UmanDiagnostics
A Quanterix Company

REF 20-8002

Instructions for Use – NF-light™ (Neurofilament light) Serum ELISA

1. Limited use statement

BY USE OF THIS PRODUCT, RESEARCHERS AGREE TO BE BOUND BY THE TERMS OF THIS LIMITED USE STATEMENT. Researchers may use this product **for research use only**, not for diagnostic procedures, no commercial use is allowed. Commercial use means any and all uses of this product and derivatives by a party for monetary or other consideration and may include but is not limited to use in: (1) product manufacture; and (2) to provide a service, information or data; and/or (3) resale of the product or its derivatives, whether or not such product or derivatives are resold for use in research.

2. Intended use

Serum NF-light™ ELISA is a **Research Use Only** assay for quantitative measurements of human Neurofilament light (NF-L) protein in serum samples. The kit is intended for professional use.
The NF-light™ Serum ELISA can also be used for research on samples containing NF-L from rat, mouse, bovine or macaque sources as the antibodies in the assay recognizes NF-L from these species as well.

3. Notice to user

If a serious incident occurs in relation to this device, the incident should be reported to the manufacturer. See contact details in the end of this instruction.

4. Background

Neurofilaments are the main cytoskeletal constituents in neuronal cells. They are important for the maintenance of the axonal caliber and morphological integrity, which affect the velocity and fidelity of neuronal transmissions. Three different neurofilament chains exist, named according to their size. These are Neurofilament light, medium and heavy respectively. The Neurofilament light constitutes the backbone to which the heavier chains co-assemble, forming the neurofilament fiber. Following injuries of nerve cells due to direct trauma or slow degenerative processes, the content of the cell is released into the surrounding compartment allowing quantitative determinations of the axonal proteins [1,2].

5. Method description

The UmanDiagnostics NF-light™ Serum ELISA assay is an enzymatic immunoassay designed for quantitative measurements of NF-L in human serum. The test uses two highly specific, non-competing, monoclonal antibodies [3]. The capture antibody is coated on a solid surface and binds the sample NF-L. The secondary/detection antibody is biotin conjugated and addition of HRP-conjugated Streptavidin allows for quantitative determinations by enzymatic turn-over of a colorless substrate (TMB) to a colored product. The absorbance value can be correlated to the amount of NF-L in the sample by the use of a calibrator curve.

Recommended quantification range:	4 pg/mL – 100 pg/mL
Calibrator curve range:	0.5 pg/mL – 40 pg/mL
LLoD:	0.4 pg/mL
LLoQ:	0.8 pg/mL
Incubation time:	4 hours and 15 minutes
Sample size:	50 µL/replicate
Minimum dilution of sample:	4 x

Precision:

NF-L level	Intra-assay precision mean (range)
5-10 pg/mL	6.6 % (4.2-11.1)
10-125 pg/mL	4.3 % (2.9-5.9)

NF-L level	Inter-assay precision mean (range)
10-15 pg/mL	10 % (8.7-11.2)
15-130 pg/mL	5.7 % (3.9-6.8)

6. Warnings, handling precautions and important notes

- In case of severe damage to the kit package please contact your supplier in written form no later than one week after receiving the kit. Do not use damaged components. Please keep the damaged components stored for complaint related issues. Lost vacuum for the plate has no negative effect on assay performance.
- The NF-light™ Serum ELISA is for **Research Use Only** and is not for in vitro diagnostic use.
- The product should be used strictly in accordance with this instruction for use (IFU).
- All samples to be analyzed should be considered potentially contagious, therefore, take appropriate safety measures when handling and disposing of biological samples.
- The kit has been designed to be able to be used at two separate analysis occasions. If the kit is intended to be used at two occasions, not more than 500 µL extra volume of conjugate and detector antibody working solutions should be prepared, to allow for sufficient reagent volumes at the second analysis occasion.
- Do not mix reagents from different Assay lots.
- It is advised to run samples and Calibrators in duplicate. If large deviations occur between replicates, please re-assay.
- Make sure to allow all reagents to reach room temperature before use.
- All incubation steps should be performed at room temperature (18-25°C).
- Keep all components and reagents away from heat and direct sunlight.
- A plate lid is provided with the kit. This should be used to cover the plate during incubation steps, to protect from contamination.

- During the incubation steps, use an **orbital ELISA tabletop shaker** at **800 rpm**. **Agitation of the plate at 800 rpm is of UTMOST IMPORTANCE to obtain reliable results.** Incubation at a lower frequency will cause lowered absorbances and unreliable results.

