研究用試薬

Neurofilament (pNf-H) ELISA
Test instruction

ORDER NO.	ANTIGEN	SUBSTRATE	FORMAT
EQ 6561-9601	Phosphorylated neurofilament heavy chain	Ab-coated microplate wells	96 x 01 (96)

Indication: The enzyme immunoassay (ELISA) provides quantitative in vitro determination of phosphorylated neurofilament heavy chain (pNf-H) in cerebrospinal fluid (CSF) or serum samples of human origin. The product is designed for use as IVD.

Principle of the test: In the first analysis step, the calibrators and patient samples are diluted with monoclonal peroxidase-labelled anti-pNf-H antibodies and added to microplate wells coated with polyclonal anti-pNf-H antibodies. In this process pNf-H is bound in a complex. In a second incubation using the peroxidase substrate tetramethylbenzidine (TMB), the bound peroxidase promotes a colour reaction. 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, pNf-H, ready for use	red	1 x 1.0 ml	CAL 1
3. Calibrator 2, pNf-H, ready for use	red	1 x 1.0 ml	CAL 2
4. Calibrator 3, pNf-H, ready for use	red	1 x 1.0 ml	CAL 3
5. Calibrator 4, pNf-H, ready for use	red	1 x 1.0 ml	CAL 4
6. Calibrator 5, pNf-H, ready for use	red	1 x 1.0 ml	CAL 5
7. Calibrator 6, pNf-H, ready for use	red	1 x 1.0 ml	CAL 6
8. Control 1, pNf-H, ready for use	green	1 x 1.0 ml	CONTROL 1
9. Control 2, pNf-H, ready for use	blue	1 x 1.0 ml	CONTROL 2
10. Enzyme conjugate peroxidase-labelled anti-pNf-H antibody, ready for use	blue	1 x 12 ml	CONJUGATE
11. Wash buffer, 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
12. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
13. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
14. Test instruction	---	1 booklet	
15. Quality control certificate	---	1 protocol	
16. Protective foil	---	3 pieces	

LOT	Lot description	CE	☐ Storage temperature
IVD	In vitro diagnostic medical device		☐ Unopened usable until

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.

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.

Updates with respect to the previous version are marked in grey.

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

- **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 individual 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:** The reagents must be mixed thoroughly before use.

- **Enzyme conjugate:** Ready for use. Mix 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 for use.

Warning: Some of the reagents contain preserving agents in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human cerebrospinal fluid (CSF) or serum samples.

Notes on sample handling: It is of particular importance that CSF be filled directly into polypropylene tubes. If the analysis is not to be performed immediately following puncture, the samples should be stored at -20°C and subjected to preferably no more than one, maximal two, freeze/thaw cycles.

Incubation

(Partly) manual test performance

Sample incubation: (1st step)

Pipette **100 µl** of enzyme conjugate and **25 µl** of calibrators, controls and undiluted patient samples into each of the reagent wells.
Cover the reagent wells with the provided protective foil.
Incubate for **120 minutes** on an **orbital shaker (450 rpm)** at room temperature (+18°C to +25°C).

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.

Note: Residual liquid (>10 µl) in the reagent wells after washing can interfere with the substrate and lead to false low extinction readings.
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.
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.

Substrate incubation: (2nd 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 the reaction:

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, slightly shake the microplate to ensure a homogeneous distribution of the solution.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an analysis device. 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

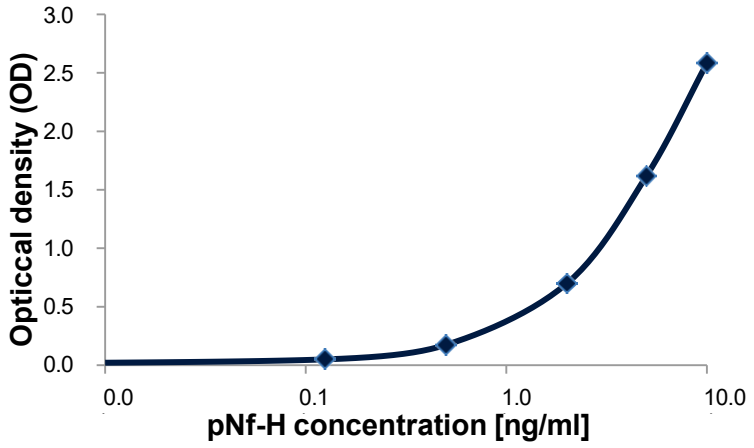
	1	2	3	4	5	6	7	8	9	10	11	12
A	C 1	P 1	P 9									
B	C 2	P 2	P 10									
C	C 3	P 3	P 11									
D	C 4	P 4	P 12									
E	C 5	P 5	P 13									
F	C 6	P 6	P 14									
G	Co 1	P 7	P 15									
H	Co 2	P 8	P 16									

The pipetting protocol for microplate strips 1 to 3 is an example for the **quantitative analysis** of 16 patient samples (P 1 to P 16).

