TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

AlphaLISA Insulin (Human) Detection Kit

Product number: AL350HV/C/F

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

Contents

Page

Product Information……………………………………………………………………………………..2

Quality Control………………………………………………………………………………………...2

Analyte of Interest…………………………………………………………………...…………………3

Description of the AlphaLISA Assay ……………………………………………………….........3

Precautions……………………………………………………………………………………………3

Kit content: Reagents and Materials…………………………………………………………….…4

Recommendations………………………………………………………………………….….…5

Assay Procedure...…………………………………………………………………………….……..5

Data Analysis………………………………………………………………………………....……8

Assay Performance Characteristics……………………………..……………………………….…………9

Human Serum and Human Plasma Experiments………………………..……………………………....10

Troubleshooting Guide……………………………………………………………………….…………..11

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Product Information

Application: This kit is designed for the quantitative determination of human Insulin (Ins) in cell culture supernatants, serum, and plasma using a homogeneous AlphaLISA assay (no wash steps). This assay can also be used to quantify mouse and rat insulin using an appropriate standard.

Sensitivity: Lower Detection Limit (LDL): 0.21 µIU/mL

Lower Limit of Quantification (LLOQ): 0.55 µIU/mL

EC_{50}: 324 µIU/mL

Dynamic range: 0.21 – 10,000 µIU/mL (Figure 1).

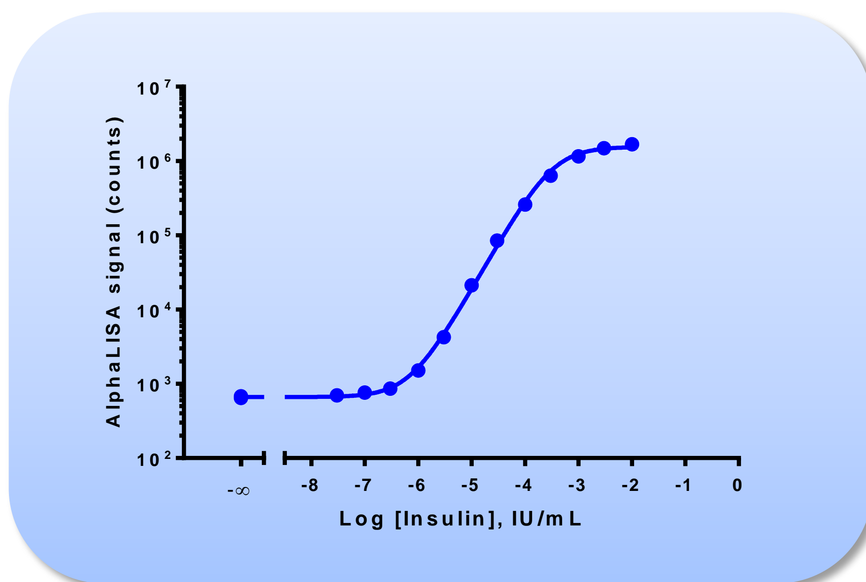


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4˚C. Store analyte at -20°C after reconstitution**.** Limit the number of 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, 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

Insulin is synthesized as a proinsulin hormone of 110 aa by Beta-cells of the islets of Langherans in the pancreas. After removal of the precursor signal peptide, proinsulin is post-translationally cleaved into two chains (peptide A of 21 aa and peptide B of 30 aa) that are covalently linked via two disulfide bonds and secreted upon increased glucose concentration in blood. Blood concentration increases from around 50 pmol/L to 300-400 pmol/L 30 min after glucose uptake. Insulin is a key player in the control of both carbohydrate and lipid metabolism and has been implicated in various diseases including diabetes, heart disease and obesity.

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.

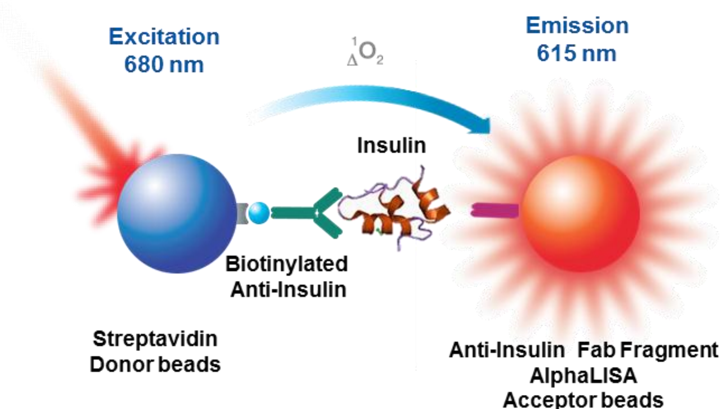


Figure 2. AlphaLISA assay principle.

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. The analyte included in this kit is from a source.

