MANUAL

AlphaLISA® Human and Mouse Fibronectin Detection Kit

Product number: AL3174

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of **human and mouse Fibronectin** using a homogeneous no wash AlphaLISA assay.

Kit contents: The kit contains 6 components: AlphaLISA Acceptor beads coated with anti-Fibronectin Antibody, Streptavidin-coated Donor beads, Biotinylated anti-Fibronectin antibody, Lyophilized hFibronectin 10X AlphaLISA Immunoassay Buffer and 5X AlphaLISA Lysis buffer.

Sensitivity: Lower Detection Limit (LDL): **210 pg/mL**
Lower Limit of Quantification (LLOQ): **773 pg/mL**
EC_{50}: 300 ng/mL

Dynamic Range: **210 – 3 000 000 pg/mL**

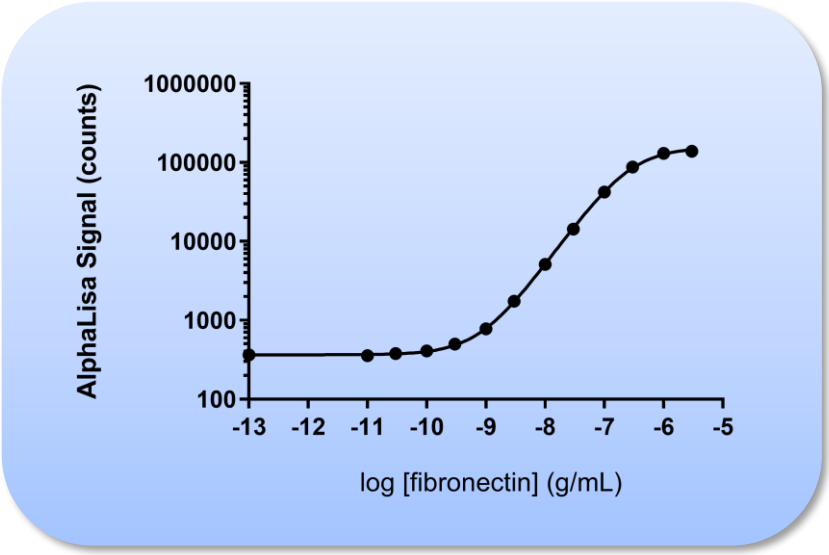


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a grey AlphaPlate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at 4 ºC. For reconstituted analyte, aliquot and store at -20 ºC for up to 1 month. **Avoid freeze-thaw cycles as fibronectin protein is prone to aggregation**.

Stability: This kit is stable for at least 6 months from the date of manufacture when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Fibronectin is a very large (500+ kDa) protein dimer that plays a critical role in maintaining the extracellular matrix in tissues by binding both to integrins and to collagen fibers via different domains. The protein also modulates cell migration and differentiation and is involved in wound healing and development. Aberrant fibronectin expression has been shown in cancer and fibrosis models.

The AlphaLISA Fibronectin Detection Kitis designed to detect all variants of human and mouse fibronectin (including ED-A/ED-B splice domains) in both plasma and cell culture medias.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in culture medias (DMEM, and RPMI 1640), in human serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-hFibronectin antibody binds to the streptavidin coated AlphaLISA Donor beads, while another anti-hFibronectin antibody is conjugated to AlphaLISA Acceptor beads. In the presence of hFibronectin, the beads come into proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in emission with λ_{max} at 615 nm (Figure 2).

