AlphaLISA Human Frataxin Detection Kit

Product number: AL322HV/C/F

Caution: For Laboratory Use. A research product for research purposes only.

Contents

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Product Information

Application: This kit is designed for the quantitative determination of human Frataxin (hFXN) in cell and tissue lysates using a homogeneous AlphaLISA assay (no wash steps). The assay has shown reactivity with full length (1-210), intermediate (42-210), and the mature form (81-210) of Frataxin.

Sensitivity: Lower Detection Limit (LDL): 8 pg/mL

Lower Limit of Quantification (LLOQ): 30 pg/mL

EC_{50}: 139 ± 30 ng/mL

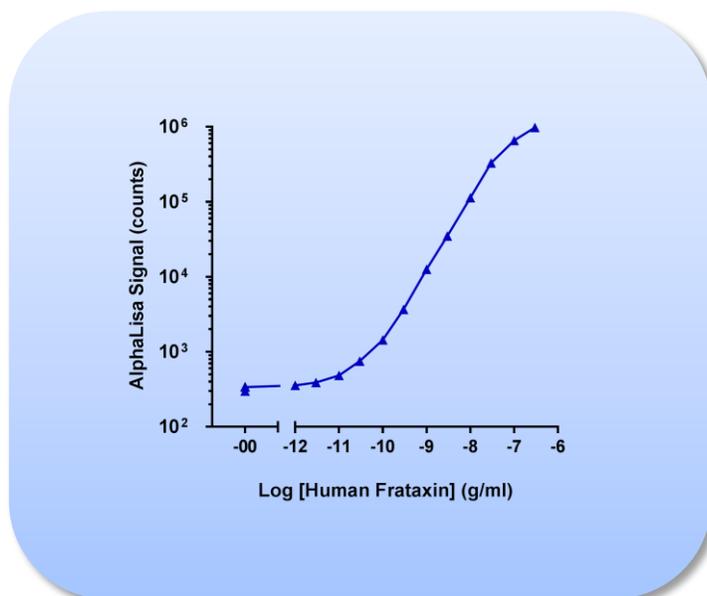**Dynamic range:** 8 – 300,000 pg/mL (Figure 1).

Figure1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate[TM] -384 microplate and the EnVision[®] Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4°C. Store reconstituted analyte at -20°C.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the hFXN analyte is stable for at least 6 months when stored at -20°C.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals, EC_{50} and LDL were measured on the EnVision Multilabel Plate Reader with Alpha option using the protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum counts may vary between bead lots and the instrument used, with no impact on LDL measurement.

Analyte of Interest

Frataxin is expressed as a 210 AA, 23 kDA protein from the FXN gene located on chromosome 9. Upon expression, the FXN protein is directed to the mitochondrion by its 41 AA N-terminal mitochondrial targeting sequence. In the mitochondrion, the protein is cleaved by the mitochondrial processing peptidase (MPP) to its intermediate form of 42 – 210 AA. Later on, MPP cleaves the protein to its mature form of 81-210 AA. Although the function of the FXN protein is not clearly defined, it is thought to be vitally important for Iron-Sulfur cluster biogenesis, heme biosynthesis, and chelation and transportation of iron specifically involved with the mitochondria. The Frataxin protein is the primary culprit for a debilitating neurodegenerative disease called Friedreich's Ataxia. Due to similarities in the diseases, there is some evidence to suggest that FXN may be involved in other neurodegenerative diseases such as Parkinson's, Multiple Sclerosis, and Amyotrophic Lateral Sclerosis. This AlphaLISA kit has been designed for the detection and quantification of FXN from cell and tissue lysates.

