 1.0 ml	CAL 1
3. Calibrator 2, ready for use		1 x 1.0 ml	CAL 2
4. Calibrator 3, ready for use		1 x 1.0 ml	CAL 3
5. Calibrator 4, ready for use		1 x 1.0 ml	CAL 4
6. Calibrator 5, ready for use		1 x 1.0 ml	CAL 5
7. Calibrator 6, ready for use		1 x 1.0 ml	CAL 6
8. Control 1, ready for use	green	1 x 1.0 ml	CONTROL 1
9. Control 2, ready for use	blue	1 x 1.0 ml	CONTROL 2
10. Biotin biotin-labelled anti-pNf-H antibody, ready for use	green	1 x 7 ml	BIOTIN
11. Enzyme conjugate peroxidase-labelled streptavidin, ready for use	blue	1 x 12 ml	CONJUGATE
12. Wash buffer, 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
13. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
14. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
15. Instruction for use	---	1 booklet	
16. Quality control certificate	---	1 protocol	
17. Protective foil	---	3 pieces	FOIL

Additional materials and equipment (not supplied in the test kit)

- Automatic microplate washer: recommended. Washing of the microplates can also be carried out manually.
- Microplate reader: wavelength of 450 nm, reference wavelength range from 620 nm to 650 nm
- Calibrated pipettes
- Centrifuge
- Pipette tips
- Calibrated stepper pipette: recommended for the pipetting of biotin, enzyme conjugate, substrate, and stop solution
- Distilled or deionised water
- Microplate shaker: Orbital shaker (450 rpm)
- 37°C incubator or bain-marie: recommended to warm the wash buffer
- Stop watch

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Storage and stability

The test kit has to be stored at a temperature between +2°C and +8°C, do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

In use stability following the first opening

After opening, the reagents are stable until the indicated expiry date when stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

Warnings and precautions

- The product must only be used by trained laboratory staff in a clinical or research laboratory.
- If the packed reagents are visibly damaged, do not use the test kit.
- Before using the product, read the instruction for use carefully. Use only the valid version provided with the product.
- The pipetting volumes, incubation times, temperatures, and preparation steps given in the instructions must be adhered to.
- Do not substitute or mix the EUROIMMUN reagents with reagents from other manufacturers.
- Wash buffers, substrate and stop solutions are exchangeable independent of the lots if they have identical article numbers (see labelling). All other reagents are lot-specific and must not be combined with other lots.
- Observe Good Laboratory Practice (GLP) and safety guidelines. Some of the reagents contain preservatives in non-declarable concentrations. Avoid eye and skin contact with samples and reagents. In case of eye or skin contact, rinse thoroughly with water. Remove and wash contaminated clothing. In case of ingestion, obtain medical advice.
- The calibrator material of bovine origin was produced in such way that it should not contain infectious agents. Nonetheless, all materials should be treated as being a potential infection hazard. They should be handled with care and decontaminated or disposed of with due care as all biological hazardous substances.

Preparation and stability of the samples

- **Samples:** Human serum or EDTA, heparin or citrate plasma.
- **Sample preparation:** The patient samples to be investigated must be brought to room temperature (+18°C to +25°C) and must be mixed thoroughly. Only clear serum or plasma should be used (if necessary, centrifuge beforehand). The patient samples are used undiluted.
- **Information on sample handling:** The patient samples to be investigated can usually be stored for up to 24 hours at room temperature and up to 14 days at +2°C to +8°C. For longer storage times, the patient samples should be stored at -80°C and undergo maximum two freeze/thaw cycles before pNf-H determination.

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antibodies can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.
- **Calibrators and controls:** Ready for use. Mix reagents thoroughly before use.
- **Biotin:** Ready for use. Mix biotin thoroughly before use.
- **Enzyme conjugate:** Ready for use. Mix enzyme conjugate thoroughly before use.
- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to +37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionised or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working-strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.
- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.
- **Stop solution:** Ready to use.

Waste disposal

Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Quality control

For every group of tests performed, the concentrations determined for the controls must lie within the limits stated for the relevant test kit lot. A quality control certificate with the respective data and the concentration information of the calibrators included in the kit is provided. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

Reference material

Since there is no international reference material for the determination of neurofilament (pNf-H) the Neurofilament (pNf-H)-high sensitive ELISA was standardised based on internal reference material. The quantification is made in picograms per milliliter (pg/ml).

