TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Research Use Only. Not for use in diagnostic procedures.

Glucagon AlphaLISA Immunoassay Detection Kit

Product No.: AL3140HV/C/F

Contents

Material Provided

Format: AL3140HV: 100 assay points AL3140C: 500 assay points AL3140F: 5 000 assay points
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.

Product Information

Application: This kit is designed for the quantitative determination of Glucagon in cell culture media, sera and plasma, using a homogeneous AlphaLISA assay (no wash steps). The assay is compatible with human, mouse, rat, and porcine species and is highly specific for pancreatic Glucagon.

Sensitivity: Lower Detection Limit (LDL): 1.3 pg/mL
Lower Limit of Quantification (LLOQ): 4.3 pg/mL
EC50: 3.41 ng/mL

Dynamic Range: 1.3 – 15 000 pg/mL

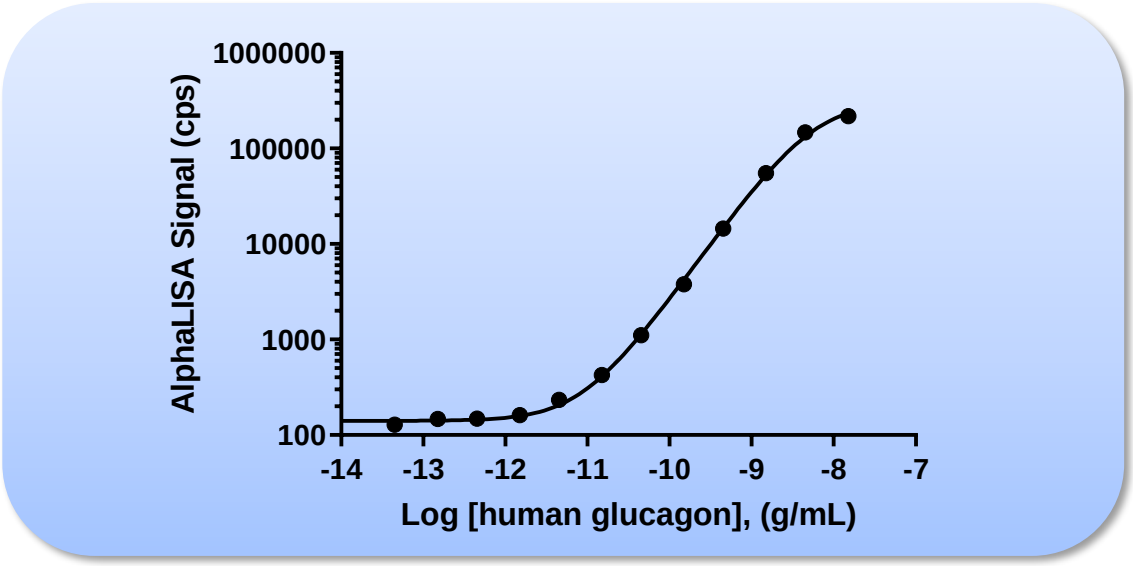

Figure 1. Typical sensitivity curve in AlphaLISA HiBlock Buffer. The data was generated using a Light Gray Alphaplate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at 4 ˚C. For reconstituted analyte aliquot and store at -20 °C. It has been demonstrated that the Glucagon analyte solution is stable for 14 days 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.

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Analyte of Interest

Glucagon is a 29 amino acid peptide hormone produced by the pancreas. Glucagon generally functions as a counter-regulatory homrone opposing the actions of insulin to maintain appropriate levels of blood glucose. Normal human serum glucagon levels range from 50-200 pg/mL. The glucagon:insulin ratio controls the rate of gluconeogenesis and glycogenolysis, disruption of this ratio can have severe metabolic implications. The present kit permits detection of glucagon (i.e. analyte) in different sample matrices and is compatible with human, mouse, rat, and porcine species.

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 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 transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2).