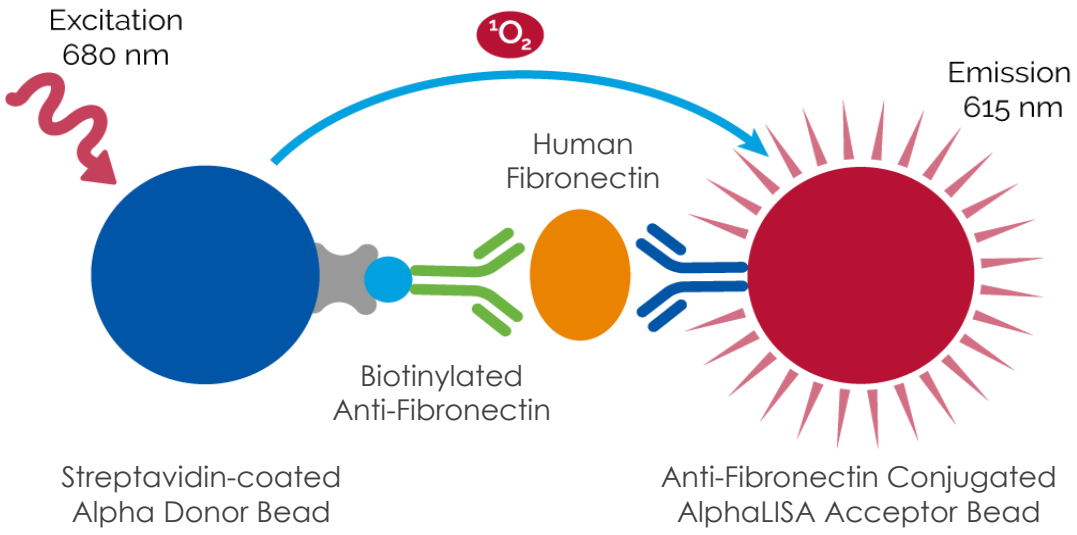


Figure 2. AlphaLISA Human and Mouse Fibronectin Detection Assay Principle.

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 (Roscolux filters #389 from Rosco are recommended) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The biotinylated anti-Fibronectin antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3174HV (100 assay points)	AL3174C (500 assay points)	AL3174F (5000 assay points)
AlphaLISA Anti-Total Fibronectin Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, 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 II, pH 7.4	40 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 Anti-Total Fibronectin 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 Recombinant Total Fibronectin	3 µg (1 tube, clear cap)	3 µg (1 tube, clear cap)	3 µg (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X)	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle
AlphaLISA Lysis Buffer (5X)	2 mL, 1 small bottle	5 mL, 1 small bottle	50 mL, 1 medium bottle

* Reconstitute lyophilized analyte in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped 0.5 mL polypropylene vials and stored at -20°C for future experiments. **The aliquoted analyte at -20°C is stable up to 1 month. Upon unfreezing and usage of reconstituted analyte, discard excess material. Avoid freeze-thaw cycles as fibronectin protein is prone to aggregation**. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3174S).

** 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 100 µL in 96-well plates or 50 µL in 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-Fibronectin 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 Plus Adhesive Sealing Film	Revvity Inc.	6050185
EnVision®-Alpha Reader	Revvity Inc.	-

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 (2000*g*, 10-15 sec). Re-suspend all reagents by vortexing before use.
- Use Milli-Q® grade H_2O to dilute 10X AlphaLISA Immunoassay 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.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment.

Assay Procedure

- The manual described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The manuals also include testing samples in 452 wells. If different amounts 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 manual 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.

Format	# of data points	Volume				Plate recommendation
		Final	Sample	AlphaLISA Acceptor Beads/biotin antibody mix	SA-Donor beads	
AL3174HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3174C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290)
	500	50 µL	5 µL	5 µL	40 µL	½ Area AlphaPlate-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) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3174F	5 000	50 µL	5 µL	5 µL	40 µL	½ Area AlphaPlate-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) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)

The following 2-Step manual 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 1X AlphaLISA Immunoassay Buffer:
Add 1 mL of 10X AlphaLISA Immunoassay Buffer to 9 mL Milli-Q® grade H_2O.