Assay procedure

(Partly) manual test performance

Sample incubation: (1st step)
According to the pipetting scheme, pipette **50 µl** of biotin solution and **50 µl** of calibrators, controls or undiluted patient samples into each of the reagent wells. Cover the reagent wells with the provided foil.
Incubate for **120 minutes at room temperature** (+18 °C to +25 °C) on an orbital shaker **at medium speed** (approx. 450 rpm).

Washing:
Manual: Remove the protective foil, empty the wells and subsequently wash 3 times using 300 µl of working-strength wash buffer for each wash.
Automatic: Remove the protective foil and wash the reagent wells 3 times with 450 µl of working-strength wash buffer (program setting: e.g. TECAN Columbus Washer “Overflow Mode”).

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Enzyme conjugate incubation: (2nd step)
Pipette **100 µl** of enzyme conjugate into each of the microplate wells. Cover the wells and incubate for **30 minutes at room temperature** (+18°C to +25°C) on a microplate shaker **at medium speed** (approx. 450 rpm).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd step)
Pipette **100 µl** of chromogen/substrate solution into each of the microplate wells.
Incubate for **15 minutes at room temperature** (+18°C to +25°C; protect from direct sunlight).

Stopping:
Pipette **100 µl** of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution**. Prior to measuring, carefully shake the microplate to ensure a homogeneous distribution of the solution.

Test performance using fully automated analysis devices

The sample dilution and subsequent test processing are performed fully automatically with an analysis instrument. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I and the EUROIMMUN Analyzer I-2P and this EUROIMMUN ELISA. Validation documents are available on enquiry.
Automated test performance using other fully automated, open-system analysis devices is possible. However, the combination should be validated by the user.

Pipetting protocol

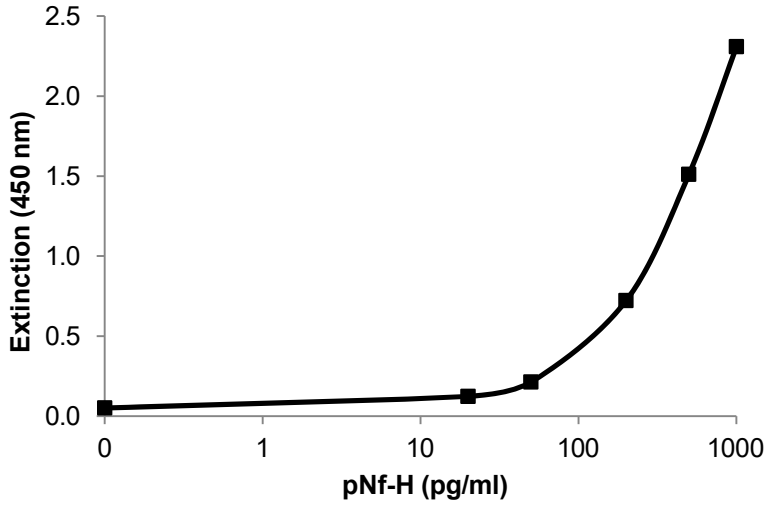
	1	2	3	4	5	6	7	8	9	10	11	12
A	C 1	P 1	P 9	P 17								
B	C 2	P 2	P 10	P 18								
C	C 3	P 3	P 11	P 19								
D	C 4	P 4	P 12	P 20								
E	C 5	P 5	P 13	P 21								
F	C 6	P 6	P 14	P 22								
G	Co 1	P 7	P 15	P 23								
H	Co 2	P 8	P 16	P 24								

The pipetting protocol for microplate strips 1 to 4 is an example for the **quantitative analysis** of 24 patient sera (P 1 to P 24).

The calibrators (C 1 to C 6), the controls (Co 1 and Co 2) and the patient samples have each been incubated in one well. The reliability of the determination can be improved by duplicate determinations for each sample.
The controls serve as internal controls for the reliability of the test procedure. They must be assayed with each test run.

Test evaluation

Quantitative: The standard curve from which the concentration of analyte in the patient samples can be taken is obtained by plotting of the extinction values measured for the 6 calibrators against the corresponding units (linear/log). For computer-aided calculation of the standard curve, the evaluation procedure “5-parameter logistics” should be selected. For correct logarithmic representation it might be necessary to set the concentration of calibrator 1 from 0 to e.g. 0.1 pg/ml. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of analyte concentrations in patient samples.



If the extinction for a patient sample lies above the extinction of calibrator 6, the result should be reported as “>1000 pg/ml”. It is recommended to pre-dilute this sample e.g. 1:4 and measure it again in a new test run. The result in pg/ml read from the calibration curve for this sample must then be multiplied by factor 4.
For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends retesting the samples.

