MANUAL

Technology: AlphaLISA™

AlphaLISA Human Neurofilament L (NF-L) Detection Kit

Part number:	**AL3199HV**	**AL3199C**	**AL3199F**
Assay points:	100	500	5,000

Storage: Store kit in the dark at 4 °C. For reconstituted analyte, aliquot and store at -80 °C. Avoid freeze-thaw cycles.

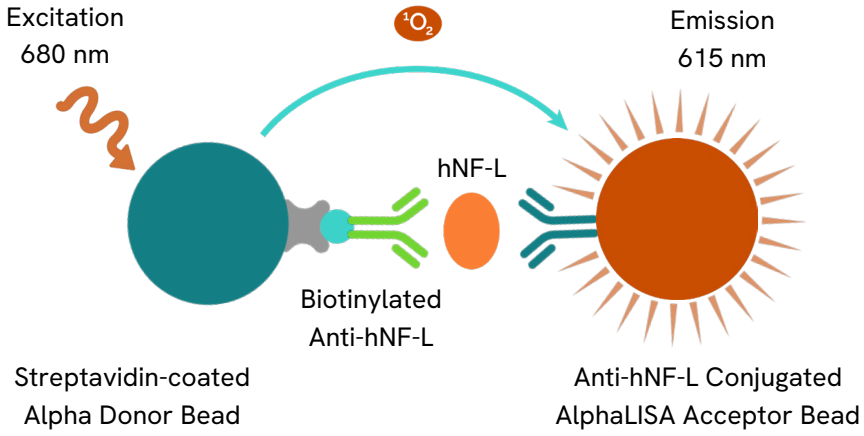
Version: 1 **Date:** October 2024

ANALYTE OF INTEREST

Neurofilament Light chain protein, also known as Neurofilament Light polypeptide, NF-L, NFL or NEFL is a member of intermediate filament protein family, selectively expressed in neurons. NF-L (68 kDa) associated with neurofilament-heavy chain (NF-H, 200 kDa), medium chain (NF-M, 125 kDa) and alpha-internexin, forms the structure of axons. Depending on conditions, different levels of NF-L are released during brain development, maturation, aging and in numerous neurodegenerative diseases proportionally to the degree of axonal damage. Many studies have demonstrated that NF-L can be used as a biomarker for disease monitoring and more broadly as a neuronal damage marker but also to check the differentiation into neurons in IPSc models for example.

DESCRIPTION OF THE ALPHALISA ASSAY

AlphaLISA technology allows the detection of molecules of interest in cell culture supernatant in a highly sensitive, quantitative, reproducible, and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-hNF-L antibody binds to the streptavidin coated AlphaLISA Donor beads, while an anti-hNF-L antibody is conjugated to AlphaLISA Acceptor beads. In the presence of hNF-L, the beads come into proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in emission with λ_{max} at 615 nm.



PRECAUTIONS

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The biotinylated anti-hNF-L antibody contains sodium azide. Contact with skin or inhalation should be avoided.

KIT CONTENT: REAGENTS AND MATERIALS

Kit components	AL3199HV***	AL3199C****	AL3199F****
AlphaLISA hNF-L Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, pH 7.2	12 µL @ 5 mg/mL (1 brown tube, white cap)	25 µL @ 5 mg/mL (1 brown tube, white cap)	250 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP II, pH 7.4	80 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 x 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Anti-hNF-L Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Lyophilized Recombinant hNF-L*	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X)	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Reconstitute lyophilized analyte in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped 0.5 mL polypropylene vials and stored at -80 °C for future experiments. Refer to the product CoA for stability information on the reconstituted analyte stored at -80°C Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3199S).

** Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates.

**** The number of assay points is based on an assay volume of 50 µL in 384-well assay plates using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001% final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the biotinylated anti-hNF-L antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Additional reagents and materials:

The following materials are recommended but not provided in the kit:

Item	Suggested source
Light gray AlphaPlate™-384	Revvity Inc.
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.
EnVision®-Alpha Reader	Revvity Inc.

RECOMMENDATONS

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000*g*, 10-15 sec).
- Re-suspend the Donor and Acceptor beads by vortexing before use.
- Use Milli-Q® grade H_2O to dilute buffers and to reconstitute the lyophilized analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Film to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multimode Plate Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate. It is recommended to avoid multiple reads of the same well of the assay plate.
- The standard curves shown in this manual are provided for information only. A standard curve must be generated for each experiment.