Description of the AlphaLISA Assay

AlphaLISA technology allows for the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfers in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2). Combining this assay with an AlphaPLEX 645- or AlphaPLEX 545 - based kit will allow the quantification of 2 (or more) analytes in the same well.
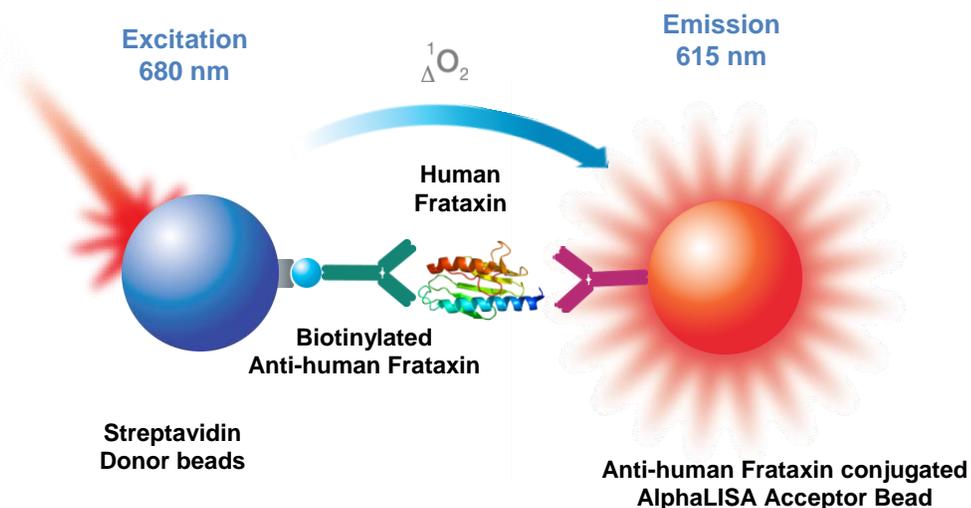

Figure 2. AlphaLISA Assay Principle.

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 (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.

- All blood components and biological materials should be handled as potentially hazardous.

- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.

- The Biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL322HV (100 assay points***)	AL322C (500 assay points***)	AL322F (5000 assay points***)
AlphaLISA Anti-hFXN Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)	50 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)	500 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	100 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	1000 µL @ 5 mg/mL (1 brown tubes, <u>black</u> caps)
Biotinylated Antibody Anti-hFXN stored in PBS, 0.1% Tween-20, 0.05% NaN$_3$, pH 7.4	30 µL @ 500 nM (1 tube, <u>black</u> cap)	75 µL @ 500 nM (1 tube, <u>black</u> cap)	750 µL @ 500 nM (1 tube, <u>black</u> cap)
AlphaLISA hFXN (0.3 µg), lyophilized analyte *	1 tube, <u>clear</u> cap	1 tube, <u>clear</u> cap	1 tube, <u>clear</u> cap
AlphaLISA Immunoassay Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute hFXN in 100 µL Milli-Q® grade H$_2$O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted hFXN is stable for at least 18 months at -20°C. One vial contains an amount of hFXN sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL322S).

** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100 and 0.5% Proclin-300. Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).
Note: 10X buffer might be slightly yellow. However, this does not affect the assay results.
Once diluted, 1X AlphaLISA Immunoassay Buffer contains 25 mM HEPES, pH 7.4, 0.1% Casein, 1 mg/mL Dextran-500, 0.5% Triton X-100 and 0.05% Proclin-300.

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL322HV) or 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 Antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	PerkinElmer Inc.	6050195
EnVision®-Alpha Reader	PerkinElmer Inc.	-

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Recommendations

- 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 (2000g, 10-15 sec). Re-suspend all reagents by vortexing before use.

- Use Milli-Q$^®$ grade H$_2$O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer 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 Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.

- The AlphaLISA signal is detected with an EnVision Multilabel 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.

- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment. The standard curve should be performed in the Immunoassay buffer for serum and/or plasma samples.

Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- 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 a different amount of samples are tested, the volumes of all reagents have to 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.

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Format	# of data points	Volume				Plate recommendation
		Final	Sample	AlphaLISA beads / Biotin Antibody MIX	Donor beads	
AL322HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL322C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	20 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL322F	5 000	50 µL	5 µL	20 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

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The protocol (2 incubation steps) described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells).
If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

Working with cell or tissue lysate samples – We recommend using AlphaLISA Lysis buffer for the extraction of the Frataxin protein. For testing samples, using a total protein sample of 40 μg generally yielded 0.5 ng/mL or greater recovery of Frataxin protein. Adding any additional components to the lysates (i.e. dyes) may require dilution of the sample for a more accurate recovery; generally a 4-10 fold dilution is sufficient when using a 5 μL sample.

Standard Preparation:

1) Preparation of 1X AlphaLISA Immunoassay Buffer:

- Add 3 mL of 10X AlphaLISA Immunoassay Buffer to 27 mL H_2O.