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

3) Preparation of Total Fibronectin analyte standard dilutions:

a. Reconstitute lyophilized Total Fibronectin (3 µg) in 100 µL Milli-Q® grade H_2O.
b. Prepare standard dilutions as follows in 1X AlphaLISA **Lysis Buffer** (change tip between each standard dilution):

Tube	Vol. of Total Fibronectin (µL)	Vol. of diluent (µL) *	[Total Fibronectin] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted Total Fibronectin	90	3.00E-06	3 000 000
B	60 µL of tube A	140	1.00E-06	1 000 000
C	60 µL of tube B	120	3.00E-07	300 000
D	60 µL of tube C	140	1.00E-07	100 000
E	60 µL of tube D	120	3.00E-08	30 000
F	60 µL of tube E	140	1.00E-08	10 000
G	60 µL of tube F	120	3.00E-09	3 000
H	60 µL of tube G	140	1.00E-09	1 000
I	60 µL of tube H	120	3.00E-10	300
J	60 µL of tube I	140	1.00E-10	100
K	60 µL of tube J	120	3.00E-11	30
L	60 µL of tube K	140	1.00E-11	10
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

Important Note: Pay attention to use AlphaLISA Lysis Buffer as diluent for standards, not the Immunoassay buffer. Immunoassay buffer is used for preparation of reagents working solutions only.

* Dilute standards in **diluent** (e.g., 1X AlphaLISA **Lysis 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).

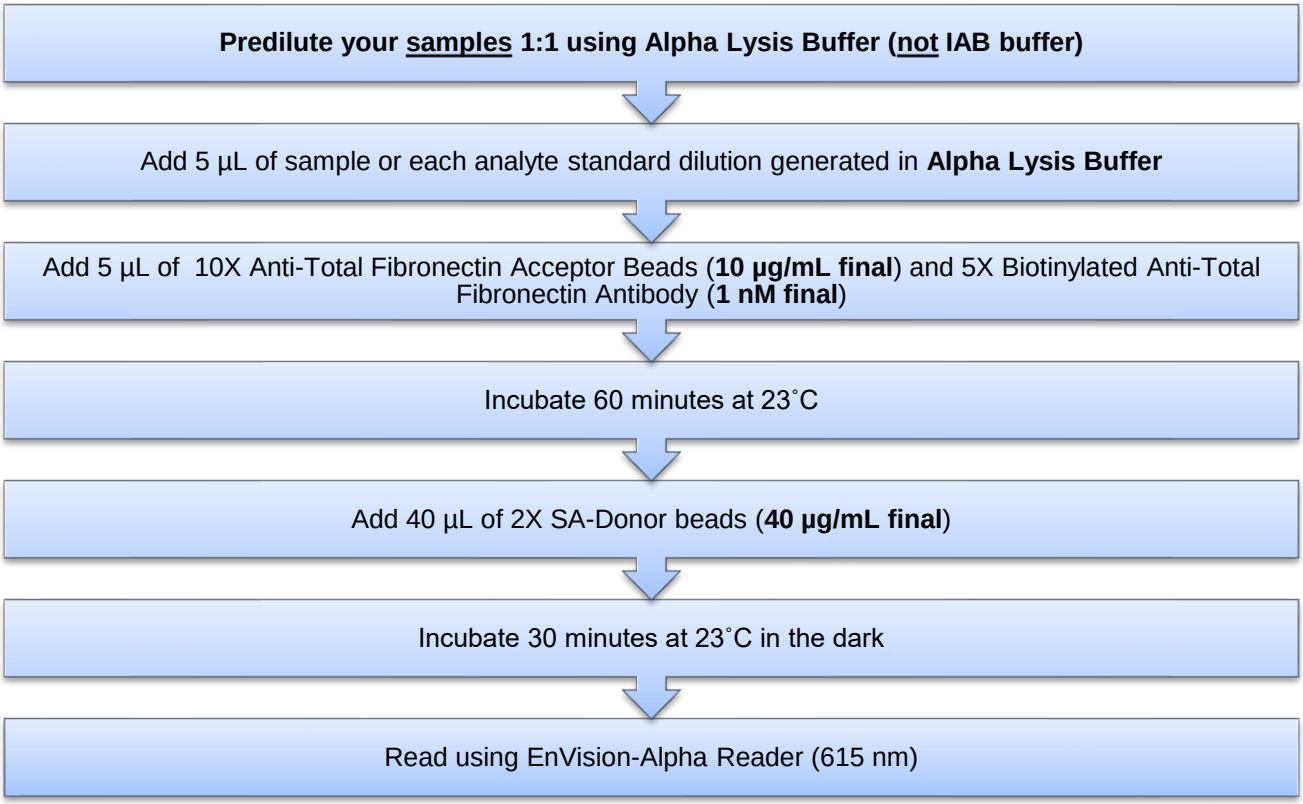
4) Preparation of 10X AlphaLISA Anti-Total Fibronectin Antibody Acceptor beads (100 µg/mL) and Biotinylated Anti-Total Fibronectin Antibody (10 nM):