ASSAY PROCEDURE

- The protocol described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The protocols also include testing samples in 452 wells. If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The standard dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

Format	# of data points	Volumes for 2-Step Protocol				Plate recommendation*
		Final	Sample	AlphaLISA Acceptor Beads + biotinylated Ab Mix	SA-Donor beads	
AL3199HV	**100**	**100 µL**	**10 µL**	**40 µL**	**50 µL**	**White OptiPlate-96 White ½ AreaPlate-96**
AL3199C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96
	500	**50 µL**	**5 µL**	**20 µL**	**25 µL**	**½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate™-384**
	1 250	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 ProxiPlate™-384 Plus White OptiPlate-384
	2 500	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536
AL3199F	**5 000**	**50 µL**	**5 µL**	**20 µL**	**25 µL**	**½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate-384**
	12 500	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 ProxiPlate-384 Plus White OptiPlate-384
	25 000	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536

***Light gray AlphaPlates were specifically designed for use with AlphaLISA assays and are strongly recommended for best assay performance**.

The 2-Step protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly*.

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL Milli-Q® grade H_2O.

2) Preparation of hNF-L analyte standard dilutions:

   a. Reconstitute lyophilized hNF-L (1 µg) in 100 µL Milli-Q® grade H_2O. The remaining reconstituted analyte should be aliquoted immediately and stored at -80 °C for future assays (see page 2 for more details).
   b. A standard curve must be generated for each experiment. The standard curve should be performed in a similar matrix diluent as the samples (e.g. cell culture media for cell supernatant samples, FBS for serum samples). Use of the 1X AlphaLISA Immunoassay Buffer is recommended as a diluent to confirm assay performance.
   c. Prepare standard dilutions as follows (change tip between each standard dilution).

Tube	Vol. of hNF-L (µL)	Vol. of diluent (µL)*	[hNF-L] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted hNF-L	90	1.00E-06	1 000 000
B	60 µL of tube A	140	3.00E-07	300 000
C	60 µL of tube B	120	1.00E-07	100 000
D	60 µL of tube C	140	3.00E-08	30 000
E	60 µL of tube D	120	1.00E-08	10 000
F	60 µL of tube E	140	3.00E-09	3 000
G	60 µL of tube F	120	1.00E-09	1 000
H	60 µL of tube G	140	3.00E-10	300
I	60 µL of tube H	120	1.00E-10	100
J	60 µL of tube I	140	3.00E-11	30
K	60 µL of tube J	120	1.00E-11	10
L	60 µL of tube K	140	3.00E-12	3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Dilute standards in appropriate diluent (e.g. 1X AlphaLISA Immunoassay Buffer).

At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL/LLOQ is calculated. If LDL/LLOQ does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 2.5X MIX AlphaLISA Anti-hNF-L Acceptor beads (12.5 µg/mL) + Biotinylated Anti-hNF-L Antibody (2.5 nM):

   a. Prepare just before use.
   b. Add 25 µL of 5 mg/mL AlphaLISA Anti-hNF-L Acceptor Bead and 50 µL of 500 nM Biotinylated Anti-hNF-L Antibody to 9 925 µL of 1X AlphaLISA Immunoassay Buffer.