2) Preparation of hFXN analyte standard dilutions:

- hFXN analyte is provided at 0.3 μg in lyophilized form. Reconstitute with 100 μL H_2O to create a 3 μg/mL solution. Prepare standard dilutions as follows (change tip between each standard dilution):
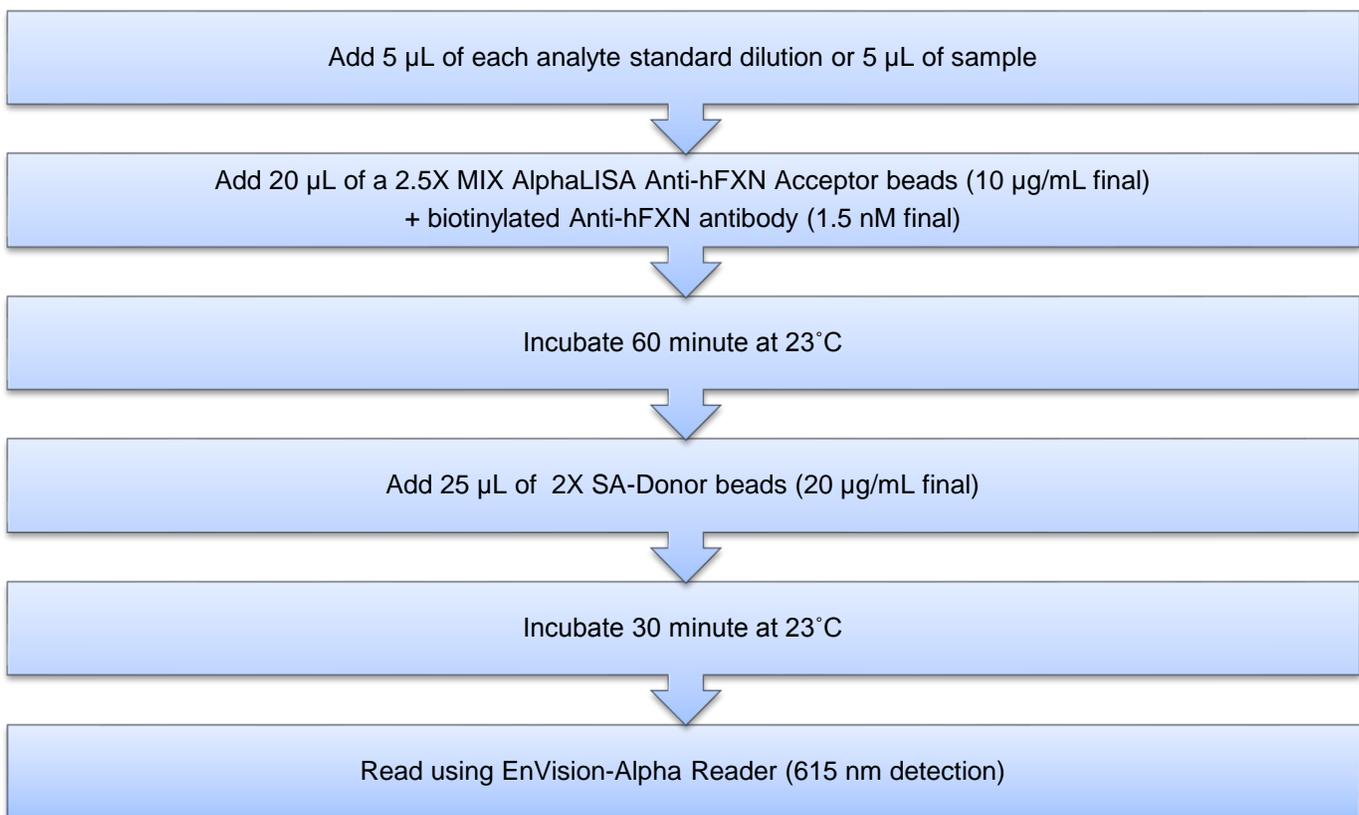
Tube	Vol. of hFXN (μL)	Vol. of diluent (μL) *	[hFXN] in standard curve		Final [hFXN] in well
			(g/mL in 5 μL)	(pg/mL in 5 μL)	(g/mL in 50 μL)
A	10 μL of provided hFXN	90	3.00E-07	300000	3.00E-08
B	60 μL of tube A	120	1.00E-07	100000	1.00E-08
C	60 μL of tube B	140	3.00E-08	30000	3.00E-09
D	60 μL of tube C	120	1.00E-08	10000	1.00E-09
E	60 μL of tube D	140	3.00E-09	3000	3.00E-10
F	60 μL of tube E	120	1.00E-09	1000	1.00E-10
G	60 μL of tube F	140	3.00E-10	300	3.00E-11
H	60 μL of tube G	120	1.00E-10	100	1.00E-11
I	60 μL of tube H	140	3.00E-11	30	3.00E-12
J	60 μL of tube I	120	1.00E-11	10	1.00E-12
K	60 μL of tube J	140	3.00E-12	3	3.00E-13
L	60 μL of tube K	120	1.00E-12	1	1.00E-13
M ** (background)	0	100	0	0	0
N ** (background)	0	100	0	0	0
O ** (background)	0	100	0	0	0
P ** (background)	0	100	0	0	0