- **Use a supplied 15 mL Sarstedt tube** (art. 62.554.016) **when preparing the Conjugate S solution. Other tubes may have a negative impact on Conjugate S activity causing the overall absorbance level to drop and the sample read-outs to be unreliable.** Note however that reagent reservoirs can be used **to facilitate** pipetting in all steps.

- Make sure there are no bubbles in the wells prior to measuring the absorbance.
  Follow good laboratory practice and safety guidelines. Wear lab coats, disposable gloves and protective glasses when necessary.
- **CAUTION:** Avoid contact with Stop Solution S. It may cause skin irritations and burns. A Material Safety Data Sheet for this product is available upon request directly from UmanDiagnostics AB.

7. Shelf life and storage of reagents

Store the kit at +(2-8)°C and keep away from heat or direct sunlight. Do not freeze the components.
Reconstituted Calibrator and Positive Control S should be used immediately and cannot be re-used.
Once opened, the kit should be used within 4 weeks. An opened strip plate should be sealed with tape to avoid excess humidity and stored in +(2-8)°C.
The shelf-life of the kit is printed on the kit box label and can also be found on the included certificate of analysis.

8. Sample Storage

Samples should be kept at -80°C in polypropylene tubes. Repetitive freeze-thawing should be avoided.

9. Materials

Kit components provided:

Cap colour	Short name	Full name	Description	Quantity
N/A	Plate-S	Anti-NF-L Plate S	Pre-coated with mouse anti NF-L monoclonal antibody, covered with a lid and sealed in plastic pouch with desiccant.	12 x 8 wells
Blue	**NFL-Cal-S**	Human recombinant NF-L Calibrator S	Recombinant Human NF-L, to be reconstituted according to the vial label.	2 vials
Blue	**ELISA-Dil-S**	ELISA Diluent S	Aqueous buffered solution with detergent and interference blocker.	1 x 45 mL
Green	**Det-S**	Detector Ab S	Biotin labelled anti NF-L monoclonal antibody in aqueous buffered biotin-free stabilizing solution.	1 x 450 µL
Green	**DetDil-S**	Detector Ab Diluent S	Aqueous buffered biotin free stabilizing solution.	1 x 12 mL
Purple	**Conj-S**	Conjugate S	Streptavidine Horseradish peroxidase conjugate in aqueous buffered biotin-free stabilizing solution. Dilute according to label.	1 x 800 µL
Purple	**ConjDil-S**	Conjugate Diluent S	Aqueous buffered biotin free stabilizing solution.	1 x 12 mL
White	Wash-S	Wash Buffer S	10x Aqueous buffered solution with detergent.	2 x 40 mL
Black	TMB-S	TMB Substrate S	Tetramethylbenzidine substrate	1 x 12 mL
Black	Stop-S	Stop Solution S	Diluted H_2SO_4 (8% v/v)	1 x 6 mL
Grey	**Pos Ctrl-S**	Human recombinant NF-L Positive Control S	Recombinant Human NF-L, to be reconstituted according to the vial label.	2 vials

Additional material provided:
15 mL tube for conjugate dilution, 2 pcs

Not included essential equipment:
Microtiter plate reader 450 nm (reference wavelength in the range of 620 - 650 nm)
Micropipettes 10-1000 µL
Vortex mixer
Orbital ELISA tabletop shaker ***(800 rpm)***
Deionized water
Wash bottle, automated or semi-automated microtiter plate wash system
Pipette tips and timer
Polystyrene or polypropylene tubes for calibrator and sample dilution

10. Assay procedure

Preparations:

- All assay reagents should be brought to room temperature prior to use.
- **Preparation of Wash buffer S, 1x;** Dilute the total content of one Wash Buffer S (Wash-S) bottle with deinonized water to a final volume of 400 mL. Diluted, unused Wash buffer S, 1x can be stored at room temperature and should be used within two months. The Wash-S 10x can appear opalescent due to high salt concentration (no effect on assay performance).

Preparation of Positive Control S:
Reconstitute the lyophilized Positive Control S (**Pos Ctrl-S**) with the volume indicated on the vial, using ELISA Diluent S (**ELISA-Dil-S**). Positive Control S should not be diluted further.

Preparation of Calibrator dilution series (calibrators):
Reconstitution and preparation of calibrator dilution series should be performed directly before use.
Label eight micro-tubes, one for each calibrator point (that is 40 pg/mL, 25 pg/mL, 12.5 pg/mL, 5 pg/mL, 2.5 pg/mL, 1 pg/mL, 0.5 pg/mL) and one for the zero calibrator. The lyophilized NF-L Calibrator S (**NFL-Cal-S**) should be reconstituted with the volume of ELISA Diluent S (**ELISA-Dil-S**) indicated on the vial label.