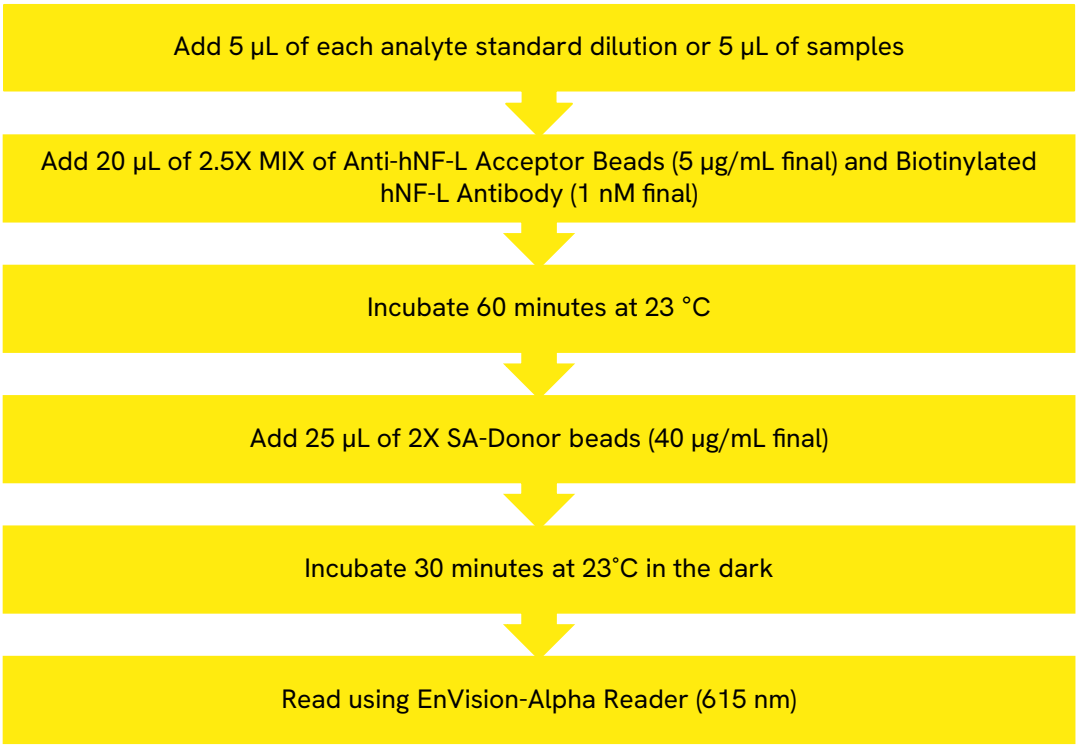
4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):

   a. Prepare just before use.

   b. Keep the beads under subdued laboratory lighting.

   c. Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA Immunoassay Buffer.

5) In a light gray AlphaPlate (384 wells):



DATA ANALYSIS

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + [3xSD]) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + [10xSD]) on the

standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).

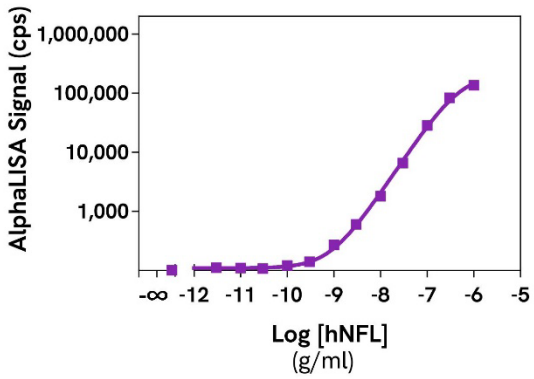
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

ASSAY PERFORMANCE CHARACTERISTICS

AlphaLISA assay performance described below was determined by using the kit protocol using 1X AlphaLISA Immunoassay Buffer as an assay buffer. The analytes (standards) were prepared in different matrix diluents depending on sample type. All other components were prepared in 1X AlphaLISA Immunoassay Buffer.

Standard curve:

A typical sensitivity curve is shown below, using the protocol described on page 4, using 1X AlphaLISA Immunoassay Buffer to dilute the standard.



Assay sensitivity:

The LDL was calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + [3xSD]) on the standard curve. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL of sample using the recommended assay conditions.

The LLOQ was calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + [10xSD]) on the standard curve. The values correspond to the lowest concentration of analyte that can be accurately quantified in a volume of 5 µL of sample using the recommended assay conditions.

Analyte diluent	LDL (pg/mL)	LLOQ (pg/mL)
AlphaLISA Immunoassay buffer 1X	120.5	356.6
DMEM+10% FBS	60.2	197.8
RPMI+10% FBS	71.7	238.9
Neurobasal medium containing N2	84.4	262.9
KO-DMEM/F12 medium + 1% B-27	118.4	371.9

Assay precision:

Samples (SH-SY5Y neuroblastoma cell supernatant) containing different concentrations of analyte were prepared in DMEM+10%FBS. All other components were prepared in AlphaLISA Immunoassay Buffer. The assays were performed in a 384-well plate format.

Intra-assay precision:

The intra-assay precision was determined using a total of 24 replicates per sample in one assay. Shown as CV% of measured concentration.

hNF-L CONCENTRATION	DMEM + 10% FBS
Sample A, 28944 pg/mL	12.2%
Sample B, 15517 pg/mL	5.4%
Sample C, 3180 pg/mL	5.5%

Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with three measurements per sample in each assay. Shown as CV% as measured concentration.

hNF-L	DMEM+10%FBS
Sample A, 18144 pg/mL	8.7%
Sample B, 9827 pg/mL	9.2%
Sample C, 2556 pg/mL	8.4%

Dilution linearity

A cell supernatant collected from SH-SY5Y neuroblastoma cells and containing a known concentration of analyte was serially diluted in DMEM + 10% FBS. The assay was performed on serially diluted samples, along with a standard curve prepared in the same cell culture medium. Concentrations of hNF-L in samples were determined by interpolating from the standard curve. The other components of the assays (anti-hNF-L Acceptor beads, biotinylated anti-hNF-L antibody, and SA-Donor beads) were prepared in 1X AlphaLISA Immunoassay Buffer.