The calibrators (C 1 to C 6), the controls (Co 1, Co 2), and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimises reagent wastage.
The controls serve as internal controls for the reliability of the test procedure. They must be assayed with each test run.

Calculation of results

Quantitative: The standard curve from which the concentration of pNf-H in the unknown patient samples can be taken is obtained by plotting of the extinction readings measured for the 6 calibration sera against the corresponding units (linear/log). For computer-aided calculation of the standard curve, the evaluation procedures 4-PL, 5-PL (PL= parameter logistics) or alternatively cubic spline shall be used.
For correct logarithmic representation it might be necessary to set the concentration of calibrator 1 from 0 to e.g. 0.01 ng/ml. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of concentrations in patient samples.



If the extinction of a patient sample lies above the value of calibrator 6, the result should be reported as “>10 ng/ml”. It is recommended that the sample be re-tested in a new test run with an additional dilution of e.g. 1:4. The result in ng/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 values from a duplicate determination deviate substantially from one another, EUROIMMUN recommends retesting the samples.

Therapeutic decisions should not be made on the basis of results from this test, but only under consideration of clinical findings and further diagnostic values.

Test characteristics

Calibration: For every group of tests performed, the values of the concentrations must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

Antibodies: The reagent wells are coated with polyclonal anti-pNf-H antibodies.

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 pNf-H concentration. The detection limit of the Neurofilament (pNf-H) ELISA was found to be 0.027 ng/ml.

Cross-reactivity: This ELISA specifically detects phosphorylated neurofilament heavy chain (pNf-H). While cross-reactions with related compositions were not observed, it cannot be excluded that pNf-H from other species, e.g. bovines, might be detected.

Interference: Contamination of CSF samples with blood up to a concentration of 10% (v/v) did not cause interference with the ELISA. Nevertheless, red tint of the sample indicates significant contamination with blood. The sample should not be used. Haemolytic, lipaemic and icteric serum samples showed no influence on the result up to a concentration of 10 mg/ml haemoglobin, 20 mg/ml triglycerides and 0.4 mg/ml bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 CSF and 3 serum samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on duplicates performed in 10 different test runs on 5 different days. The inter-lot CVs are based on duplicates in 2 different test runs performed for each of 3 kit lots.

Intra-assay variation, n = 20		
CSF sample	**Mean value (ng/ml)**	**CV (%)**
1	0.8	3.4
2	1.9	2.2
3	5.5	3.1

Intra-assay variation, n = 20		
Serum sample	**Mean value (ng/ml)**	**CV (%)**
1	0.6	2.6
2	1.0	2.2
3	3.3	2.5

Inter-assay variation, n = 10 x 2		
CSF sample	**Mean value (ng/ml)**	**CV (%)**
1	0.8	4.1
2	2.0	6.4
3	5.2	7.9

Inter-assay variation, n = 10 x 2		
Serum sample	**Mean value (ng/ml)**	**CV (%)**
1	0.7	4.6
2	0.8	11.9
3	2.8	11.3

Inter-lot variation, n = 3 x 2 x 2		
CSF sample	**Mean value (ng/ml)**	**CV (%)**
1	0.8	6.6
2	2.0	4.4
3	5.2	5.1

Inter-lot variation, n = 3 x 2 x 2		
Serum sample	**Mean value (ng/ml)**	**CV (%)**
1	0.7	7.2
2	0.8	8.4
3	2.9	12.0

