TECHNICAL DATA SHEET

AlphaPlex™ 645 Research Reagents

AlphaPlex™ 645 Human Insulin Immunoassay Kit

Product number: **AP204SM-HV/C/F**

Research Use Only. Not for use in diagnostic procedures.

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Product Information

Application: This kit is designed for the quantitative determination of human Insulin in cell culture media, sera and plasma, using a homogeneous AlphaPlex 645 assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 2.8 µIU/mL

Lower Limit of Quantification (LLOQ): 6.7 µIU/mL

EC_{50}: 402.8 µIU/mL

Dynamic range: 2.8 - 10000 µIU/mL(Figure 1).

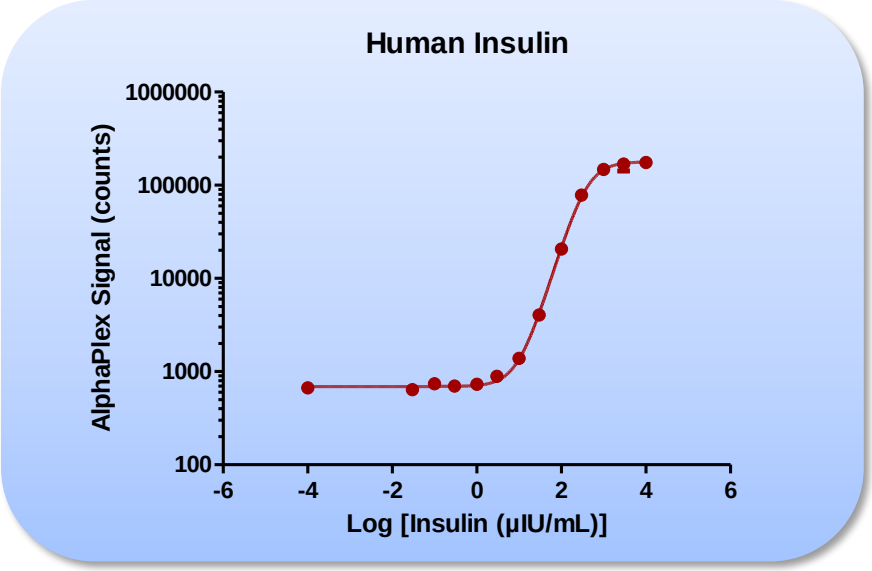


Figure. 1. Typical sensitivity curve in HiBlock buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2102.

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

Stability: This kit is stable for at least 12 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 AlphaPlex 645 assay. Maximum and minimum signals, EC_{50} and LDL were measured on the EnVision Multilabel Plate Reader with Alpha option 2102 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 comprising 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 ~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 AlphaPlex 645 Assay

AlphaPlex 645 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 AlphaPlex 645 assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-Analyte Antibody is conjugated to AlphaPlex 545 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
645 nm (Figure 2).

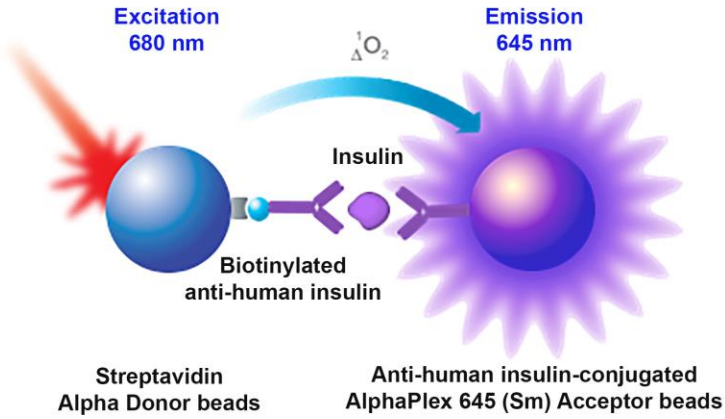


Figure 2. AlphaPlex 645 Assay principle.