a. Prepare just before use.
b. Add 50 µL of 5 mg/mL AlphaLISA Anti-Total Fibronectin Antibody Acceptor Beads and 50 µL of 500 nM Biotinylated Anti-Total Fibronectin Antibody to 2400 µL of 1X AlphaLISA Immunoassay Buffer (**IAB**).

5) Preparation of 1.25X Streptavidin (SA) Donor beads (50 µg/mL):

a. Prepare just before use.
b. Keep the beads under subdued laboratory lighting.
c. Add 200 µL of 5 mg/mL SA-Donor beads to 19800 µL of 1X AlphaLISA Immunoassay Buffer (**IAB**).

6) In an AlphaPlate (384 wells):



Important note: Fibronectin is a protein that tends to aggregate in solution. To prevent this, **biological assay samples (not prepared standards) must be diluted 1:1 in AlphaLISA Lysis buffer** before plating in the assay. After interpolation of your samples on the standard curve, multiply the read concentration by a factor of 2 to take into consideration this predilution step.

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.
- **As there is a predilution step (1:1) in lysis buffer prior of the assay, multiply the read concentrations of your samples by a factor of x2.**
- If samples have been further diluted, the concentration read must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined by a 2-step manual using AlphaLISA Lysis buffer as the analyte diluent and Immunoassay Buffer 1X as assay buffer. The analytes (standards) were prepared in Lysis Buffer 1X, DMEM, DMEM + 10% FBS, RPMI, RPMI 1640 + 10% FBS, 100% FBS and 10% FBS in Lysis buffer. All media samples were diluted 1:1 in Lysis buffer. All other components were prepared in AlphaLISA Immunoassay Buffer 1X.

- Assay Sensitivity:

AlphaLISA sensitivity was determined by a 2-step manual using AlphaLISA Lysis buffer as the analyte diluent and Immunoassay Buffer 1X as assay buffer. The analytes (standards) were prepared in Lysis Buffer 1X, DMEM, DMEM + 10% FBS, RPMI, RPMI 1640 + 10% FBS, 100% FBS and 10% FBS in Lysis buffer. All media samples were diluted 1:1 in Lysis buffer. All other components were prepared in AlphaLISA Immunoassay Buffer 1X.
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
210	AlphaLISA Lysis buffer 1X
129	DMEM
697	DMEM + 10% FBS
384	RPMI
392	RPMI 1640 + 10% FBS
120	100% FBS
159	10% FBS

- 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 Lysis buffer 1X, DMEM, DMEM + 10% FBS, RPMI, RPMI 1640 + 10% FBS, and 100% FBS and 10% FBS. All media samples were diluted 1:1 in Lysis buffer. All other components were prepared in AlphaLISA Immunoassay Buffer 1X. 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 6 independent determinations in triplicate. Shown as CV%.

hFibronectin	AlphaLISA Lysis 1X	DMEM	DMEM + 10% FBS	RPMI 1640	RPMI 1640 + 10% FBS	100% FBS	10% FBS
Intra-CV (%)	1.9	4.2	10.6	4.1	4.6	1.9	3.4

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 determinations. Shown as CV%.

hFibronectin	AlphaLISA Lysis Buffer 1X	DMEM	DMEM + 10% FBS	RPMI 1640	RPMI 1640 + 10% FBS	100% FBS	10% FBS
Inter-CV (%)	6.4	3.2	7.7	14.8	11.9	5.8	6.6

- Spike Recovery:

Three known concentrations of analyte were spiked into Lysis Buffer 1X, DMEM, DMEM + 10% FBS, RPMI, RPMI 1640 + 10% FBS, 10% FBS and 100% FBS. All media samples were diluted 1:1 in Lysis buffer. All samples, including non-spiked diluents were measured in the assay. Standard curves were generated in same media than the samples, diluted 1:1 in lysis buffer. For FBS samples, standard curves were generated in 1:1 FBS and Lysis buffer. All other assay components were diluted in AlphaLISA Immunoassay Buffer 1X.