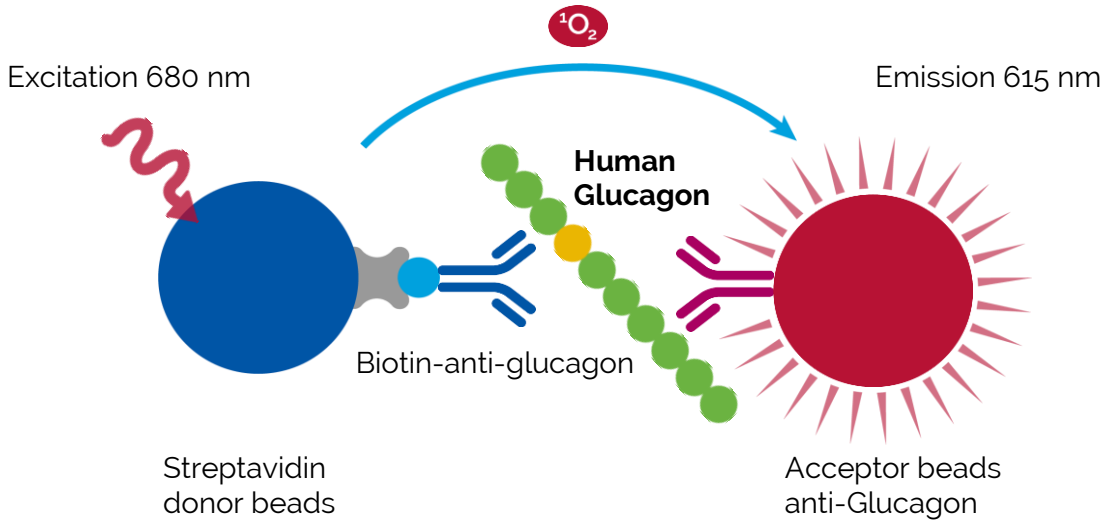

Figure 2. AlphaLISA Glucagon Detection Kit Assay Principle.

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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-Glucagon antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3140HV (100 assay points)	AL3140C (500 assay points)	AL3140F (5000 assay points)
AlphaLISA Anti-Glucagon Acceptor beads stored in PBS, 0.05% Kathon CG/ICP, 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% Kathon CG/ICP, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	1 000 µL @ 5 mg/mL (2 brown tubes, black cap)
Biotinylated Anti-Glucagon 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 Glucagon *	15 ng (1 tube, clear cap)	15 ng (1 tube, clear cap)	15 ng (1 tube, clear cap)
AlphaLISA HiBlock Buffer (10X)	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* The thawed 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 the Glucagon analyte solution is stable for 14 days at -20°C. One vial contains an amount of Glucagon sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3140S).

** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100, 5% Blocking reagent, 5% BSA and 0.5% Kathon. Extra buffer can be ordered separately (cat # AL004C: 10 mL, cat # AL004F: 100 mL).

Note: 10X buffer is slightly tan. If not fully in suspension when diluted to the final 1X solution, it is recommended to centrifuge it for 5 min at 1000 rpm and use the supernatant. It should be noted however, that the appearance of the buffer does not affect its efficacy.

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL3140HV) 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 anti-Glucagon 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:
*PP: Polypropylene

Item	Suggested source	Catalog #
TopSeal™-A Plus Adhesive Sealing Film	PerkinElmer Inc.	6050185
EnVision®-Alpha Reader	PerkinElmer Inc.	-
StorPlate-384V, PP*, 384 well, V-bottom *or* StorPlate-96V, PP*, 96 well, V-bottom	PerkinElmer Inc.	6008590 *or* 6008290

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 H_2O to dilute 5X AlphaLISA HiBlock 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.
- This Glucagon AlphaLISA assay can be performed in cell culture media (DMEM, RPMI) or secretion buffers such as KREBS with or without phenol red and plasma or serum samples. It is important to have a protein source in the cell culture medium (i.e. 1% BSA or 10% FCS) to prevent analyte sticking to cell culture plastic surfaces.
- For Glucagon analysis in blood-derived products, it is recommended to use regular specimen type for this analyte: EDTA plasma (+/-Trasylol). Aprotinin supplementation in samples before storage can be beneficial in preventing glucagon degradation by specific proteases. Aprotinin supplementation has been tested (0.6 TIU/mL) and does not interfere with the assay.
- **IMPORTANT! This kit protocol includes a mandatory 4-fold predilution step for samples (whatever their type). It is important to strictly follow the predilution step for samples to insure accuracy and robustness. A polypropylene container has to be used for sample predilution (tube or plate) to avoid Glucagon sticking.**
- The standard curves shown in this technical data sheet are provided for information only. **A standard curve must be generated for each experiment in the 1X AlphaLISA HiBlock Buffer. There is no need to perform standard curve in your sample matrix**. The recommended fitting model is a 4-Parameter Logistic regression with a $1/Y^2$ weighting.