Indeed, the presence of two acceptor beads allow for the following assays:

- Two unrelated analyte measurements.
- Total versus modified analyte.
- Two different modifications on same analyte.
- Cascade effects.
- Protein-protein interactions

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	AP204Sm-HV (100 assay points***)	AP204Sm-C (500 assay points***)	AP204Sm-F (5 000 assay points***)
AlphaPlex 645 Anti-Human Insulin Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	25 µL @ 5 mg/mL (1 brown tube, purple cap)	50 µL @ 5 mg/mL (1 brown tube, purple cap)	500 µL @ 5 mg/mL (1 brown tube, purple cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	100 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 mL @ 5 mg/mL (1 brown tube, black caps)
Biotinylated Antibody Anti-Human Insulin stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	25 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Human Insulin Analyte Lyophilized (100 µIU/mL)	1 tube, clear cap	1 tube, clear cap	1 tube, clear cap
AlphaLISA HiBlock Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small 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 Human Insulin analyte solution is stable for at least 6 months at -20°C. One vial contains an amount of Human Insulin sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AP201S).

** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100, 5% BSA and 0.5% Proclin-300. 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 (AP201HV) 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 AlphaPlex 645 signal. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaPlex 645 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.	-

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 HiBlock 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.
- AlphaPLEX 645 signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 1000 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D670as (Barcode# 605), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 224).
- AlphaPlex 645 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 a similar matrix as the samples (e.g. FBS for serum 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.

		Volume				
Format	**# of data points**	**Final**	**Sample**	**AlphaPlex 645 beads / Biotin Antibody MIX**	**SA-Donor beads**	**Plate recommendation**
AP204Sm HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AP204Sm C	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)
AP204Sm F	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)

High sensitivity protocol (3 incubation steps) – Dilution of standards in 1X AlphaLISA HiBlock Buffer

The protocol described below is recommended when generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations with manual pipetting). *If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

Notes: This protocol requires AlphaLISA HiBlock Buffer and it is a 3 step assay!

1) Preparation of 1X AlphaLISA HiBlock Buffer:

- Add 1 mL of 10X AlphaLISA HiBlock Buffer to 9 mL H_2O.

2) Preparation of Insulin analyte standard dilutions:

Reconstitute lyophilized human Insulin (0.01 IU, 28 units/mg) in 100 µL H_2O. Prepare standard dilution as follows (change tip between each standard dilution):

Tube	Vol. of Human Insulin (µL)	Vol. of diluent (µL) *	[human Insulin] in standard curve	
			(µIU/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted Human Insulin	90	10000	357000
B	60 µL of tube A	140	3000	107000
C	60 µL of tube B	120	1000	35700
D	60 µL of tube C	140	300	10700
E	60 µL of tube D	120	100	3570
F	60 µL of tube E	140	30	1070
G	60 µL of tube F	120	10	357
H	60 µL of tube G	140	3	107
I	60 µL of tube H	120	1	36
J	60 µL of tube I	140	0.3	10
K	60 µL of tube J	120	0.1	3
L	60 µL of tube K	140	0.03	1
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 HiBlock 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 5X AlphaPlex 645 Anti-Human Insulin Acceptor beads (50 µg/mL):
Add 15 µL of 5 mg/mL AlphaPlex 645 Anti-Human Insulin Acceptor beads to 1485 µL of 1X AlphaLISA HiBlock Buffer. Prepare just before use.