Antigen Spike Recovery (%)							
Spiked hFibronectin (ng/mL)	**Lysis Buffer 1X**	**DMEM**	**DMEM + 10% FBS**	**RPMI**	**RPMI 1640 + 10% FBS**	**100% FBS**	**10% FBS**
1 000	93	82	88	87	99	80	82
100	106	118	102	117	102	112	101
10	122	102	125	129	124	94	111

- Specificity:

Cross-reactivity of the AlphaLISA Fibronectin Detection Kit was tested using the various proteins in a series of assays. The cross reactivities were calculated using the signals of 1000 ng/mL Fibronectin as 100%.

Proteins	Cross Reactivity (%)	Interference 5 µg/mL
Human plasma fibronectin EDA-	100%	-
Fibronectin EDA+	100%	-
Fibronectin EDB+	100%	-
Mouse fibronectin	100%	-
Rat fibronectin	100%	-
Bovine fibronectin**	None	-95% at 3000 ng/mL Fibronectin
10% FBS**	None	-70% at 1 000 ng/mL fibronectin
Fibronectin EDA fragment	None*	None
Fibronectin EDB fragment	None*	None
Integrin $\alpha_1\beta_5$	None*	None*
Anastellin	None*	-10% at 700 ng/mL Fibronectin*
Collagen (mix)	-	None*
Heparin	None	-15% at 1000 ng/mL Fibronectin

** Information based on data generated with the same antibodies established with another homogeneous technology*.

***** FBS commonly used for cell culture does contain fibronectin concentrations in the µg/mL range that interferes with the assay. If FBS is present in samples, a standard curve must be generated in a medium having the same FBS concentration than your samples to measure the correct Fibronectin concentration. See recommendations in the cell culture experiments below when using FBS.***

- Human Cells Experiments:

Human **DU-145 prostate cancer** cells were used for testing Fibronectin secretion. DU-145 cells Treated with TGF- β1 are known to secrete significant amounts of Fibronectin, upon activation of the TGF- β1/SMAD pathway.

- Cell culture
  - DU-145 cells were thawed at 37°C for 2 minutes, spun down and resuspended in 1 mL of RPMI 1640 + 10% FBS.
  - The cells were added to a T-75 flask and left to grow until confluence in a 5% CO_2 37C incubator.
  - Cells were harvested using 0.25% trypsin solution.
  - Cells were washed twice with cold sterile PBS.
  - Cells were counted on a CellEx counter and were plated in 8-times replicas in a 96-well CulturPlate at the following amounts: 100000, 50000, 25000, 12500, 6250, 3125, 1560, 780, 390, 195, 98 and 0 cells per well (multiply by 10 to obtain concentration in cells per mL) in 100 µL additions in RPMI + 10% FBS.
  - Cells were incubated for 24 hours.