- 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	AL350HV (100 assay points***)	AL350C (500 assay points***)	AL350F (5000 assay points***)
AlphaLISA Anti-Ins Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white 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, 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 Antibody Anti-Ins 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 µL @ 500 nM (1 tube, black cap)
Human Insulin Lyophilized*	0.01 IU (1 tube, clear cap)	0.01 IU (1 tube, clear cap)	0.01 IU (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X)**	10 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* The lyophilized analyte is to be reconstituted with 100 µL of H_2O. One vial contains an amount of Human Insulin sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL204S).

** 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 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 Adhesive Sealing Film	PerkinElmer Inc.	6050185
EnVision®-Alpha Reader	PerkinElmer Inc.	-

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_2O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer.
- 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.
- AlphaLISA signal is detected using an EnVision Multilabel Reader 2103 equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).
- 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 AlphaLISA Immunoassay buffer.

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) and 452 samples. The protocols also include testing samples in 384 well plates. 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 reagents 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.

		Volume				
Format	# of data points	Final	Sample	AlphaLISA beads / Biotin Antibody MIX	SA-Donor beads	Plate recommendation
AL 350C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL350F	5 000	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)

Protocol for Insulin AlphaLISA Assay
2 Step High Concentration Protocol – Dilution of standards in 1X AlphaLISA Immunoassay Buffer or cell culture medium. The protocol described below is for one standard curve (48 wells) and 452 sample wells. *If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

Steps for Preparing Reagents

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
   Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL H_2O.

2) Preparation of Insulin analyte standard dilutions**:**
   1. Reconstitute 0.01 IU of Insulin in 100 µL of H_2O.
      a. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer or cell culture medium (change tip between each standard dilution):

Tube	Vol. of Ins (µL)	Vol. of diluent (µL) *	[Ins] in standard curve		
			(µIU/mL in 5 µL)	(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of provided Ins	90	10000	3.47E-07	347000
B	60 µL of tube A	140	3000	1.04E-07	104100
C	60 µL of tube B	120	1000	3.47E-08	34700
D	60 µL of tube C	140	300	1.04E-08	10410
E	60 µL of tube D	120	100	3.47E-09	3470
F	60 µL of tube E	140	30	1.04E-09	1041
G	60 µL of tube F	120	10	3.47E-10	347
H	60 µL of tube G	140	3	1.04E-10	104
I	60 µL of tube H	120	1	3.47E-11	35
J	60 µL of tube I	140	0.3	1.04E-11	10
K	60 µL of tube J	120	0.1	3.47E-12	3
L	60 µL of tube K	140	0.03	1.04E-12	1
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

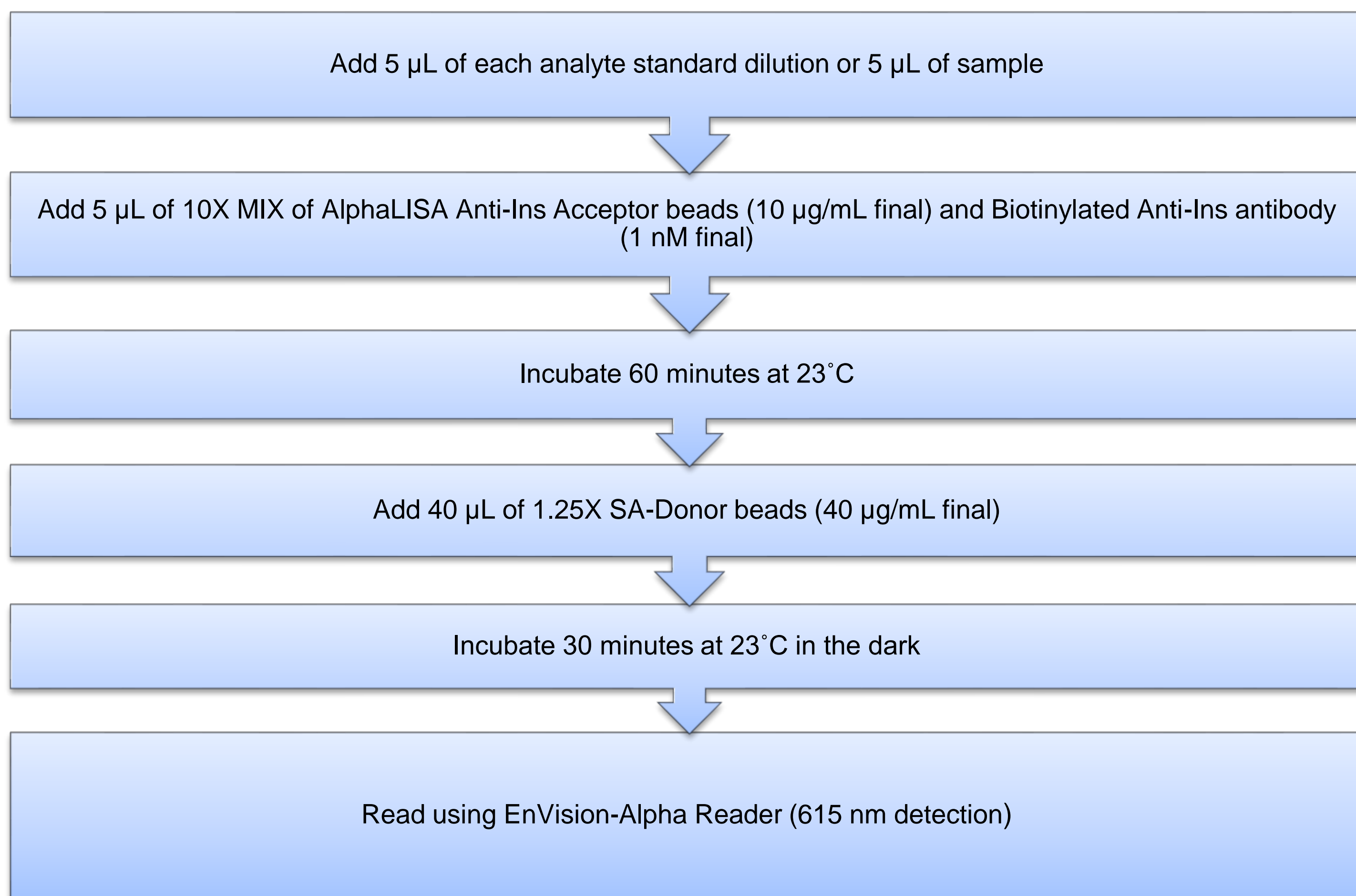
* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** 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).