Analytical performance

- **Linearity:** The linearity of the Neurofilament (pNf-H)-high sensitive ELISA was determined by dilution series of human serum and citrate plasma, mixed with bovine pNf-H in high concentrations. The Neurofilament (pNf-H)-high sensitive ELISA is linear in the measurement range from 0 pg/ml to 1000 pg/ml.
- **Detection limit:** The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable concentration of antigen. The lower detection limit of the Neurofilament (pNf-H)-high sensitive ELISA is 1.7 pg/ml.
- **Reproducibility:** The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 samples. The intra-assay coefficients of variation are based on 20 determinations, the inter-assay coefficients of variation on duplicate determinations, analysed in 10 different runs on 5 different days. The inter-lot coefficients of variation are based on duplicate determinations in 2 different runs, measured in three different lots.

Intra-assay precision, n = 20		
Sample	**Mean value (pg/ml)**	**CV (%)**
1	71.4	4.0
2	351.8	6.0
3	816.0	4.7

Inter-assay precision, n = 2 x 10		
Sample	**Mean value (pg/ml)**	**CV (%)**
4	70.4	4.7
5	311.5	6.9
6	614.9	10.6

Inter-lot precision, n = 2 x 2 x 3		
Sample	**Mean value (pg/ml)**	**CV (%)**
7	67.2	3.6
8	338.4	3.9
9	600.4	6.5

- **Cross-reactivity:** The ELISA specifically detects pNf-H. Due to the pronounced structural homologies of the neurofilaments, however, cross-reactions with the neurofilament medium chain (Nf-M) cannot be excluded. Apart from that, no further cross-reactivities are known.
- **Interference:** Haemolytic, lipaemic and icteric samples showed no influence on the result up to concentrations of 10 mg/ml haemoglobin, 20 mg/ml triglycerides and 0.4 mg/ml bilirubin in this ELISA.
- **Method comparison (accuracy):** 72 pre-characterised patient samples (origin: Germany and USA, reference method: EUROIMMUN Neurofilament (pNf-H) ELISA) were investigated with the EUROIMMUN Neurofilament (pNf-H)-high sensitive ELISA. The sensitivity amounted 93.8%, with a specifity of 95.0%. Borderline results were not included in the calculation.

n = 72		**EUROIMMUN Neurofilament (pNf-H) ELISA**		
		positive	borderline	negative
EUROIMMUN Neurofilament (pNf-H)-high sensitive ELISA	positive	15	1	2
	borderline	2	5	8
	negative	1	0	38

Clinical performance

Diagnostic sensitivity and specifity: 20 precharacterised patient samples evaluated as positive after neurological investigation were analysed with the Neurofilament (pNf-H)-high sensitive ELISA. For determination of specificity, 23 samples from Alzheimer patients, 7 samples from patients with other neurological diseases and 69 samples from healthy blood donors were tested. The ALS panel originated from the US, all other samples from Germany. Within the borderline range of 72.8 – 134.5 pg/ml, a sensitivity of 93.3% at a specificity of 94.2% was obtained (borderline values were not included in the calculation).

n = 119	EUROIMMUN Neurofilament (pNf-H)-high sensitive ELISA		
Clinical evaluation	**Positive** (>134.5 pg/ml)	**Borderline** (72.8 – 134.5 pg/ml)	**Negative** (<72.8 pg/ml)
Positive	14	5	1
Negative	5	13	81