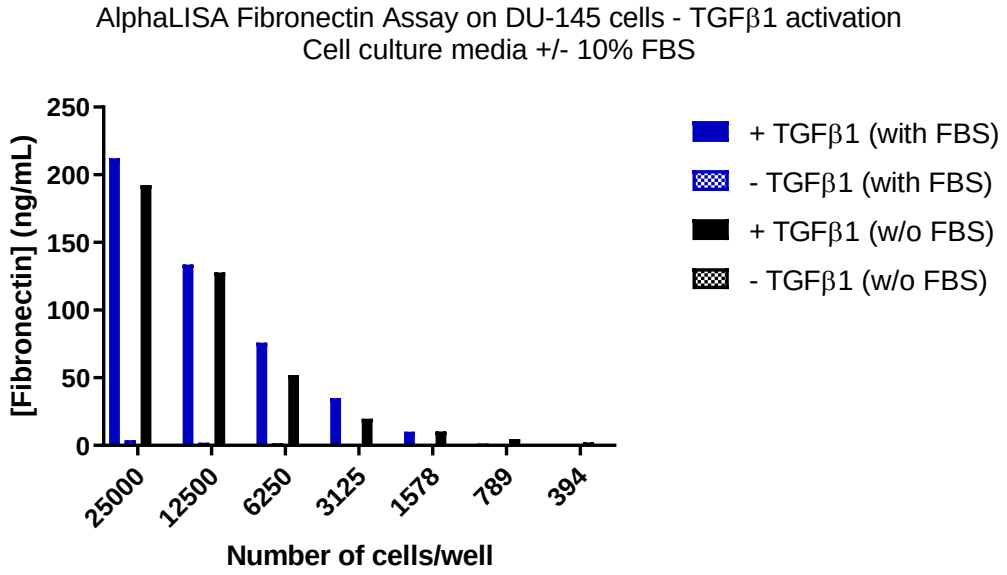
- Pharmacological treatment

  - **IMPORTANT RECOMMENDATIONS FOR FBS USE**
    i) *Bovine TGF*β1 *is present in cell culture grade FBS and has a similar potency to induce cells than human TGF*β1*, the protein being highly conserved among species.*
    It is not recommended to use FBS for final stages of cell treatments to avoid having it increasing basal levels of biomarkers downstream of the TGF/SMAD pathway. Depending on cell passage, cell type and pharmacological treatment, this effect is not always noticeable (see data below).

    ii) *Bovine fibronectin in present in very high levels (µg/mL range) in cell culture grade FBS. As a result, it is not recommended to use it in your final samples. However, it is possible to precisely measure Fibronectin concentration in 10% FBS containing samples but in this case the assay standard curve must be diluted in the same media than your samples, having the same FBS concentration.*

  - Supernatant was removed and discarded.
  - In half of the cell rows, 100 µL of RPMI without FBS were added.
  - In the other half, 100 µL of RPMI + 10% FBS were added.
  - Cells were incubated for 24 hours.
  - Supernatant was removed and discarded.
  - The following additions were made:
    i) Row 1: 100 µL of RPMI without FBS
    ii) Row 2: 100 µL of RPMI with FBS
    iii) Row 3: 100 µL of RPMI without FBS with 5 ng/mL of TGF-β1
    iv) Row 4: 100 µL of RPMI with FBS with 5 ng/mL of TGF-β1
  - Rows 5 to 8 were treated as above but with 10% FBS present.
  - Cells were incubated for 24 hours.
- Supernatant was removed and collected in a polypropylene 96-wells storage plate.
- 100 µL of 1X AlphaLISA Lysis buffer was added to each well.
- The plate was incubated at 23°C for 30 minutes with agitation.
- Both supernatant and lysate were placed at -80°C until testing.

Both supernatant and lysate were tested using the 2-step high concentration assay. For this, all supernatants were diluted 1:1 in Lysis buffer, and a standard curve of RPMI (without or with 10% FBS) was used. For the lysates, a standard curve of analyte in Lysis buffer was used.



Results show that fibronectin secretion in DU-145 cells is dependent of TGF- β1.

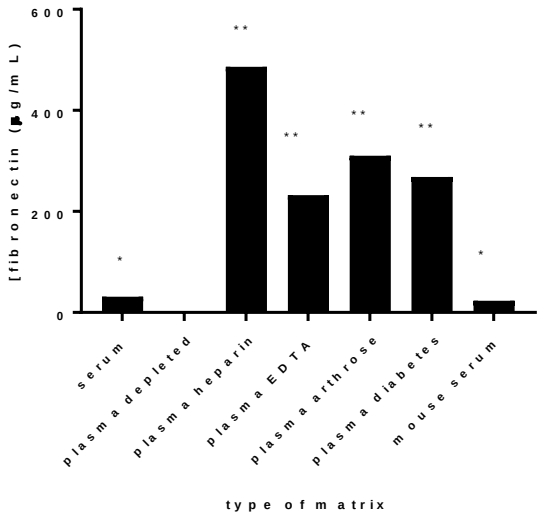
*Note: Fetal bovine serum has been shown to contain bovine TGF-*β1 *that can act on human cells. This explains the slightly higher secretion levels shown with cell stimulated in 10% FBS.*

