TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

AlphaLISA Human TGF-β1 Detection Kit

Product number: AL336 HV/C/F

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

Contents

Product Information

Application: This kit is designed for the quantitative determination of Human TGF-β1 (hTGF-β1) in human serum, plasma, and cell culture supernatants using a homogeneous AlphaLISA assay (no wash steps). The assay shows negligible cross-reactivity with human hTGF-β2 and hTGF-β3. The assay does detect mouse and porcine hTGF-β1. Other species have not been tested.

Sensitivity: Lower Detection Limit (LDL): 9 pg/mL

Lower Limit of Quantification (LLOQ): 28 pg/mL

EC_{50}: 25 ng/mL

Dynamic range: 9 – 100 000 pg/mL (Figure 1).

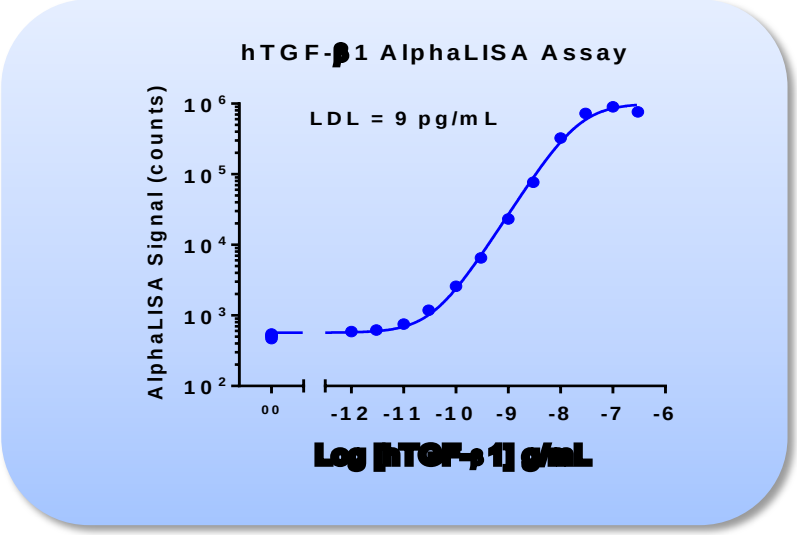


Figure 1. Typical sensitivity curve in AlphaLISA Simple Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4˚C. Store 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 hTGF-β1 analyte is stable for at least 18 months when stored at -20°C.

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

Transforming growth factor beta 1 or TGF-β1, part of the TGF β cytokine superfamily, is a 25 kD disulfide-linked homodimer. TGF-β1 is produced by many cell types including immune cells and controls cell growth, proliferation, differentiation, and apoptosis by modulating many other cytokines and cytokine receptors. Serum and urine concentrations of TGF β1 are a useful marker for determining the status of patients with diabetic nephropathy in type II. The present kit permits the detection of hTGF-β1 (i.e. analyte) in human serum, plasma, and cell culture supernatants.

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).

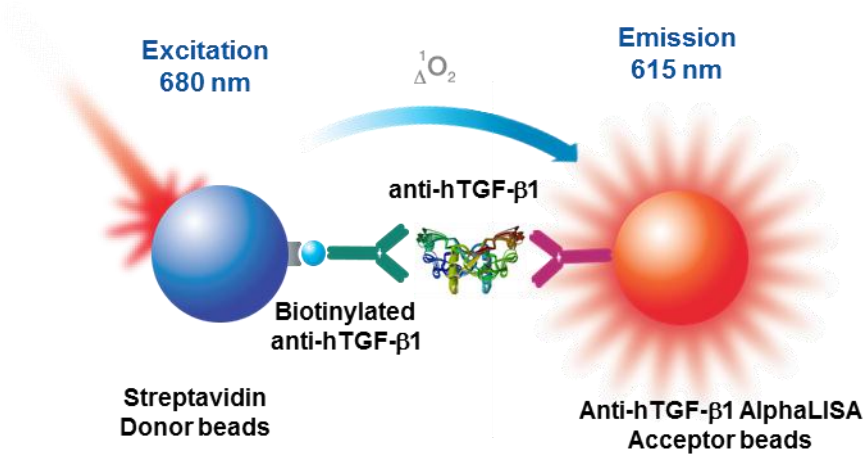


Figure 2. AlphaLISA Assay principle.

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 human 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	AL336HV (100 assay points***)	AL336C (500 assay points***)	AL336F (5000 assay points***)
AlphaLISA Anti-hTGF-β1 Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	15 µL @ 5 mg/mL (1 brown tube, white cap)	25 µL @ 5 mg/mL (1 brown tube, white cap)	250 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	100 µL @ 5 mg/mL (1 brown tube, black cap)	210 µL @ 5 mg/mL (1 brown tube, black cap)	2.1 mL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated Antibody Anti-hTGF-β1 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)
hTGF-β1 Analyte*	0.3 µg; lyophilized solid (1 tube, clear cap)	0.3 µg; lyophilized solid (1 tube, clear cap)	0.3 µg; lyophilized solid (1 tube, clear cap)
AlphaLISA Simple Immunoassay Buffer	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute hTGF-β1 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 hTGF-β1 is stable for at least 18 months at -20°C. One vial contains an amount of hTGF-β1 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL336S).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL336HV) 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 Adhesive Sealing Film	PerkinElmer Inc.	6050195
EnVision®-Alpha Reader	PerkinElmer Inc.	-
1N HCl*	various	
1.2N NaOH/0.5M HEPES*	various	