Make a dilution series as described below.

Tube	Concentration, pg/mL	ELISA Diluent S, µL	Calibrator from tube, µL
NFL-Cal-S Vial	500	Reconstitute using ELISA Diluent S according to the calibrator vial label	
A	40	920	80 (vial)
B	25	950	50 (vial)
C	12.5	550	250 (A)
D	5	700	100 (A)
E	2.5	450	50 (B)
F	1	480	20 (B)
G	0.5	980	20 (B)
H	0	500	-

Detailed Assay protocol:

1. The Calibrators reconstituted and diluted according to the Calibrator dilution table are ready to use (i.e. no further dilution should be made).

2. Dilute the serum samples a minimum of 4 times (1+3) with ELISA Diluent S (**ELISA-Dil-S**) to a total minimum volume of 420 µL.

3. Wash the wells to be used with Wash buffer S (1X), (3x300 µL). Washing could be performed either by an automated washer or by manual pipetting. If washing manually, make sure to remove excess wash buffer between each wash by tapping the plate against absorbent paper. Remaining wash buffer and/or insufficient washing could affect the reactivity of the subsequent reagent.

4. Add **200 µL of each Calibrator (A-G),** Pos-Ctrl-S**,** serum **sample** and zero calibrator (**H**, **ELISA-Dil-S**) to each well. Duplicates are recommended.
   → Incubate 2 hours at RT with agitation (800 rpm).

5. Wash the wells with Wash buffer S (1X), (3x300 µL), see point 3.

6. Directly before use, dilute the Detector Ab S (**Det-S**) 40 times (1+39) to a 1X working concentration using Detector Ab Diluent S (**DetDil-S**). Mix thoroughly by inverting the tube or by vortexing.
   → Add **100 µL** of freshly diluted **Detector Ab S** to each well.

→ Incubate 90 minutes at RT with agitation (800 rpm).

7. Wash the wells with Wash buffer S (1X), (3x300 µL), see point 3.

8. **Use a supplied 15 mL tube to prepare the conjugate solution:**
   → Directly before use, dilute the Conjugate S (**Conj-S**) to a 1X working concentration according to the vial label using Conjugate Diluent S (**ConjDil-S**). Mix thoroughly by inverting the tube or by vortexing.
   → Add **100 µL** of newly diluted **Conjugate S** to each well
   → Incubate 30 minutes at RT with agitation (800 rpm).

9. Wash the wells with Wash buffer S (1X), (**6x300 µL**).

10. Add **100 µL of TMB Substrate S** (**TMB-S**) to each well.
    → Incubate 15 minutes at RT with agitation (800 rpm).
    *The plate does **not** need to be protected from light.*

11. Add **50 µL of Stop Solution S** (**Stop-S**) to each well and read the absorbance at 450 nm (reference wavelength 620-650 nm).
    ⚠ ***The Stop Solution S contains diluted sulfuric acid and is corrosive*.**

Assay overview:

<table>
<tr><td></td><td colspan="4">Washing 3 x 300 µL</td></tr>
<tr><td rowspan="2">Samples, Calibrators and Controls</td><td>Serum sample 1+3 dilution</td><td>Calibrators A-G</td><td>Zero calibrator (ELISA-Dil-S)</td><td>Pos-Ctrl-S, directly from vial</td></tr>
<tr><td>200 µL</td><td>200 µL</td><td>200 µL</td><td>200 µL</td></tr>
<tr><td rowspan="2"></td><td colspan="4">Incubation 2 hours, 800 rpm</td></tr>
<tr><td colspan="4">Washing 3 x 300 µL</td></tr>
<tr><td>Detector Ab S, working solution</td><td colspan="4">100 µL</td></tr>
<tr><td rowspan="2"></td><td colspan="4">Incubation 90 minutes, 800 rpm</td></tr>
<tr><td colspan="4">Washing 3 x 300 µL</td></tr>
<tr><td>Conjugate S, working solution</td><td colspan="4">100 µL</td></tr>
<tr><td rowspan="2"></td><td colspan="4">Incubation 30 minutes, 800 rpm</td></tr>
<tr><td colspan="4">Washing 6 x 300 µL</td></tr>
<tr><td>TMB Substrate S</td><td colspan="4">100 µL</td></tr>
<tr><td></td><td colspan="4">Incubation 15 minutes, 800 rpm</td></tr>
<tr><td>Stop Solution S</td><td colspan="4">50 µL</td></tr>
<tr><td colspan="5">Read the plate at 450 nm (using reference wavelength 620-650 nm) directly after adding the Stop Solution S</td></tr>
</table>