Excellent dilution linearity (R^2 > 0.99, slope = 1.0) and dilution recovery were achieved in the diluted samples **with a dilution ≥ 2 fold necessarily** (in the range of 2- down to 6-fold dilution). The results are shown in the table below.

Sample dilution factor (2)	Expected hNF-L (pg/mL)	Observed hNF-L (pg/mL)	Dilution Recovery (%)
1/2	2826	2825.9	-
1/4	1412.9	1361.9	96%
1/8	706.5	675.2	96%
1/16	353.2	296.2	84%
1/32	176.6	163.5	93%
1/64	88.3	97.0	110%

Specificity:

Cross-reactivity of the AlphaLISA Human NF-L Detection Kit was tested using recombinant Human Neurofilament Medium and Heavy Chain as analytes or mouse neuroblast Neuro-2a cell supernatant in an assay using the protocol as described above. The cross reactivities were established using the related proteins below at a test concentration of 3 to 1 000 000 pg/mL or pure cell supernatant from T175 flask at 80% confluence. Percentage recovery was computed by comparing the measured interpolated concentration versus the theoretical one.

Proteins	Cross reactivity
Human NF-L	100 %
Human NF-M	0 %
Human NF-H	0 %
Mouse NF-L	0 %

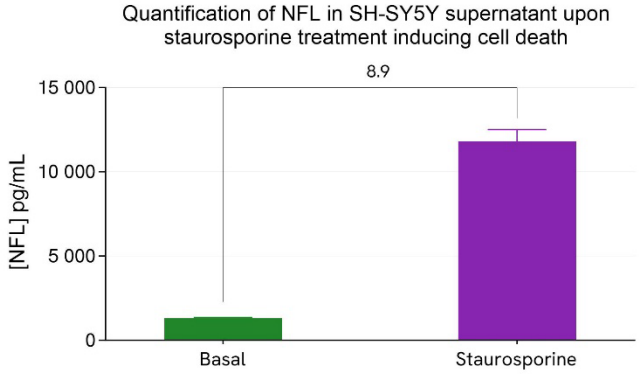
Cell experiments:

Cell culture conditions

The human neuroblastoma cell line SH-SY5Y was plated in a 96-well plate (25,000 cells/well). The day after, cells were treated overnight with 50 µL of Staurosporine (0.5 µM) diluted in complete culture medium compared to control.

Pharmacological treatment

Cell supernatants were collected for the quantitative measurement of secreted NF-L levels. The NF-L concentration in the sample was estimated using the AlphaLISA Human NF-L Detection Kit. As expected, Staurosporine treatment resulted to cellular death, mimicking neurodegeneration, increasing NF-L release into the supernatant compared to basal condition.



Spike recovery

Three known amounts of hNF-L were spiked into three SH-SY5Y supernatant 2330 -110925 and 783161 pg/mL). The spiked samples were diluted to 2- fold, using cell culture medium, resulting in 19676 -9562 and 5218 pg/mL in 2x diluted supernatant samples. The samples were assayed along with the standard prepared in cell culture medium. Other components (anti-hNF-L Acceptor beads, biotinylated anti-hNF-L antibody, and SA-Donor beads) of the assays were prepared in AlphaLISA Immunoassay Buffer. The spike recoveries of hNF-L were determined. The results shown in the table below indicate that excellent recoveries were achieved for all three spikes tested.

[hNF-L]	Diluent: Cell Culture medium	
	Spiked sample (SH-SY5Y supernatant)	
Spike (pg/mL)	Concentration (pg/mL)	Recovery (%)
No spike	**19676**	N/A
2330	21016	96%
110925	125936	96%
783161	793628	99%
No spike	**9562**	N/A
2330	12076	102%
110925	113659	95%
783161	770292	97%
No spike	**5218**	N/A
2330	7315	97%
110925	110742	95%
783161	787334	100%

TROUBLESHOOTING

To find detailed recommendations for common situations you might encounter with your AlphaLISA assay kit, please visit our website at www.revvity.com.

www.revvity.com

revvity

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