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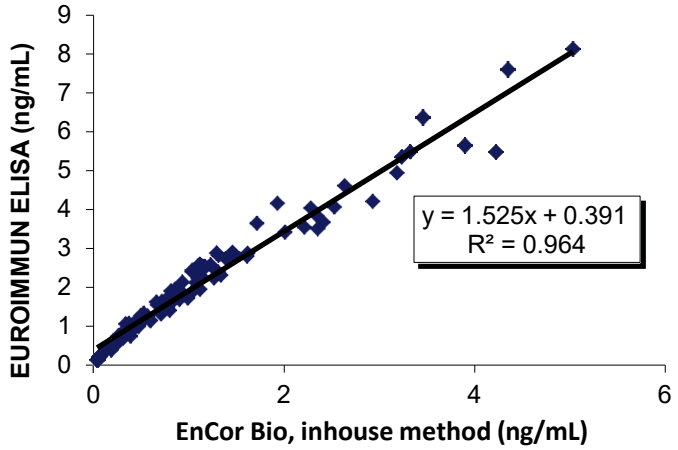
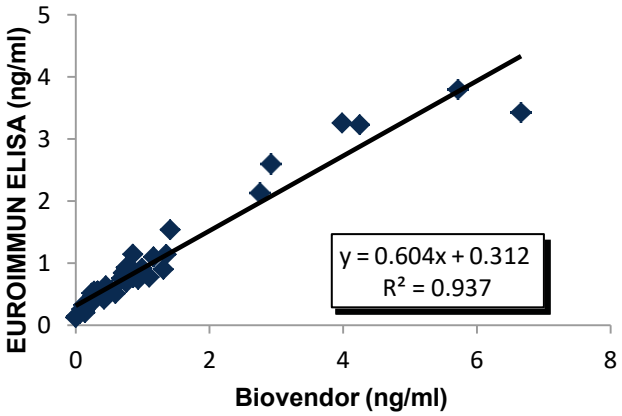
Linearity: The linearity of the test was investigated by linearly diluting two CSF samples (5.2, 11.5 ng/ml) and two serum samples (1.9, 8.6 ng/ml) in 10% steps with sample buffer and measuring them in triplicates. Linearity is given for CSF samples in the range of 0.6 ng/ml to 11.5 ng/ml, the agreement with the expected value ranged from 90% to 118% with a mean correlation coefficient of r = 0.991. Linearity is given for serum samples in the range of 0.4 ng/ml to 8.6 ng/ml, the agreement with the expected value ranged from 100% and 122% with a mean correlation coefficient of r = 0.993.

Method comparison: The EUROIMMUN ELISA was compared with other available immunoassays and showed the following correlation:

IronHorse, pNf-H in-house method MSD platform	EI = 1.525 x EnCor + 0.39 ng/ml; n = 100; R^2 = 0.964
Biovendor, Human Phosphorylated Neurofilament H ELISA Cat. No. RD191138300R Research use only	EI = 0.604 x Biovendor + 0.31 ng/ml: n = 36; R^2 = 0.937





Expected values for CSF samples: To evaluate the clinical performance of the Neurofilament (pNf-H) ELISA, 159 clinically characterised CSF samples were evaluated (origin: Europe; 80 with ALS, 79 controls with other neurological diseases).

Panel	Statistics	pNf-H [ng/ml]
ALS (n = 80)	**5% percentile**	**0.69**
	mean value	2.46
	95% percentile	5.49
Controls (n = 79)	5% percentile	0.16
	mean value	0.65
	95% percentile	**1.52**

For the interpretation of findings from clinically characterised patient samples, EUROIMMUN recommends the interpretation of findings on the basis of a limit range (5% percentile of ALS panel to 95% percentile of control panel):

negative:	**≤0.69 ng/ml**
borderline:	**>0.69 to <1.52 ng/ml**
positive:	**≥1.52 ng/ml**

Every laboratory should use their own normal values established under specific ambient conditions.

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Excluding the borderline cases, there was a sensitivity of 93.7% at a specificity of 93.9% for the patient samples described above. With borderline cases excluded, the positive predictive value (PPV) amounted to 93.7% and the negative predictive value (NPV) to 93.9%.

		Clinical characterisation		
		ALS	no ALS	total
EUROIMMUN test system **Neurofilament (pNf-H) ELISA**	positive	59	4	63
	borderline	17	13	30
	negative	4	62	66
	total	80	79	159

Expected values for serum samples: To evaluate the clinical performance of the Neurofilament (pNf-H) ELISA, 64 clinically characterised serum samples were evaluated (origin: Europe; 20 with ALS, 40 controls with other neurological diseases and healthy persons).

Panel	Statistics	pNf-H [ng/ml]
ALS (n=80)	**5% percentile**	**0.17**
	mean value	0.52
	95% percentile	1.04
Controls (n=79)	5% percentile	0.02
	mean value	0.08
	95% percentile	**0.29**

For the interpretation of findings from clinically characterised patient samples, EUROIMMUN recommends the interpretation of findings on the basis of a limit range (5% percentile of ALS panel to 95% percentile of control panel):

negative:	**≤ 0.17 ng/ml**
borderline:	**> 0.17 to ≤ 0.29 ng/ml**
positive:	**≥ 0.29 ng/ml**

Every laboratory should use their own normal values established under specific ambient conditions.

Excluding the borderline cases, there was a sensitivity of 93.3% at a specificity of 92.7% for the patient samples described above. With borderline cases excluded, the positive predictive value (PPV) amounted to 82.4% and the negative predictive value (NPV) to 97.4%.

		Clinical characterisation		
		ALS	no ALS	total
EUROIMMUN test system **Neurofilament (pNf-H) ELISA**	positive	14	3	17
	borderline	5	3	8
	negative	1	38	39
	total	20	44	64

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].

Disease course

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].

Diagnosis

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].

Literature

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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.