3) Preparation of 10X Anti-Ins AlphaLISA Acceptor beads (100 µg/mL) and biotinylated Anti-Ins Antibody (10 nM):
   a. Add 50 µL of 5 mg/mL **AlphaLISA Anti-Ins Acceptor beads and** 50 µL of 500nM **Biotinylated Anti-Ins Antibody** to 2400 µL of 1X AlphaLISA Immunoassay Buffer .
   b. Prepare just before use.

4) Preparation of 1.25X Streptavidin (SA) Donor beads (50 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 200 µL of 5 mg/mL SA-Donor beads to 19800 µL of 1X AlphaLISA Immunoassay Buffer.
   c. Prepare just before use.

5) In a white Optiplate (384 wells):



Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader 2103 equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

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 High Concentration 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.

LDL (µIU/mL)	LLOQ (µIU/mL)	Buffer/Serum	# of experiments
0.21	0.55	AlphaLISA Immunoassay Buffer	6
0.47	1.1	DMEM + 10% FBS	6
0.57	1.3	RPMI + 10% FBS	6

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

** Only the analytes were prepared in Cell Culture media. All of other components were prepared in Immunoassay Buffer.

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 (IAB), DMEM + 10% FBS, or RPMI + 10% FBS. Each assay consisted of one standard curve comprising 12 data points in triplicate and 12 background wells containing no analyte. The assays were performed in a 384-well format using AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using 3 independent experiments for a total of 16 independent determinations in triplicate. CV% were calculated for each individual experiment then averaged. Shown is the average intra-experimental CV%.

Ins	IAB	DMEM	RPMI
CV%	8	8	7

- Inter-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 was then averaged. Shown is the inter-experimental CV%.

Ins	IAB	DMEM	RPMI
CV%	14	15	21

- Spike Recovery:

Three known concentrations of Ins were spiked into AlphaLISA Immunoassay Buffer (IAB), DMEM, or RPMI medium. All samples, including non-spiked Immunoassay Buffers were measured in the assay. The average recovery was reported from 4 measurements in triplicate.

Spiked Ins (µIU/mL)	% Recovery		
	IAB	DMEM	RPMI
100	108	90	103
30	114	100	106
10	126	92	101

- Specificity for Insulin:

Cross-reactivity of the AlphaLISA Insulin Kit was tested against the following proteins and was calculated at the Human standard curve EC_{50} point when performed in AlphaLISA Immunoassay Buffer.

Protein	% Cross-reactivity	LDL, µIU/mL
Mouse Insulin	58	0.65
Rat Insulin	64	0.52

Human Serum and Plasma Experiments

Pooled normal Human Serum and Human Plasma were utilized and AlphaLISA Immunoassay Buffer (IAB) was used as the diluent. Insulin was detected in both normal Human serum and Human plasma (data not shown). Insulin was also detected in biological matrices of mouse and rat (data not shown).

- Dilutional Linearity:

Dilutional linearity was determined by serial dilutions of Human Serum and Human Plasma supplemented with 1000 µIU/mL of Human Insulin then diluted with IAB and compared to standard curve prepared in IAB.

Dilution Factor	% Recovery	
	Human Serum	Human Plasma
2	61	68
4	84	81
8	100	100
16	110	117
32	113	119
64	104	117

- Spike Recovery:

Spike recovery in Human Serum and Human Plasma was performed by first diluting Human Serum and Human plasma samples 8-fold then spiking them with 100, 30, and 10 µIU/mL. Spike samples were compared to standard curve prepared in IAB. All samples dilutions were with IAB.

Spiked amount, µIU/mL	% Recovery	
	Human Serum	Human Plasma
100	91	88
30	108	131
10	118	116

Troubleshooting Guide

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

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

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