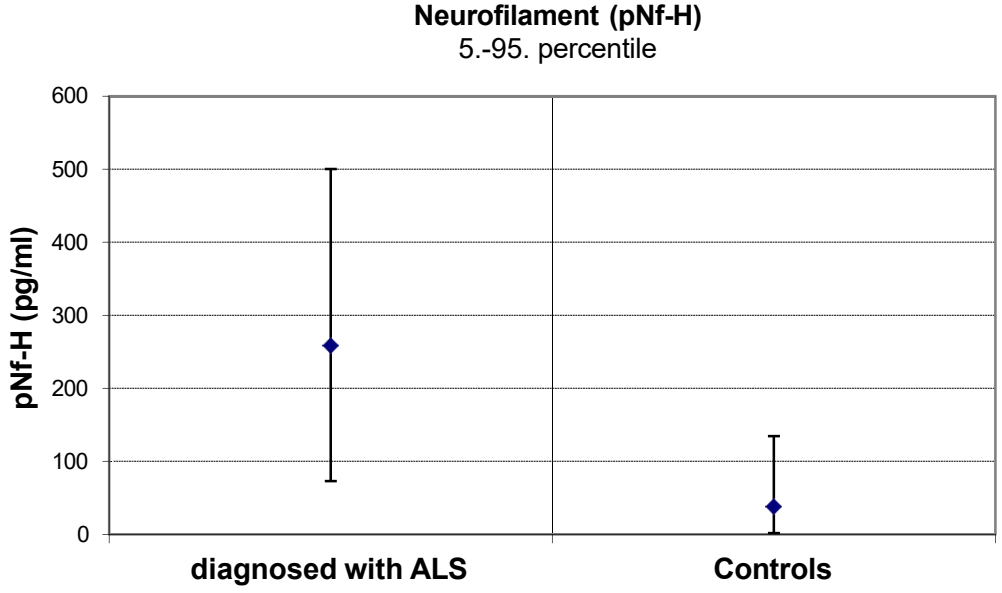
Reference range

The pNf-H concentration was determined in healthy blood donors with the Neurofilament (pNf-H)-high sensitive ELISA. 2 out of 157 samples had a concentration of over 134.5 pg/ml and were thus evaluated as positive.

Expected values

Patients diagnosed with ALS	5% percentile	**72.8**
	Mean	258.8
	95% percentile	500.3
Apparently healthy blood donors and patients with other neurological disorders	5% percentile	1.7
	Mean	38.1
	95% percentile	**134.5**

Values in pg/ml
Using a borderline zone of 72.8 to 134.5 pg/ml
(5% percentile of ALS to 95% percentile of healthy controls)



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Limitations of the procedure

- The result of this test is no proof of the presence or absence of a disease. The test kit is used to support diagnostics. The results should always be interpreted together with clinical findings and further diagnostic tests.
- A negative result does not exclude the presence of a neurological disorder.
- Contamination of samples with blood may lead to false positive results.
- Correct performance of sample collection and storage is crucial for the test results.
- The test system is validated for the determination of neurofilament (pNf-H) in human serum and plasma only.
- The binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostatically adjusted ELISA incubator in all incubation steps. The higher the room temperature during the incubation steps, the greater will be the extinction. The same variations also apply to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.
- Insufficient washing (e.g. less than 3 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction readings.
- Residual liquid (>10 µl) in the reagent wells after washing can interfere with the substrate and lead to false low extinction readings.
- The partial or complete adjustment of the test system to the use of instruments for automated sample processing or other liquid handling devices may result in differences between the results obtained with automated processing and those obtained with manual procedure. It is the responsibility of the user to validate the instruments used so that they yield test result within the reliable range.
- A negative result does not exclude a disease. In case of a borderline result, a clear evaluation is not possible. With a clinical suspicion and a negative or borderline serum result, clarification by other diagnostic methods and/or serological testing of a follow-up sample is recommended.

Literature

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8. Lu CH et al. (2015). **Plasma neurofilament heavy chain levels and disease progression in amyotrophic lateral sclerosis: insights from a longitudinal study.** J Neurol Neurosurg Psychiatry 86(5): 565-573.
9. De Schaepdryver M et al. (2019). **Analytical performance of a CE-marked immunoassay to quantify phosphorylated neurofilament heavy chain**s. Clin Chem Lab Med.

Technical support

In case of technical problems you can ask for support via the EUROIMMUN website (www.euroimmun.com/contact).

Meaning of the symbols

Symbol	Meaning	Symbol	Meaning
STRIPS	Microplate strips	STOP SOLUTION	Stop solution
CAL 1	Calibrator 1	FOIL	Protective foil
CAL 2	Calibrator 2	IVD	In vitro diagnostic medical device
CAL 3	Calibrator 3	LOT	Lot description
CAL 4	Calibrator 4		Storage temperature
CAL 5	Calibrator 5		Unopened usable until (YYYY-MM-DD)
CAL 6	Calibrator 6		CE-labelled
CONTROL 1	Control 1		Protect from direct sunlight
CONTROL 2	Control 2		Manufactoring date (YYYY-MM-DD)
BIOTIN	Biotin		Manufacturer
CONJUGATE	Enzyme conjugate		Observe instructions for use
WASH BUFFER 10x	Wash buffer, 10x concentrate	REF	Order number
SUBSTRATE	Substrate		Contents suffice for <n> analyses