11. Calculation of results

The results can be calculated automatically by using an immunoassay software package.
A $1/y^2$-weighted 4-parameter logistic curve (4PL) provides the best curve fit (see a typical calibrator curve below).
The zero calibrator absorbance should neither be included in the calibrator curve, nor should it be subtracted from the measurement data.
If a nonlinear regression software tool is unavailable, a linear regression model in, e.g., Excel, may be applied. *Please note that such a model will not be as accurate in the lower end of the curve as a 4PL model.*
For linear regression, plot a lin-log calibrator curve from the absorbance (y-axis) and the concentration (x-axis). Add a linear trendline and use the trendline equation to manually calculate the concentration of your samples from their respective absorbance.

For all calculations, please note the following:

→ The zero calibrator absorbance should not be subtracted from the measurement data. It should be used only as an indicator of the background levels which should be compared to expected values on the Assay CoA.

→ The concentration calculations should be based on *difference data*, i.e., the absorbance data where the absorbance of the reference wavelength have been subtracted from the λ450 absorbance.

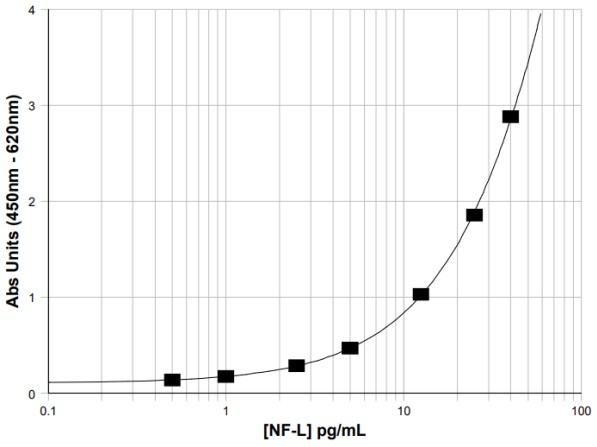
→ **The sample read-outs from the curve must be multiplied by the dilution factor of the sample in order to obtain the concentration in the original sample**.

→ The quantification interval of the curve is between 1 pg/mL and 25 pg/mL. Samples that fall above this interval should be diluted accordingly and re-analyzed. Samples that fall below this interval are too low to be accurately quantified by this method. See section 13 for details.

12. Quality control

In order to confirm proper assay performance, the following criteria should be fulfilled. Note that absorbances refer to *difference data* (λ450 minus the reference absorbance);

- The curve should have an appearance as shown in the figure below.
- The maximum absorbance for 40 pg/mL should be > 2.0 Absorbance units (AU).
- The background (the average zero calibrator absorbance of at least 2 replicates) should be <0.2 AU.
- The relative absorbance of the calibrator points should be approximately according to the table below.
- The control sample (**Pos Ctrl-S**) should fall within the acceptance interval stated on the CoA.



Calibrator (pg/mL)	Relative Absorbance (AU)	CV (%)
40	100.0 %	0.0
25	64.9 %	3.3
12.5	36.2 %	4.3
5	16.4 %	5.0
2.5	10.3 %	5.9
1	6.0 %	8.0
0.5	4.5 %	9.6

Typical calibrator curve. The Difference data (λ450 minus the reference absorbance) is plotted against the calibrator concentration (pg/mL). Expected relative absorbance of each calibrator point in the table to the right.

13. Measuring range

The calibrator curve covers the interval 0.5 pg/mL to 40 pg/mL NF-L. Quantification of NF-L should be done in the interval between calibrator points 1.0 pg/mL and 25 pg/mL for highest accuracy. For a 4-time diluted sample, that is

a functional quantification range of 4 pg/mL to 100 pg/mL. Extrapolation beyond the curve is not allowed with the implication that samples above the highest calibrator point must be further diluted and re-measured.

14. Additional dilution of samples

Diluted samples containing NF-L concentrations above the 25 pg/mL calibrator point should be further diluted and re-assayed for higher accuracy. The concentration read from the curve needs to be multiplied by the final dilution factor of the sample to obtain the concentration in the sample.

15. Assay limitations

Potential interference from heterophilic antibodies might cause erroneous results. Other factors that may affect the serum matrix of a sample and thus the result of the assay are, for example, regular exposure to animals, immunotherapy or other procedures utilizing immunoglobulins or immunoglobulin fragments and biotin therapy. Carefully evaluate research results if the sample donors are suspected of having these types of interferences.

