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Human Lactate Dehydrogenase A subunit (LDHA) AlphaLISA Detection Kit

Product No.: AL3077C/F

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Product Information

Application:	This kit is designed for the quantitative determination of human LDHA in serum, culture media and cell lysate using a homogeneous AlphaLISA assay (no wash steps).
Sensitivity:	Lower Detection Limit (LDL): 503 pg/mL Lower Limit of Quantification (LLOQ): 1685 pg/mL EC ₅₀ : 790 ng/mL
Dynamic range:	503 – 1 000 000 pg/mL

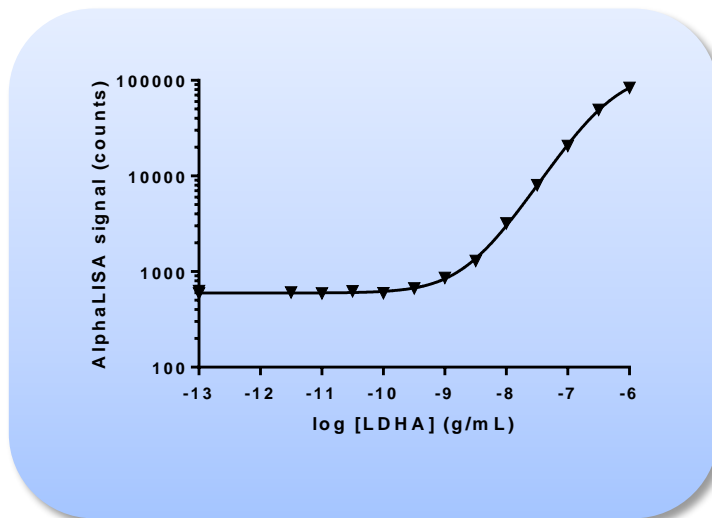


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage:	Store kit in the dark at 4 °C. For reconstituted analyte aliquot and store at -20 °C. Avoid freeze-thaw cycles.
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.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals, EC₅₀ 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:

Lactate Dehydrogenase A subunit (LDHA) is one subunit of lactate dehydrogenase, which is a tetramer of A and B subunits. Subunit A is mainly present in muscles, where it plays a key role in energy metabolism by catalyzing the oxidation of pyruvate into lactate to produce energy in absence of oxygen. The enzyme is ubiquitous in cells playing the same metabolic functions. Elevation of the enzyme levels in cells is a known marker of many cancers, while its elevation in cell culture media is indicative of cell death. This detection kit is designed to quantify LDHA in serum and cell lysates.

Description of the AlphaLISA Assay

AlphaLISA technology allows 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 this AlphaLISA assay, a Biotinylated Anti-LDHA Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-LDHA Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the LDHA, 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 transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2).

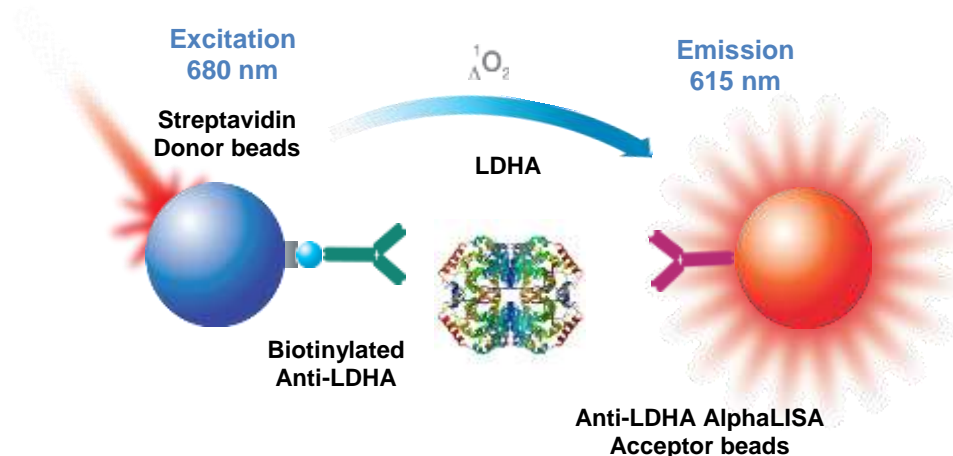


Figure 2. AlphaLISA Assay Principle.