* These are recommended when working with biological samples (see page 8 below)

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 AlphaLISA Simple Immunoassay 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.
- The AlphaLISA signal is detected with an EnVision Multilabel 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. The standard curve should be performed in the AlphaLISA Simple Immunoassay Buffer 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 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**	**AlphaLISA Acceptor beads**	**Biotinylated Antibody**	**SA-Donor beads**	**Plate recommendation**
AL336HV	100	100 µL	10 µL	10 µL	10 µL	70 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL336C	250	100 µL	10 µL	10 µL	10 µL	70 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	5 µL	5 µL	35 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	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	1 µL	7 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL336F	5 000	50 µL	5 µL	5 µL	5 µL	35 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	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	1 µL	7 µL	Light gray AlphaPlate-1536 (cat # 6004350)

3 Step High Sensitivity Protocol described below is for 500 assay 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.*

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

2) Preparation of hTGF-β1 analyte standard dilutions:
   a. Reconstitute lyophilized hTGF-β1 (0.3 µg) in 100 µL H_2O.
   b. Prepare standard dilutions as follows in 1X AlphaLISA Simple Immunoassay (change tip between each standard dilution):

Tube	Vol. of hTGF-β1 (µL)	Vol. of diluent (µL) *	[hTGF-β1] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of provided hTGF-β1	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 Simple 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 AlphaLISA Anti-hTGF-β1 Acceptor beads (50 µg/mL):
   a. Prepare just before use.
   b. Add 25 µL of 5 mg/mL AlphaLISA Anti-hTGF-β1 Antibody Acceptor to 2475 µL of 1X AlphaLISA Simple Immunoassay Buffer.

4) Preparation of 10X Biotinylated Anti-hTGF-β1 Antibody (10 nM):
   a. Prepare just before use.
   b. Add 50 µL of 500 nM Biotinylated Anti- hTGF-β1 Antibody to 2450 µL of 1X AlphaLISA Simple Immunoassay Buffer.

5) Preparation of 1.43X Streptavidin (SA) Donor beads (60 µg/mL):
   a. Prepare just before use.
   b. Keep the beads under subdued laboratory lighting.
   c. Add 210 µL of 5 mg/mL SA-Donor beads to 17290 µL of 1X AlphaLISA Simple Immunoassay Buffer.

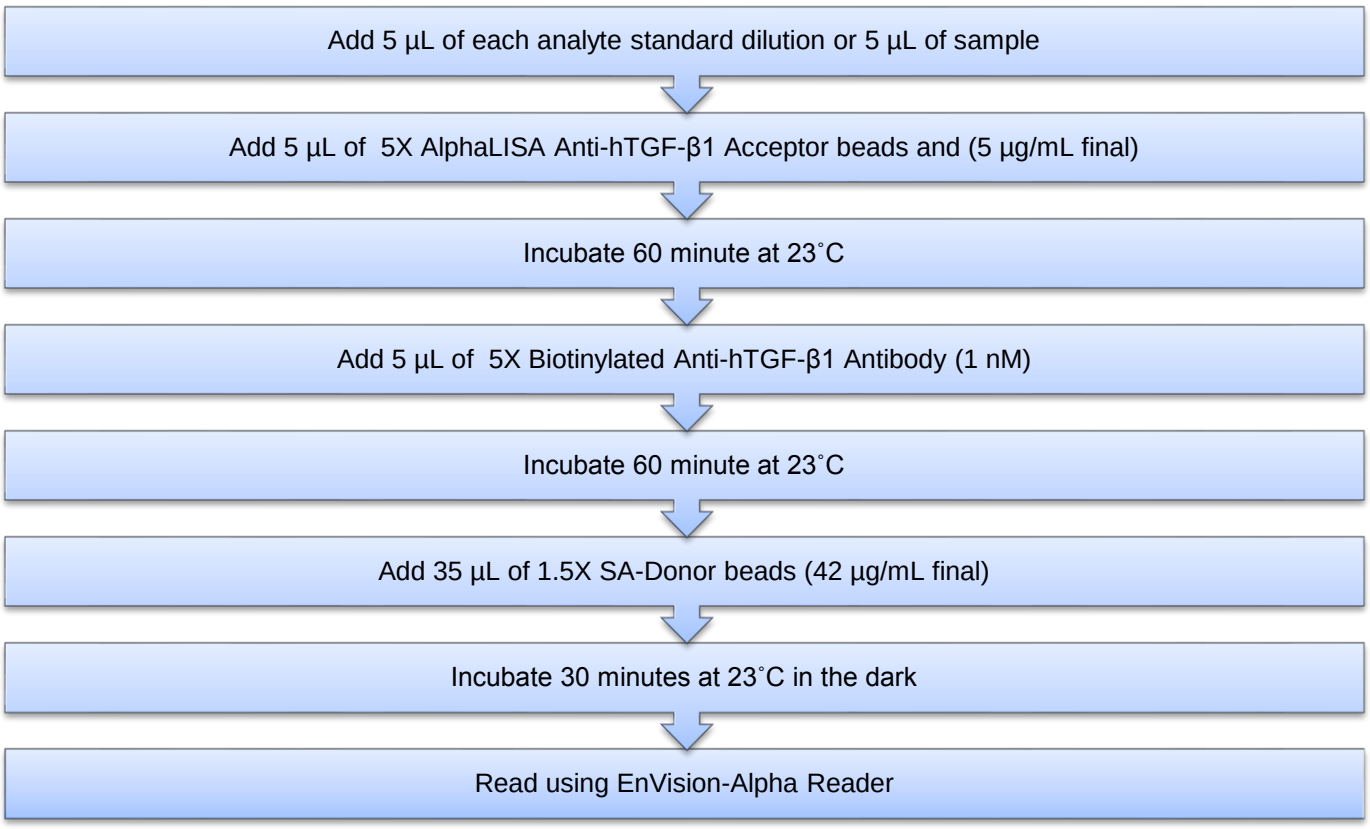
6) Test Sample Preparations:

Test samples such as serum, plasma, cell culture supernatant containing FBS require an acid activation and neutralization procedures to activate latent TGF- β1 to an immnuoreactive form. Sample activation needs to be performed in polypropylene tubes using 2 parts sample and one part 1N HCI and one part 1.2N NaOH/0.5M HEPES.

For example,

a. To 50 µL of sample add 25 uL 1N HCI. Mix well and incubate for 10 minutes at room temperature to complete acid activation.
b. The activated samples are then mixed with 25 uL of 1.2N NaOH/0.5M HEPES to neutralize the samples.
c. All activated samples should be assayed immediately following activation and neutralization.
d. If cell culture supernatants are tested, preparing the standard curve in cell culture media containing 10% non-activated FBS (determination of TGF-β1 in FBS is required in assay) is recommended.
e. All other components of the assay are prepared using 1X AlphaLISA Simple Immunoassay Buffer.

7) 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 3 step high sensitivity protocol using AlphaLISA Simple Immunoassay Buffer (SIAB).

- 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/Medium*	# of experiments
9	BIAB with 0.1%BSA	17
9	DMEM+ 10% FBS	8
17	RPMI + 10% FBS	8

* The standard was prepared in these diluents. Note that LDL/ LLOQ can be decreased (i.e. sensitivity increased) by preparing standards in different matrixes.

- 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 SIAB, DMEM, or RPMI. 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 SIAB.

- Intra-assay precision:

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

hTGF-β1	**BIAB** with 0.1%BSA	DMEM	RPMI
CV(%)	5.5	7.4	5.0

- 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%.

hTGF-β1 (3 ng/ml)	SIAB	DMEM	RPMI

CV (%)	19.2	19.8	17.2

- Spike Recovery:

Three known concentrations of analyte were spiked in SIAB, or in cell culture media containing 10% FBS. All samples, including non-spiked buffer or media were measured in the assay. The average recovery from three independent measurements is reported. Note that the standard curves were prepared in SIAB, DMEM, and RPMI.

Spiked hTGF-β1 (ng/mL)	% Recovery*		
	BIAB with 0.1%BSA	DMEM	RPMI
10	111	100	100
1	95	90	95
0.1	104	100	103

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- Specificity:

Cross-reactivity of the hTGF-β1 Kit was tested using the following proteins at 100 ng/mL in SIAB. Reactivity to hTGF-β1 is 100%.

Protein	% Cross-reactivity
Human TGF-β2	0
Human TGF-β3	0
Mouse TGF-β1	67
Porcine TGF-β1	87

Human Serum Experiments

To validate the assay kit, commercially available human serum samples (acid activated) with unknown concentrations of hTGF-β1 were used to examine dilution linearity and spike recovery.

To determine the basal levels of hTGF-β1 in activated serum, the samples were serially diluted and the concentrations were determined using a standards curve made in SIAB.

As shown in the table, when samples are diluted 32 fold or greater, 4 to 8 ng/mL of hTGF-β1 was detected in the serum.

Dilution Factor	hTGF-β1 Detected in Activated serum (ng/mL)
16	2.7
32	4.5
64	5.5
128	5.8
256	6.5
512	7.2
1024	8.4
2048	5.5

For the spike recovery, activated human serum (2x diluted following acid activation and neutralization procedures) containing 300 ng/mL hTGF-β1 was serially diluted using a standard prepared SIAB. Optimal recovery was achieved with

greater than 32 fold dilution.

Spiked in activated serum (ng/mL)	Dilution Factor	% Recovery
18.8	16	65
9.4	32	80
4.7	64	84
2.3	128	83
1.2	256	83
0.6	512	94
0.3	1024	99
0.1	2048	109

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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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

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