TECHNICAL DATA SHEET

AlphaPlex™ Research Reagents

AlphaPlex 645 Human Interferon Gamma-induced Protein 10 Sm Detection Kit

Product number: AP326SM-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 IP-10 in serum, plasma, and cell culture supernatants using a homogeneous AlphaPlex 645 assay (no wash steps). The assay shows negligible cross-reactivity with other cyotkines.

Sensitivity: Lower Detection Limit (LDL): 26 pg/mL

Lower Limit of Quantification (LLOQ): 96 pg/mL

EC_{50}: 35 ± 6 ng/mL

Dynamic range: 26 – 300 000 pg/mL (Figure 1).

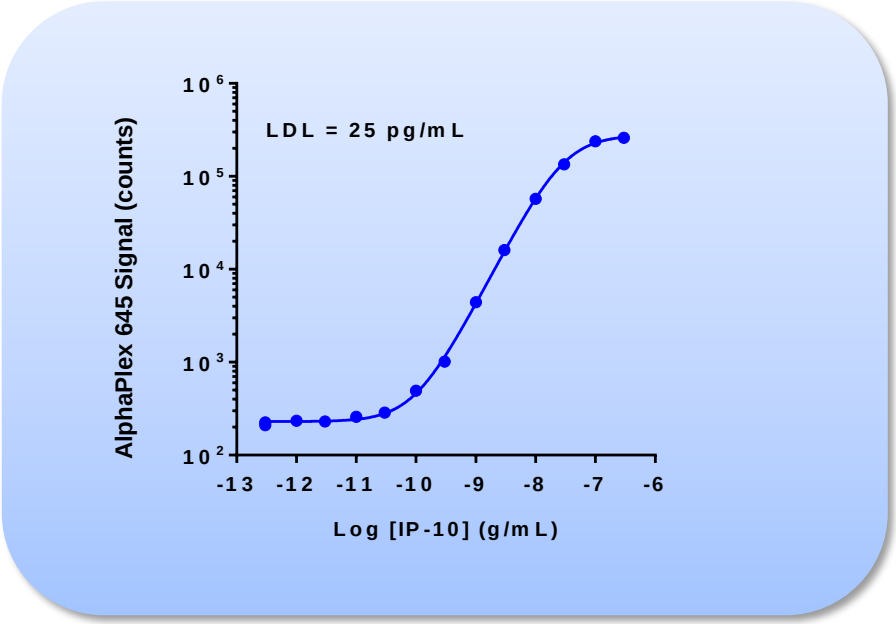

Figure 1. Typical sensitivity curves in HiBlock Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2104.

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 TNF-alpha analyte is stable for at least 18 months when stored at -20°C.

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 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

Interferonγ-induced protein 10 (IP-10) is also known as IC-X-C motif chemokine 10 (CXCL10) or small-inducible cytokine B10. It is an 8.7 kDa protein encoded by the CXCL10 gene and belongs to the CXC chemokine family. IP-10 is secreted by several types of immune cells in response to IFNγ stimulation. Increased levels appear to be a pre-treatment marker for interferon/ribavirin therapy in HCV and HIV infected patients. The present kit permits the detection of human IP-10 (i.e. analyte) in human serum, plasma, and immune cell culture supernatants.

Description of the AlphaPlex Assay

AlphaPlex 645 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 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 645 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 645 nm (Figure 2).

Combining this assay with an AlphaLISA or AlphaPlex 545- based kit will allow the quantification of 2 (or more) analytes in the same well. 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-molecule interactions.

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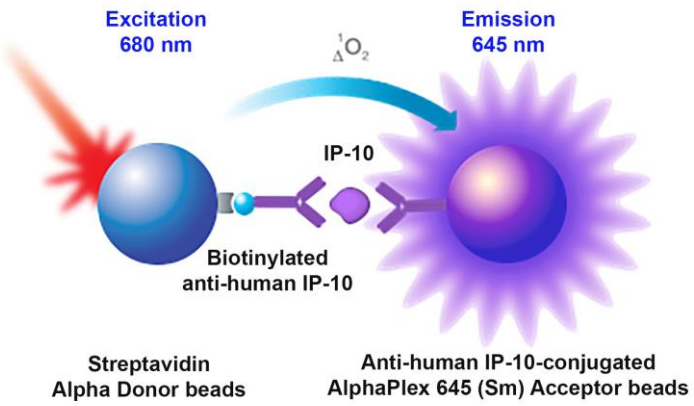


Figure 2. AlphaPlex 645 assay principle

Precautions

- The AlphaScreen® 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	AP326SM-HV (100 assay points***)	AP326SM-C (500 assay points***)	AP326SM-F (5000 assay points***)
AlphaPlex 645 Anti-hIP-10Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	50 µL @ 5 mg/mL (1 brown tube, purple cap)	100 µL @ 5 mg/mL (1 brown tube, purple cap)	1 mL @ 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 (2 brown tube, black caps)
Biotinylated Antibody Anti-hIP-10 stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	50 µL @ 500 nM (1 tube, black cap)	100 µL @ 500 nM (1 tube, black cap)	1 mL µL @ 500 nM (1 tube, black cap)
AlphaPlex 645 hIP-10 (0.3 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap	1 tube, clear cap
HiBlock Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute hIP-10 in 100 µL Milli-Q® grade H_2O. 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 hIP-10 is stable for at least 18 months at -20°C. One vial contains an amount of hIP-10 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL326S).