16. Performance data

Specificity
The anti-NF-L antibodies used in this assay have been used extensively in other methods. It is the antibody-pair in the NF-light® ELISA (art.no 10-7001) for CSF samples which has been available for many years without any reports about unspecific binding. The same antibodies are also used in the NF-L quantification assays provided by Quanterix (articles 102153, 102258 and 103186).

Analytical Sensitivity
Lowest Limit of Detection (LLoD) = 0.4 pg/mL
Lowest Limit of Quantitation (LLoQ) = 0.8 pg/mL
The calculations were based on the method described in the NCCLS definition EP17-4.

Precision
For intra-precision: Four serum samples ranging from 5 – 125 pg/mL were analysed in six replicates at three different occasions. The CV (%) for each sample was calculated for each separate occasion (n=6) and the intra-precision is reported as the mean CV (%) of its respective concentration range.
For inter-precision: 10 serum samples ranging from 10 – 130 pg/mL were analysed in duplicate on nine or ten separate occasions. The CV (%) for each sample was calculated (n= 9 or 10) and the inter-precision was reported as the mean CV (%) of its respective concentration range.

NF-L level	Intra-assay precision
5-10 pg/mL	6.6 % (4.2-11.1)
10-125 pg/mL	4.3 % (2.9-5.9)

NF-L level	Inter-assay precision
10-15 pg/mL	10 % (8.7-11.2)
15-130 pg/mL	5.7 % (3.9-6.8)

Recovery
The recovery interval tested for human recombinant NF-L was 2 to 18 pg/mL. For recovery testing of spiking with endogenous human NF-L, the interval was 7 to 14 pg/mL.

NF-L origin	Lowest recovery	Highest recovery	Average recovery
Recombinant NF-L	47.5%	90.6%	79.9%
Endogenous NF-L	77.9%	95.5%	90.2%

Dilution linearity
One hundred nine serum samples were spiked with endogenous NF-L and serially diluted 4 – 64 times. The read-out (pg/mL) from each measurement was multiplied by its respective dilution factor. Linearity was calculated for each measurement by relating it to the respective mean of all dilutions considered to be within a linear interval. Measurements within 80 – 120% of this mean were considered linear.

Percentage of linear and nonlinear measurements			
Dilution factor	**<80% (nonlinear)**	**80 – 120% (linear)**	**>120% (nonlinear)**
4x	17.4	82.6	0.0
8x	4.6	95.4	0.0
32x	0.0	100.0	0.0
64x	0.0	100.0	0.0

Parallelism

Eight serum samples with high endogenous levels of NF-L were 2-fold serially diluted, three times, from an original 4-time dilution to a 32-time dilution. Each measurement was multiplied by its respective dilution factor and the CV (%) for each sample was calculated (n=4). Parallelism was defined as sample CV:s below 20 %.

Parallelism (CV < 20 %)	Mean = 9.2 % Range: 6.3 – 13.5 %

Accuracy

There is no available reference material for NF-L as of yet. The accuracy has instead been determined by comparing with another commercially available method, the NF-light™ Advantage Simoa® Assay provided by Quanterix. 45 identical samples were analysed twice by the NF-light™ Serum ELISA and by the Simoa® assay. Correlation was evaluated. The R^2 value was 0.90, with the NF-light™ Serum ELISA method having a mean of 27% higher quantified NF-L concentration than the Simoa® Assay.

17. Warranty

The performance data presented here was obtained using the procedure indicated. Any change or modification in the procedure not recommended by UmanDiagnostics AB may affect the results, in which event UmanDiagnostics AB disclaims all warranties, expressed, implied or statutory, including the implied warranty of merchantability and fitness for use. UmanDiagnostics AB and its authorized distributors, in such event, shall not be liable for any damages, whether direct, indirect or consequential.

18. Bibliography

1. Yuan A., et al., *Neurofilaments and Neurofilament Proteins in Health and Disease.* Cold Spring Harb Perspect Biol, 2017. **9**(4).
2. Gaetani L., et al., *Neurofilament light chain as biomarker in neurological disorders.* J Neurol Neurosurg Psychiatry, 2019;90:870-881.
3. Norgren N., et al., *Monoclonal antibodies selective for low molecular weight neurofilaments.* Hybrid Hybridomics. 2002 Feb; 21(1): 53-9.

19. Symbols used

REF	Cat.-No.:
	Use by:
LOT	Lot-No.:
	No. of Tests:
	Read instructions before use.
	Keep away from heat or direct sunlight.
	Store at:
	Manufacturer:
	Country of manufacture:
	Caution!

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