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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 to insure best results. If needed, the number of replicates or the range of concentrations covered can be modified but without extending the concentration of the high standard above 15 000 pg/mL.
- 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**	**MIX AlphaLISA AccBeads + biotinylated Ab**	**SA-Donor beads**	**Plate recommendation**
AL3140HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6002290) Light gray ½ AreaPlate-96 (cat # 6002350)
AL3140C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6002290) Light gray ½ AreaPlate-96 (cat # 6002350)
	500	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6002290) Light gray ½ AreaPlate-96 (cat # 6002350) 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) Shallow Well AlphaPlate-384 (cat # 6008350) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3140F	5 000	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) Light gray ½ AreaPlate-96 (cat # 6002350) 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) Shallow Well AlphaPlate-384 (cat # 6008350) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)

3-Steps Protocol (3 incubation steps) – Preparation of standards must be done in 1X AlphaLISA HiBlock Buffer. Predilution of samples must be done in 1X AlphaLISA HiBlock Buffer. 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 have to be adjusted accordingly*

1) Preparation of AlphaLISA HiBlock Buffer 1X:
Add 1 mL of 10X HiBlock buffer to 9 mL of Milli-Q® grade H_2O.

2) Preparation of Glucagon analyte standard dilutions:

a. Reconstitute lyophilized Glucagon in 100 µL Milli-Q® grade H_2O. The remaining reconstituted analyte should be aliquoted immediately and stored at -20 ºC for future assays.
b. The reconstituted Glucagon stock solution concentration is 150 000 pg/mL.
c. Prepare standard dilutions as follows in 1X AlphaLISA **HiBlock** Buffer (change tip between each standard dilution):

Tube	Vol. of Human Glucagon (µL)	Vol. of diluent (µL) *	[human Glucagon] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted human Glucagon	90	1.50E-08*	15 000
B	60 µL of tube A	140	4.50E-09	4 500
C	60 µL of tube B	120	1.50E-09	1 500
D	60 µL of tube C	140	4.50E-10	450
E	60 µL of tube D	120	1.50E-10	150
F	60 µL of tube E	140	4.50E-11	45
G	60 µL of tube F	120	1.50E-11	15
H	60 µL of tube G	140	4.50E-12	4.5
I	60 µL of tube H	120	1.50E-12	1.5
J	60 µL of tube I	140	4.50E-13	0.45
K	60 µL of tube J	120	1.50E-13	0.15
L	60 µL of tube K	140	4.50E-14	0.045
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 1X AlphaLISA HiBlock Buffer diluent exclusively
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.

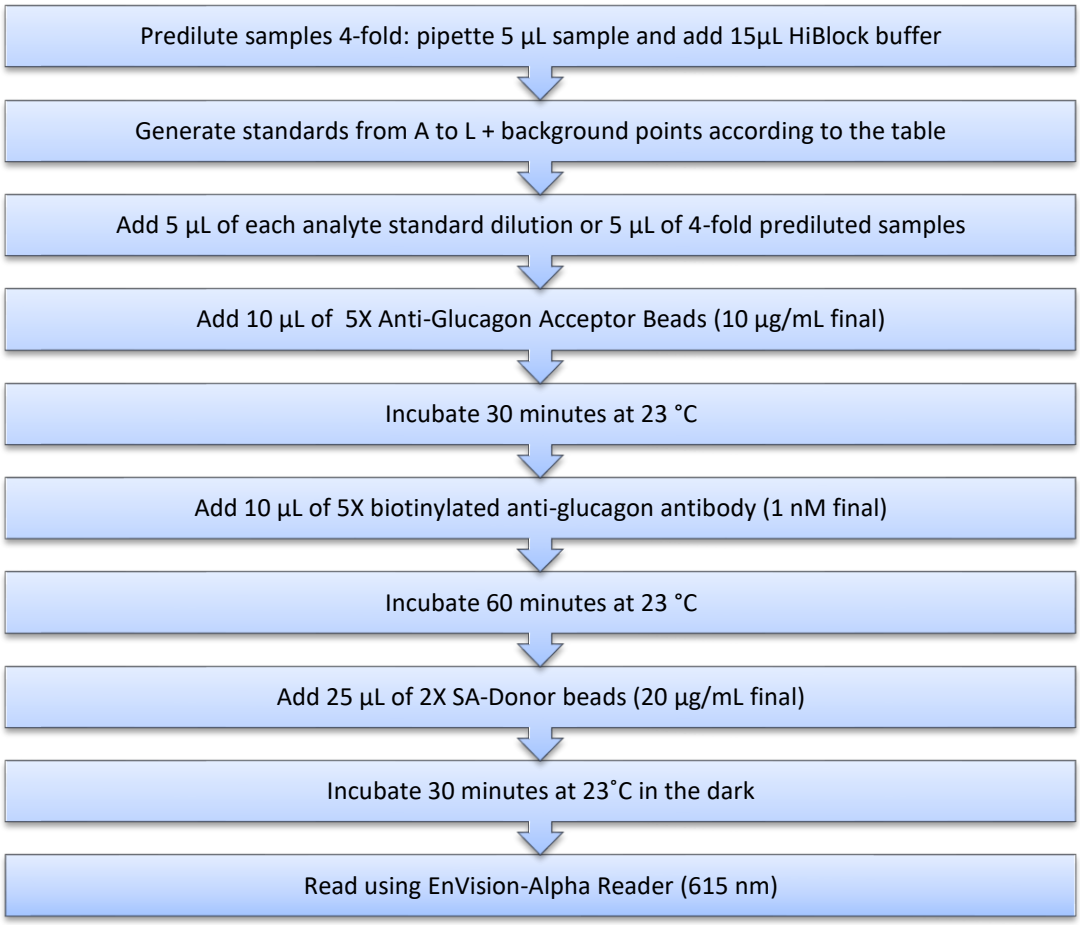
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X AlphaLISA Anti-Glucagon Antibody Acceptor beads (50 µg/mL)
   a. Prepare just before use.
   b. Add 10 µL of 5 mg/mL AlphaLISA Anti-Glucagon Antibody Acceptor Bead to 990 µL of 1X AlphaLISA HiBlock Buffer.