- Test of serum and plasma specimen

  The following biological fluids were tested for concentration of fibronectin.

  - Human serum (normal from pooled donors) (Jackson Immunoresearch 009-000-001)
  - Fibronectin depleted human plasma (Innovative Research IHUFBNDP)
  - Human plasma collected by heparin treatment (Innovative Research IPLASLIH)
  - Human plasma collected by EDTA treatment (Innovative Research IPLASK2E)
  - Human plasma from single arthritic patient (Innovative Research IPLASRA)
  - Human plasma from single diabetic type 2 patient (Innovative Research IPLASDIB)
  - Mouse serum (Jackson Immunoresearch 015-000-120)

  All fluids were diluted 1:1 in Lysis buffer, and then further diluted at half-a log dilutions in Lysis buffer and tested on a standard curve of Lysis buffer. The highest results that were before the hook effect was plotted on the standard curve and then corrected for dilution factor.



* Litterature: around 20-30 µg/mL

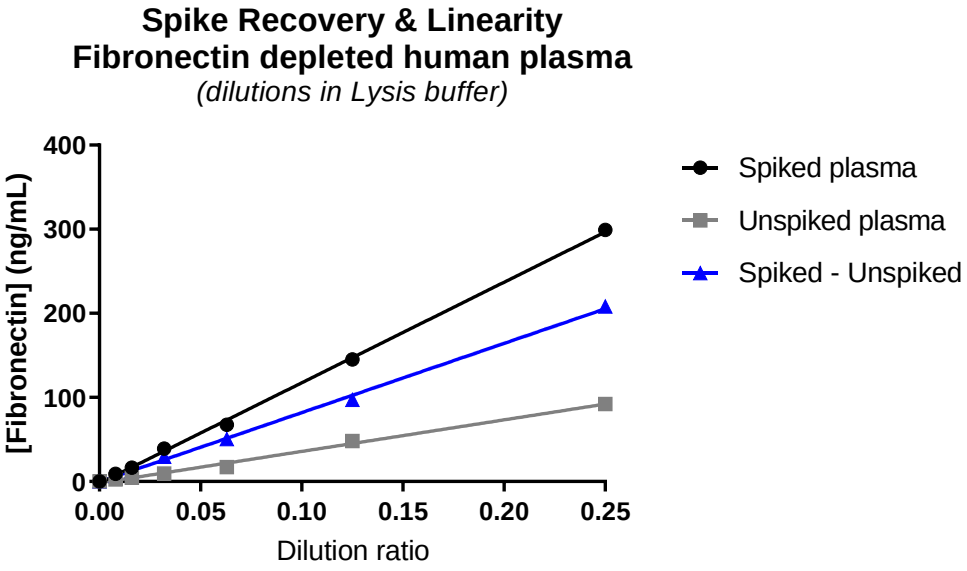
** Litterature: between 200-600 µg/mL

Matrix	[Fibronectin] µg/mL
Human serum	**31**
Human fibronectin depleted plasma	**1.85**
Human plasma (Heparin)	**486**
Human plasma (EDTA)	**282**
Human plasma from Arthritic patients	**310**
Human plasma from Diabetic patient	**268**
Mouse serum	**23**

- Fibronectin was detected in the assay at concentrations that are within accepted ranges from the literature.
- Serum samples, as shown in the literature, show much less fibronectin that plasma, indicating that preanalytical process linked to serum generation highly depletes fibronectin.
- Depleted plasma by affinity chromatography shows much lower levels of fibronectin, but the protein is still detectable with this assay.
- Mouse protein can also be detected in biological fluids.