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.
- 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	AL3077HV (100 assay points) ^{***}	AL3077C (500 assay points) ^{***}	AL3077 F (5000 assay points) ^{***}
AlphaLISA Anti-LDHA 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	80 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	200 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, <u>black</u> caps)
Biotinylated Anti-LDHA Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN ₃ , pH 7.4	20 µL @ 500 nM (1 tube, <u>black</u> cap)	50 µL @ 500 nM (1 tube, <u>black</u> cap)	500 µL @ 500 nM (1 tube, <u>black</u> cap)
Human LDHA Analyte * (10 µg/mL in PBS + 0.05% ProClin 300)	100 µL (1 tube, <u>clear</u> cap)	100 µL (1 tube, <u>clear</u> cap)	100 µL (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
AlphaLISA Lysis Buffer (5X)**	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Warm up analyte to room temperature. Remaining analyte after experiment should be aliquoted into screw-capped polypropylene vials and stored at -20 °C for future experiments. Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3077S).

** Extra buffer can be ordered separately (Immunoassay Buffer cat # AL000C: 10 mL, cat # AL000F: 100 mL; Lysis Buffer cat# AL003C: 10 mL, cat# AL003F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates or 50 µL in 96- or 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 Plus Adhesive Sealing Film	PerkinElmer Inc.	6050185
EnVision®-Alpha Reader	PerkinElmer Inc.	-

Recommendations

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 (2000g, 10-15 sec). Re-suspend all reagents by vortexing before use.
- Use Milli-Q[®] grade H₂O (18 MΩ·cm) to dilute 10X AlphaLISA Immunoassay Buffer 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 Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multilabel 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.
- The standard curves shown in this technical data sheet 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 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.

Format	# of data points	Volume				Plate recommendation
		Final	Sample	MIX AlphaLISA AccBeads + biotinylated Ab	SA-Donor beads	
AL3077HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3077C	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)
AL3077F	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)

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

- 1) Preparation of 1X AlphaLISA Immunoassay Buffer:
Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL Milli-Q[®] grade H₂O.

Preparation of 1X AlphaLISA Lysis Buffer:
Add 5 mL of 5X AlphaLISA Lysis Buffer to 20 mL of Milli-Q[®] grade H₂O.

- 2) Preparation of LDHA analyte standard dilutions:
- Allow analyte solution to warm to room temperature
 - Prepare standard dilutions as follows in **1X AlphaLISA Lysis Buffer** (change tip between each standard dilution):

Tube	Vol. of LDHA (µL)	Vol. of diluent (µL) *	[LDHA] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of stock LDHA	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 diluent (e.g. **1X AlphaLISA Lysis 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).