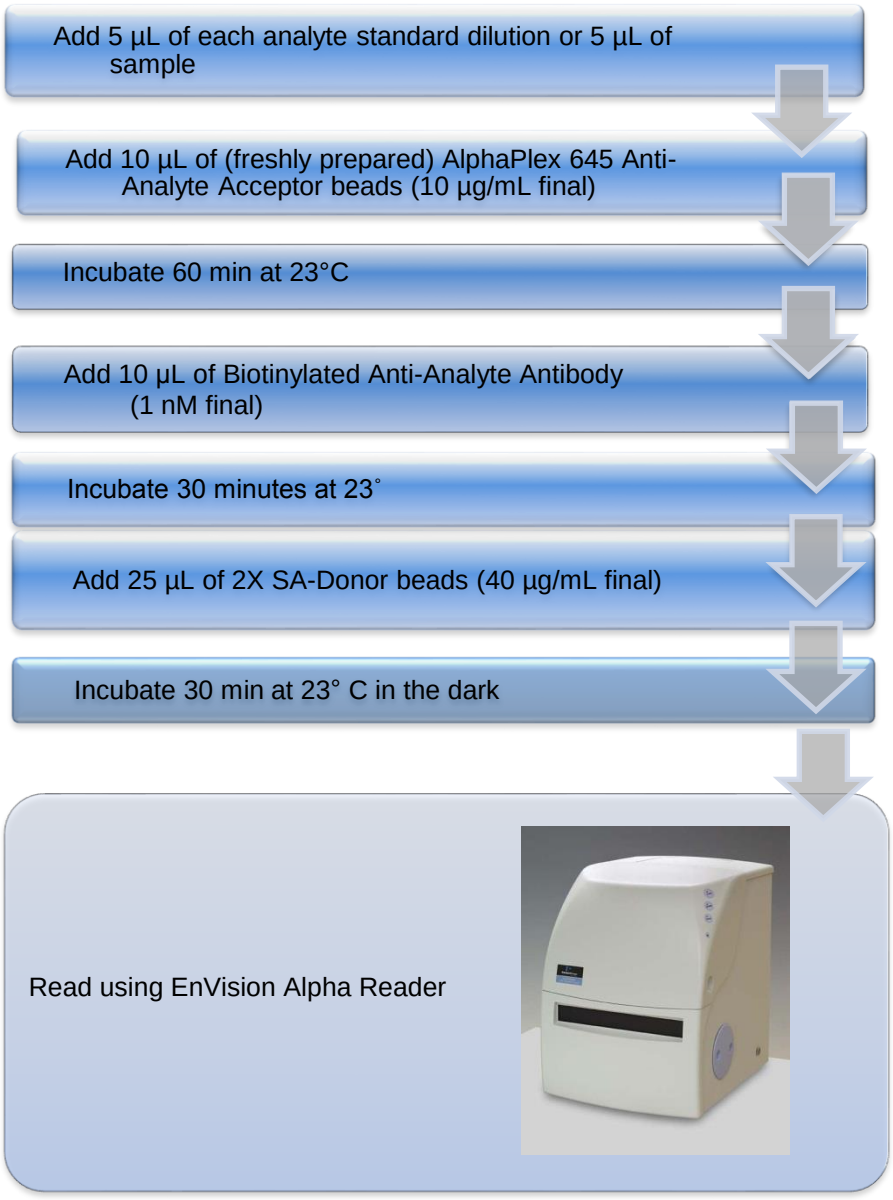
4) Preparation of 5X Biotinylated Antibody Anti-Human Insulin (5 nM):
Add 15 µL of 500 nM Biotinylated Antibody Anti-Human Insuiln to 1485 µL of 1X AlphaLISA HiBlock Buffer. Prepare just before use.

5) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting.
Add 48 µL of 5 mg/mL SA-Donor beads to 2952 µL of 1X AlphaLISA HiBlock Buffer.

6) Samples:

- If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA HiBlock Buffer).

7) In a 96- or 384-well microplate:



Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaPlex 645 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

AlphaPlex 645 assay performance described below was determined using the Hi Sensitivity protocol.

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/Media used	# of experiments
2.8	6.7	AlphaLISA HiBlock Buffer	9

* 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).

Assay precision:

The following assay precision data were calculated from the six independent assays using two different kit lots. In each lot, the analytes were prepared in AlphaLISA HiBlock Buffer (HBB). 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 HiBlock Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 8 independent determinations in triplicate. Shown CV%.

Insulin (µIU/mL)	HBB
100	5.3%

- Inter-assay precision:

The inter-assay precision was determined using a total of 5 independent determinations.

Insulin (µIU/mL)	HBB
100	10.8%

- Recovery:

Three known concentrations of analyte were spiked into cell culture media containing 10% FBS, Human Serum and AlphaLISA HiBlock Buffer (HBB). All samples were run alongside a standard curve diluted in AlphaLISA HiBlock Buffer, this standard curve was used to interpolate the concentrations of the samples. The percent recovery is defined as assay measured concentration with respect to the spiked concentration. The average recovery from two independent measurements is reported.

Spike (Human Insulin µIU/mL)	% Recovery			
	AlphaLISA HiBlock Buffer	DMEM with FBS	RPMI with FBS	Human Serum
400	112	113	89	107
200	126	113	98	85
50	111	115	60	98

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaPlex 645 Assay kit at:

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

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