4) Preparation of 5X biotinylated anti-glucagon antibody (5 nM)
   a. Prepare just before use
   b. Add 10 µL of 500 nM biotinylated antibody to 990 µL of 1X HiBlock buffer

5) Preparation of 2X Streptavidin (SA) Donor beads (40 µg/mL):
   a. Prepare just before use.
   b. Keep the beads under subdued laboratory lighting.
   c. Add 16 µL of 5 mg/mL SA-Donor beads to 1984 µL of 1X AlphaLISA HiBlock Buffer.

6) In a recommended plate (here for 384 wells):



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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 4-fold prediluted samples.**

- **IMPORTANT! Recalculate real samples concentration by multiplying interpolated value by a factor of 4.**

- If samples have been further diluted, the concentration must be multiplied by the additional dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined a 3-step protocol using AlphaLISA Hiblock as assay buffer. The analytes (standards) were prepared in HiBlock, human EDTA plasma, KREBS buffer, DMEM + 10% FBS, and RPMI + 10% FBS. All other components were prepared in HiBlock buffer.

- 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 µL sample using the recommended assay conditions.

LDL (pg/mL)	(Analyte diluent)	# of experiments
1.1	AlphaLISA HiBlock buffer	6
2.1	Human Plasma	6
1.2	DMEM + 10% FBS	6
1.3	RPMI + 10% FBS	6
0.6	KREBS buffer	6

- 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 HiBlock, human EDTA plasma, KREBS buffer, DMEM + 10% FBS, and RPMI + 10% FBS. All other components were prepared in HiBlock buffer. 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 plate format.

  - Intra-assay precision:

The intra-assay precision was determined using a total of 12 independent determinations in triplicate. Shown as CV%.

Glucagon	HiBlock	Plasma	DMEM + 10% FBS	RPMI + 10% FBS	KREBS
intraCV (%)	3	7.6	3.8	1	4.2

  - Inter-assay precision:

The inter-assay precision was determined using a total of 12 independent determinations with 6 measurements for 300 pg/mL sample. Shown as CV%.

Glucagon	HiBlock	Plasma	DMEM + 10% FBS	RPMI + 10% FBS	KREBS
interCV (%)	8.1	9.8	13.7	7.1	11.6

- Spike Recovery:

Three known concentrations of analyte were spiked into presumed healthy human plasma sample with or without 0.6 TIU/mL Aprotinin. All samples, including non-spiked sample were measured in the assay.

Spiked Glucagon (pg/mL)	% Recovery	
	Human Plasma	Human plasma + 0.6 TIU/mL Aprotinin
10 000	85.1%	89.6 %
1 000	117.6 %	111.2 %
100	116.7 %	111.7 %

The human plasma sample tested shown a measurable endogenous Glucagon concentration of 62.8 pg/mL (w/o aprotinin) and 70.4 pg/mL with 0.6 TIU/mL final Aprotinin in the initial sample.

- Specificity:

Cross-reactivity of the Glucagon AlphaLISA Detection Kit is based on data known with the same antibodies established with another homogeneous immunoassay indicating no significant cross reactivities towards oxyntomodulin, glicentin, Glucagon fragment 19-29, GLP-1 7-36 amide, GLP-1 7-37, GLP-2, nor glicentin-related pancreatic peptide (GRPP). The antibody pair is highly specific to glucagon.

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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