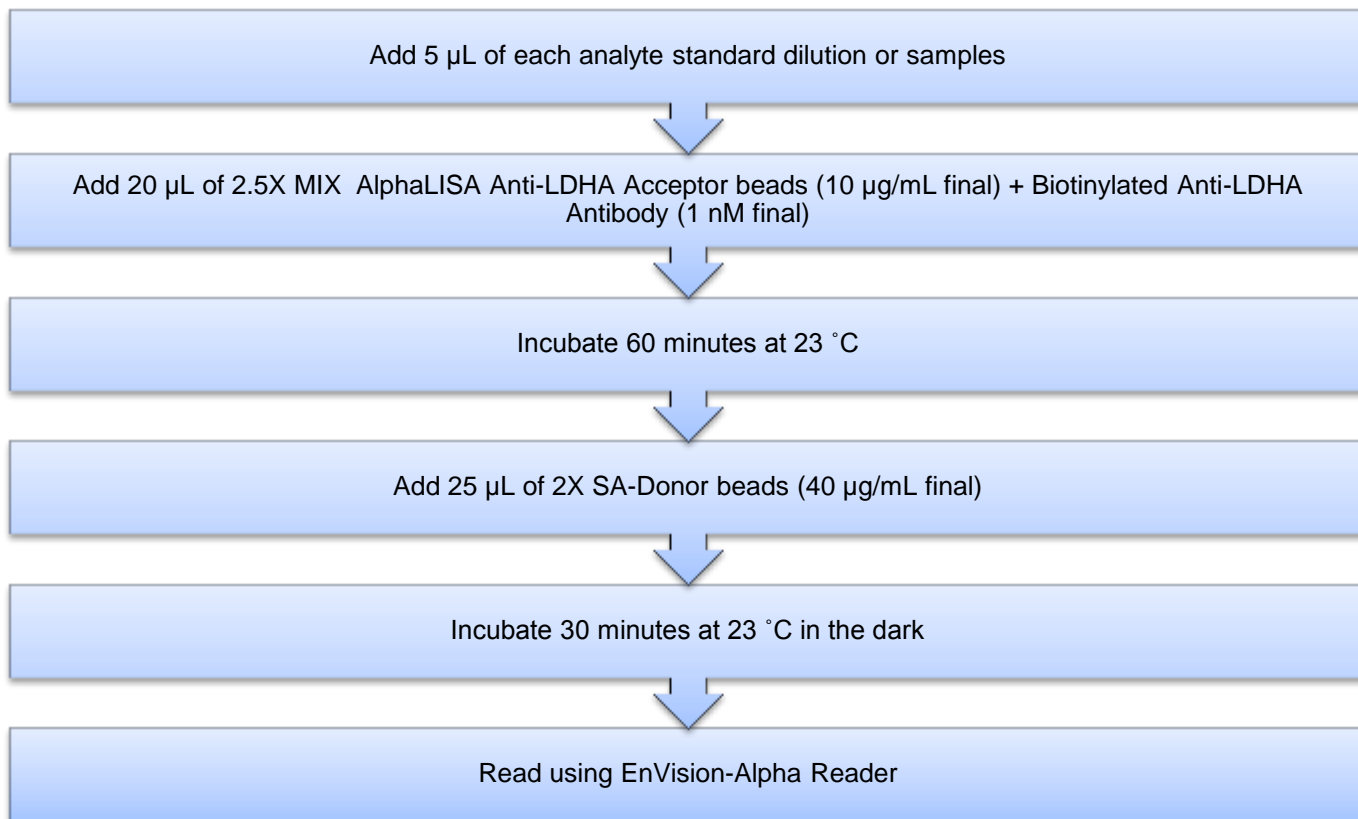
- 3) Preparation of 2.5X MIX Anti-LDHA AlphaLISA Acceptor beads (25 µg/mL) + biotinylated Anti-LDHA antibody (2.5 nM):

- Prepare just before use.
- Add 50 µL of 5 mg/mL Anti-LDHA Acceptor beads and 50 µL of 500 nM biotinylated Anti-LDHA antibody to 9 900 µL of 1X AlphaLISA Immunoassay Buffer

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

- Prepare just before use.
- Keep the beads under subdued laboratory lighting.
- Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA Immunoassay Buffer.

- 5) In a white Optiplate (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 using the 2 step protocol using AlphaLISA Immunoassay Buffer (IAB) as assay buffer. The analytes (standards) were prepared in AlphaLISA Lysis Buffer, Lysis Buffer + 10% FBS or 100% FBS, and all other components were prepared in IAB.

- Assay Sensitivity:

The LDL was calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 μ L sample using the recommended assay conditions.

LDL (pg/mL)*	(Analyte diluent)	# of experiments
500	AlphaLISA Lysis Buffer	6
3285	Lysis Buffer + 10% FBS	6
42370**	100% FBS	6

* Note that LDL can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 μ L of analyte in a final assay volume of 50 μ L).

** Assays in 100% FBS are not recommended without dilution (see below)

- 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 Lysis Buffer, and Lysis Buffer + 10% FBS. All other components were prepared in IAB. 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.

- Intra-assay precision:

The intra-assay precision was determined using a total of 16 independent determinations in triplicate. Shown as CV%.

LDHA	AlphaLISA Lysis Buffer	Lysis Buffer + 10% FBS
CV (%)	3	8

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 9 measurements for 3 ng/mL sample. Shown as CV%.

LDHA	AlphaLISA Lysis Buffer	Lysis Buffer + 10% FBS
CV (%)	15	9

- Spike Recovery:

Three known concentrations of analyte were spiked into AlphaLISA Lysis Buffer, and 10% FBS. All samples, including non-spiked diluents were measured in the assay. Note that the analytes for the respective standard curves were prepared in Lysis Buffer, and Lysis Buffer + 10% FBS. All other assay components were diluted in IAB.