*   Dilute standards in 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 is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

General Protocol (2 Steps):

1) Preparation of 2.5X MIX AlphaLISA Anti-hFXN Acceptor beads (25 µg/mL) + biotinylated Anti-hFXN Antibody (3.75 nM):
   a. Add 50 µL of 5 mg/mL AlphaLISA Anti-hFXN Acceptor beads and 75 µL of 500nM Anti-hFXN Antibody to 9875 µL of 1X AlphaLISA Immunoassay Buffer.
   b. Prepare just before use.

2) Preparation of 2X Streptavidin (SA) Donor beads (40 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 100 µL of 5 mg/mL SA-Donor beads to 12400 µL of 1X AlphaLISA Immunoassay Buffer.
   c. Prepare just before use.

3) In a white Optiplate (384 wells):

Add 5 µL of each analyte standard dilution or 5 µL of sample

Add 20 µL of a 2.5X MIX AlphaLISA Anti-hFXN Acceptor beads (10 µg/mL final)
+ biotinylated Anti-hFXN antibody (1.5 nM final)

Incubate 60 minute at 23˚C

Add 25 µL of 2X SA-Donor beads (20 µg/mL final)

Incubate 30 minute at 23˚C

Read using EnVision-Alpha Reader (615 nm detection)

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 using the 2 step protocol.

- Assay Sensitivity:

The LDL and LLOQ were calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL using the recommended assay conditions. The assays were performed in 384-well format using AlphaLISA Immunoassay Buffer (AIB).

LDL (pg/mL)	LLOQ (pg/mL)	Buffer	# of experiments
7.9	29.5	AIB	12

Note that LDL/ LLOQ can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 5 µL of analyte in a final assay volume of 50 µL).

- Assay Precision:

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the analytes were prepared in AlphaLISA Immunoassay Buffer (AIB). Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well format using AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 6 independent determinations in triplicate, shown as CV%.

hFXN	AIB
CV%	5.5

- nter-assay precision:

The inter-assay precision was determined using the data across 3 independent experiments with 16 measurements in triplicate. CV% was calculated by comparing the same measurement in each experiment. The CV% for all 16 measurements were then averaged. Shown is the inter-experimental CV%.

hFXN	AIB
CV%	10.1

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- Spike Recovery:

In four experiments, three known concentrations of hFXN were spiked into AIB or AlphaLISA Lysis Buffer (ALB) and performed triplicate. The spiked samples were referenced to the hFXN analyte curve produced in AlphaLISA Immunoassay Buffer.

Spiked hFXN (ng/mL)	% Recovery	
	AIB	ALB
30	110.7	112.8
3	127.2	88.4
0.3	113.3	96.9

- Specificity:

Cross-reactivity of the AlphaLISA hFXN Kit was tested using mouse frataxin protein at 30 ng/mL in AlphaLISA Immunoassay Buffer. Reactivity to hFXN is 100%.

Protein	% Cross-reactivity
Mouse Frataxin	9.1

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Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at:

http://www.perkinelmer.com/resources/technicalresources/applicationsupportknowledgebase/alphalisa-alphascreen-no-washassays/alpha_troubleshoot.xhtml

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