- <u>Linearity & Spike Recovery in human EDTA plasma</u>

  - Assays were made in human plasma depleted of fibronectin by gelatin affinity chromatography.
  - A sample of plasma was spiked or not at 1 µg/mL with the kit provided standard.
  - Sample was diluted 1:1 in AlphaLISA Lysis buffer.
  - Dilutions were made of the sample with a dilution factor of 1 (raw sample), 2, 4, 8, 16, 32 and 64 using lysis buffer.
  - Results were plotted on a standard curve of standard with samples diluted in lysis buffer.
  - Concentration of spiked material was measured by subtracting unspiked results from spiked sample.
  - Results were corrected for initial 1:1 dilution



Dilution Factor	Expected concentration	Measured concentration	Recovery
Neat (1:1 in lysis buffer) Spiked Fibronectin in Depleted EDTA Plasma	250	208	**83%**
2	125	97	**77%**
4	62.5	50	**80%**
8	32.125	24	**76%**
16	16	12	**75%**
32	8	7	**81%**

Note, the highest point in the dilution is not on the curve as it is outside the range of interpolation on the standard curve.

Results show acceptable linearity and recovery.

The Human and Mouse Fibronectin assay is compatible with HUMAN EDTA PLASMA SAMPLES, spike and recovery experiments being possible in fibronectin depleted EDTA plasma.

Other specimen types were not tested for spike and recovery because of limitations of extremely high endogenous fibronectin concentrations.

Troubleshooting Guide

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

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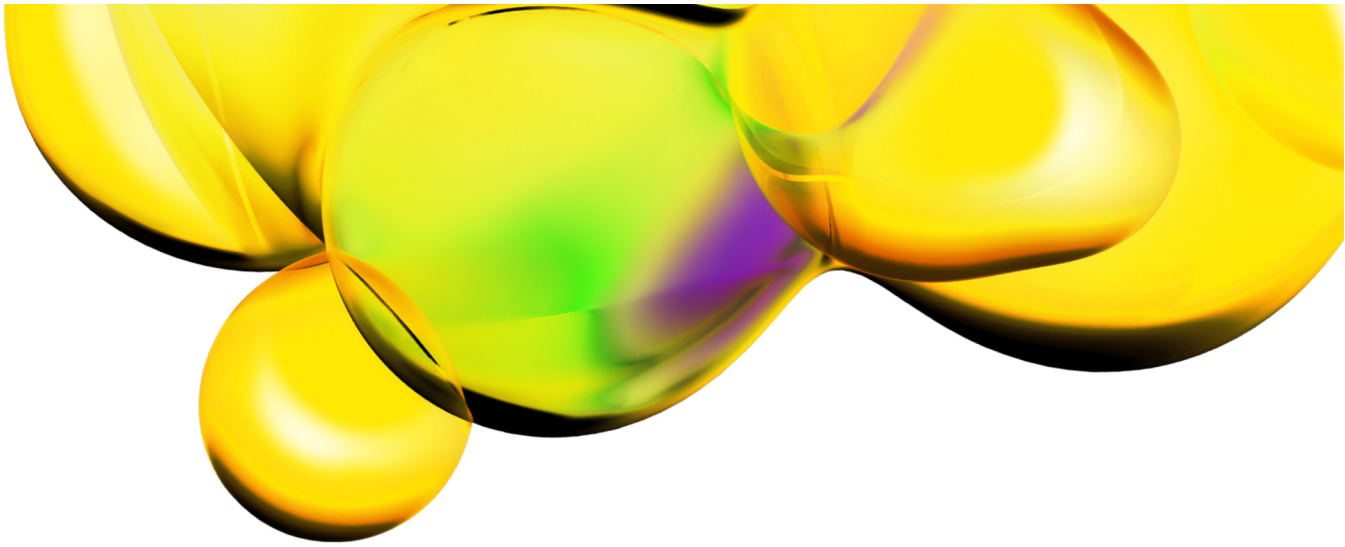

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