** Extra buffer can be ordered separately (cat # AL004C: 10 mL, cat # AL004F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AP326SM-HV) 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

General 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 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 HiBlock Buffer or in FBS 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 354 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 645beads / Biotin Antibody MIX**	**SA-Donor beads**	**Plate recommendation**
HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
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)
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)

Protocol for hIP-10 AlphaPlex 645 Assay

Quick 2 incubation steps protocol – Dilution of standards in 1X AlphaLISA HiBlock Buffer or other matrix. The protocol described below is for 500 data 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.*

Steps for Preparing Reagents

1) Preparation of 1X AlphaLISA HiBlock Buffer:
   Add 10 mL of 10X AlphaLISA HiBlock Buffer to 90 mL H_2O.

2) Preparation of hIP-10 analyte standard dilutions:

   a) Reconstitute lyophilized hIP-10 (0.3 µg) in 100 µL of H_2O.
   b) Prepare standard dilutions as follows in 1X AlphaLISA HiBlock Buffer (change tip between each standard dilution):

Tube	Vol. of hIP-10 (µL)	Vol. of diluent (µL) *	[hIP-10] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of provided hIP-10	90	3.00E-07	300000
B	60 µL of tube A	120	1.00E-07	100000
C	60 µL of tube B	140	3.00E-08	30000
D	60 µL of tube C	120	1.00E-08	10000
E	60 µL of tube D	140	3.00E-09	3000
F	60 µL of tube E	120	1.00E-09	1000
G	60 µL of tube F	140	3.00E-10	300
H	60 µL of tube G	120	1.00E-10	100
I	60 µL of tube H	140	3.00E-11	30
J	60 µL of tube I	120	1.00E-11	10
K	60 µL of tube J	140	3.00E-12	3
L	60 µL of tube K	120	1.00E-12	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.

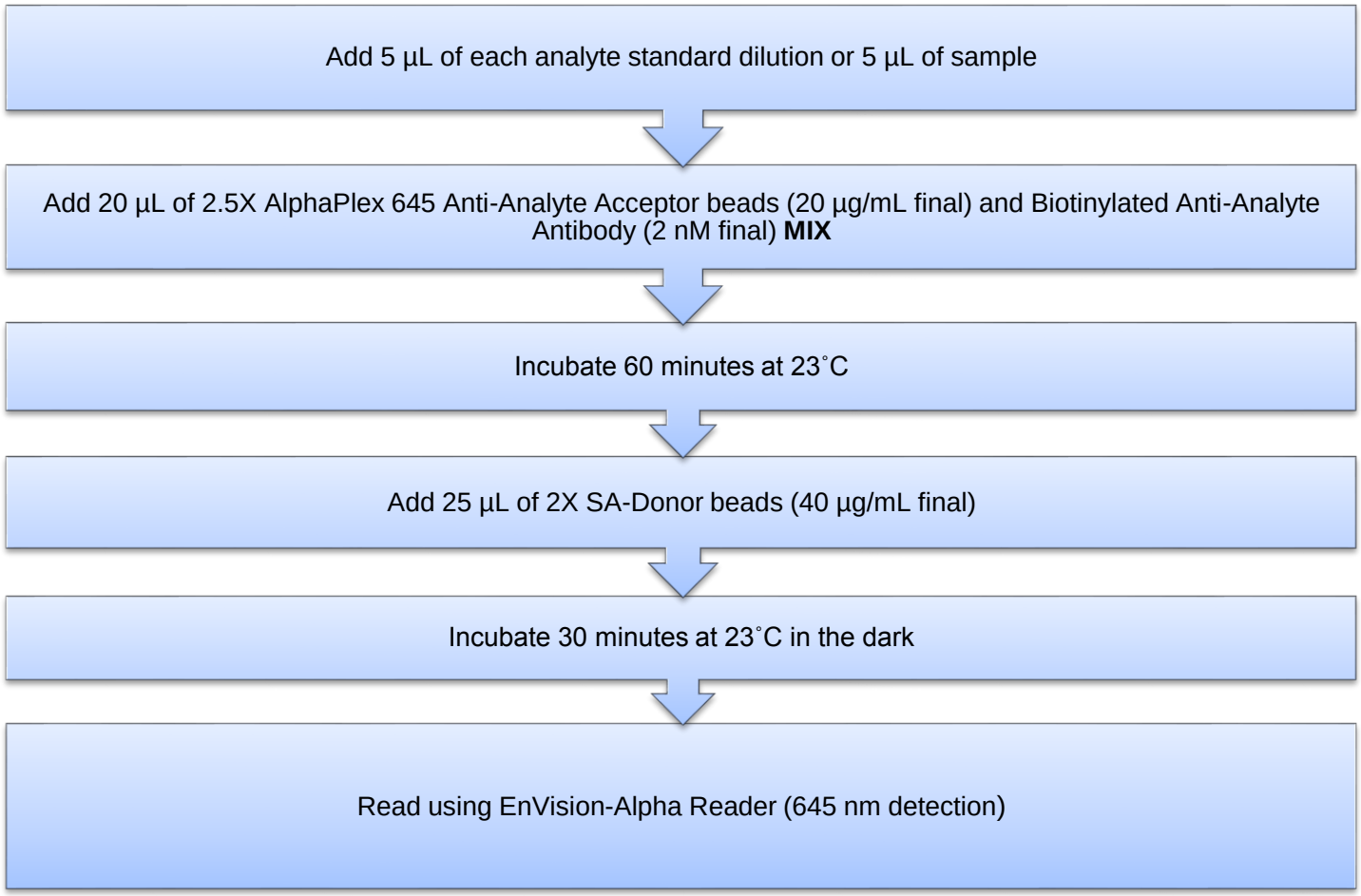
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 2.5 X AlphaPlex 645 Anti-hIP-10 Acceptor beads (50 µg/mL) and biotinylated Anti hIP-10 Antibody (5 nM) **MIX**:

   a. Add 100 µL of 5 mg/mL **AlphaPlex 645 Anti-hIP-10** Acceptor and 100 µL of 500nM **Anti-hIP-10 Antibody** to 9800 µL of 1X AlphaLISA HiBlock Buffer.
   b. Prepare just before use.

4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 200 µL of 5 mg/mL SA-Donor beads to 12300 µL of 1X AlphaLISA HiBlock Buffer.
   c. Prepare just before use.

5) In a white Optiplate (384 wells):



Read Settings: In order to read AlphaPlex 645 beads your Envision Multilabel Reader must be equipped with a D670as Mirror and 570w Filter (or similar filter around 645 nm). The read setting should be the AlphaScreen standard settings Measurement Time: 550 ms, Laser 680nm Excitation Time: 180ms.

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 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.

LDL (pg/mL)	Buffer/Serum/Media	# of experiments
26	HiBlock Buffer	12
112	FBS (undiluted)	6
289	DMEM+ 10% FBS	6
167	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 serum (i.e. FBS). All of other components were prepared in Immunoassay Buffer.

- Assay Precision:

The following assay precision data were calculated from the three independent assays. The analytes were prepared in HiBlock Buffer (HB) or Fetal Bovine Serum (FBS) or in cell culture media. 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 HiBlock Buffer.

- Intra-assay precision:

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

hIP-10	HB	FBS	DMEM	RPMI
CV%	6.0	7.9	6.2	8.4

- Inter-assay precision:

The inter-assay precision was determined using the data across 3 independent experiments with 9 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%.

hIP-10	HB	FBS	DMEM	RPMI
CV%	8.7	9.9	8.2	12.5

- Spike Recovery:

Three known concentrations of hIP-10 were spiked into HB, RPMI with 10% FBS, DMEM with 10% FBS, and FBS. Standards were prepared in HB, RPMI with 10% FBS, DMEM with 10% FBS, and 100 %FBS, All samples, including non-spiked Immunoassay Buffers were measured in the assay. The average recovery was reported from 3 independent experiments each with 3 measurements in triplicate.

Spiked hIP-10 (ng/mL)	% Recovery			
	HB	FBS	RPMI	DMEM
10	94	99	104	101
3	99	102	101	104
1	117	97	97	95

- Specificity for hIP-10:

Cross-reactivity of the AlphaPlex 645 hIP-10 Kit was tested using the following proteins at 100 ng/mL in HiBlock Buffer.

Protein	% Cross-reactivity
Mouse IP-10	3
Rat IP-10	0
Bovine IP-10	0

Serum Experiments

IP-10 is not detected in normal human serum with serial dilutions up to 2048 fold. However, serum interference with the assay performance has been observed. When serum samples are tested, it is recommended to prepare the standard curve in FBS.

- Spike recovery in human serum:

  To avoid serum interference, three known concentrations of hIP-10 were spiked into normal human serum diluted 10 fold with HiBlock buffer or into FBS diluted 10-fold with HiBlock buffer. All samples, including diluted non-spiked sera were measured in the assay. The average recovery was reported from 2 independent experiments each with 3 measurements in triplicate.

Spiked IP-10 (ng/mL)	% Recovery in diluted human serum	% Recovery in diluted FBS
10	78	97
3	85	94
1	87	94

- Dilution Linearity:

Dilution linearity was determined by serial dilutions of whole serum containing 30 ng/mL IP-10. Good linearity can be achieved with dilution factors between 16 to 128 fold.

Dilution Factor	IP-10 ng/mL	% Recovery
1	30	48
2	15	57
4	7.5	62
8	3.75	76
16	1.875	81
32	0.938	94
64	0.469	110
128	0.204	111

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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