Spiked LDHA (ng/mL)	% Recovery	
	AlphaLISA Lysis Buffer	Lysis Buffer +10% FBS
100	112	86
30	86	84
10	84	90

- Specificity and/or Selectivity:

Assay specificity and selectivity were tested with human lactate dehydrogenase B subunit (LDHB) at 1 µg/mL. The percent detected in the assay are shown in the table below.

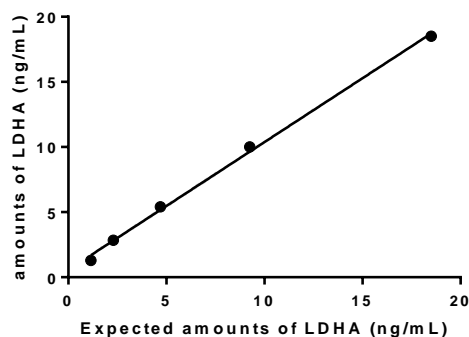
Tested Proteins	% Detected
Human LDHB	0.0

- Human Serum Experiments

- Dilution Linearity

Neat Normal Human Serum was diluted with AlphaLISA Lysis Buffer and the assay was performed along with a standard curve using the analyte prepared in AlphaLISA Lysis Buffer. Concentrations of LDHA in diluted human serum were determined by interpolating to the standard curve. Excellent dilution linearity ($R^2 \geq 0.995$) was achieved in the neat human serum sample that was diluted ≥ 8 fold. The results are shown in table and figure below.

Dilution Factor (x)	Observed LDHA (ng/mL)	Expected LDHA (ng/mL)
8	18.5	18.5
16	10	9.25
32	5.4	4.7
64	1.85	2.3
128	1.3	1.15



From these results, a concentration of 164 ng/mL of LDHA is present in human serum.

- Spike Recovery

Three known amounts of LDHA were spiked into Normal Human Serum (3, 1, and 0.5 µg/mL LDHA in spiked samples) and then the samples were diluted 8-fold into Lysis Buffer. The standard was prepared in Lysis Buffer and all other reagents were prepared in IAB. The spike recoveries of LDHA were determined and the results are shown in table below.

*Recoveries were calculated after the no spike LDHA level was subtracted (in this case, 0.164 µg/mL in Pooled Normal Human Serum). Excellent recovery was achieved for all three spikes tested.

	Diluent: 100% FBS	
	Spiked sample (Pooled Normal Human Serum)	
Spike (ng/mL)	Concentration (ng/mL)	Recovery (%)
No spike	164	N/A
3 000	2 736	91
1 000	740	74
500	416	83

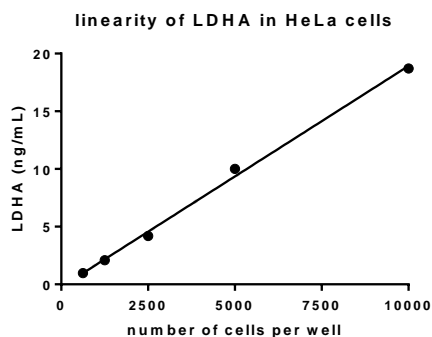
Testing show linearity (R^2 fit >0.995) for all three spiking concentrations.

Cell Lysate assay

- Dilution Linearity

To validate the assay kit, HeLa cells were tested for amounts of LDHA per cell. The cells were plated in a 96 well plate and incubated overnight in 100 µL of DMEM/F12 + 10% FBS. The cells were then washed twice with PBS and incubated 30 minutes with 50 µL of AlphaLISA Lysis Buffer. 5 µL of lysate were transferred to a 384 well plate and analyzed with the LDHA assay described above, with all dilutions of the standard curve performed in AlphaLISA Lysis Buffer

Cell number	LDHA detected in well (ng/mL)	LDH per cell (fg/cell)
10000	18.7	1.87
5000	10.0	2.0
2500	4.2	1.68
1250	2.1	1.7
625	0.98	1.59
0	0	NA



Troubleshooting Guide

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

<http://www.perkinelmer.com/lab-products-and-services/application-support-knowledgebase/alphalisa-alphascreen-no-wash-assays/alpha-troubleshooting.html>

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