MANUAL

AlphaLISA® FLT1/VEGF R1 (Human) Detection Kit

Product number: AL3018 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of Human FLT1 (hFLT1) in human cell culture supernatants and serum samples using a homogeneous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 61 pg/mL

Lower Limit of Quantification (LLOQ): 223 pg/mL

EC_{50}: 720 ng/mL

Dynamic range: 61–3 000 000 pg/mL (Figure 1).

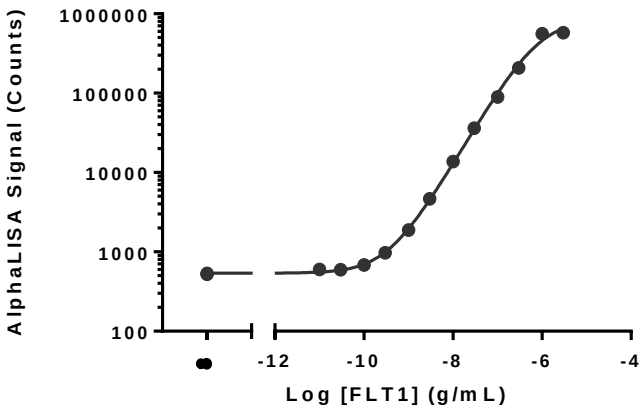


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2103.

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.

Analyte of Interest

Human FMS Related Tyrosine Kinase 1 (FLT1) also known as Vascular Endothelial Growth Factor Receptor 1 is a receptor tyrosine kinase involved in the regulation of angiogenesis and vasculogensis. FLT1 plays a roles in cell migration, as well as promotes cell proliferation. It spans the cell membrane with a cytoplasmic transmembrane domain and an extracellular ligand binding region. With its role in cell proliferation, FLT1 is studied for its effect on cancer and tumor growth, specifically its link to metastasis.

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).

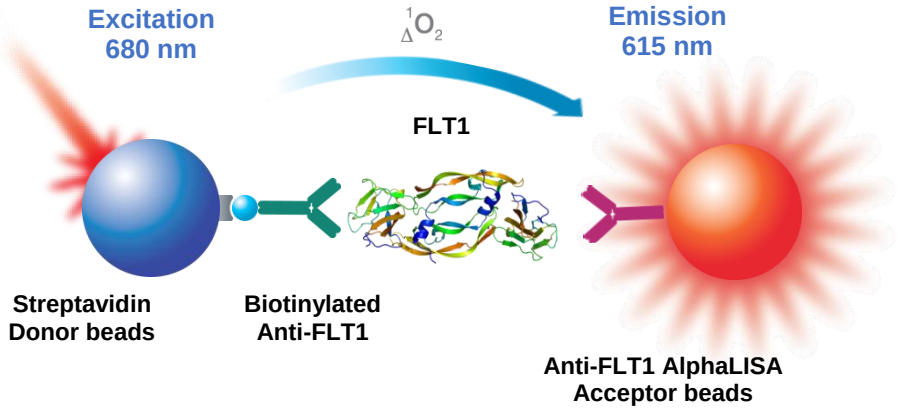


Figure 2. AlphaLISA Assay principle.

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	AL3018HV (100 assay points***)	AL3018C (500 assay points***)	AL3018F (5000 assay points***)
AlphaLISA Anti-hFLT1 Acceptor beads stored in PBS, 0.05% Kathon, 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, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	100 µL @ 5 mg/mL (1 brown tube, black cap)	1 mL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated Antibody Anti-hFLT1 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)
AlphaLISA hFLT1 (3 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap	1 tube, clear cap
AlphaLISA Immunoassay Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute hFLT1 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. One vial contains an amount of hFLT1 sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3018S).

** 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 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 Plus Adhesive Sealing Film	Revvity Inc.	6050185
EnVision®-Alpha Reader	Revvity 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 H2O (18 MΩ•cm) 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 Plus Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Plus 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 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 and Biotinylated Antibody**	**SA-Donor beads**	**Plate recommendation**
AL3018HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3018C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	5 µL	40 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	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)
AL3018F	5 000	50 µL	5 µL	5 µL	40 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	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)

2 Step High-concentration Protocol (2 incubation steps) – Dilution of standards can be done in 1X AlphaLISA Immunoassay Buffer.

Preparation of 1X AlphaLISA Immunoassay Buffer :

Add 10 mL of 10X AlphaLISA Immunoassay Buffer to 90 mL H_2O.

1) Preparation of hFLT1 analyte standard dilutions:

   a) Reconstitute lyophilized hFLT1 (3 µg) in 100 µL of H_2O.
   b) Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of hFLT1 (µL)	Vol. of diluent (µL) *	[hFLT1] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of provided hFLT1	90	3.00E-06	3 000 000
B	60 µL of tube A	120	1.00E-06	1 000 000
C	60 µL of tube B	140	3.00E-07	300 000
D	60 µL of tube C	120	1.00E-07	100 000
E	60 µL of tube D	140	3.00E-08	30 000
F	60 µL of tube E	120	1.00E-08	10 000
G	60 µL of tube F	140	3.00E-09	3000
H	60 µL of tube G	120	1.00E-09	1000
I	60 µL of tube H	140	3.00E-10	300
J	60 µL of tube I	120	1.00E-10	100
K	60 µL of tube J	140	3.00E-11	30
L	60 µL of tube K	120	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

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

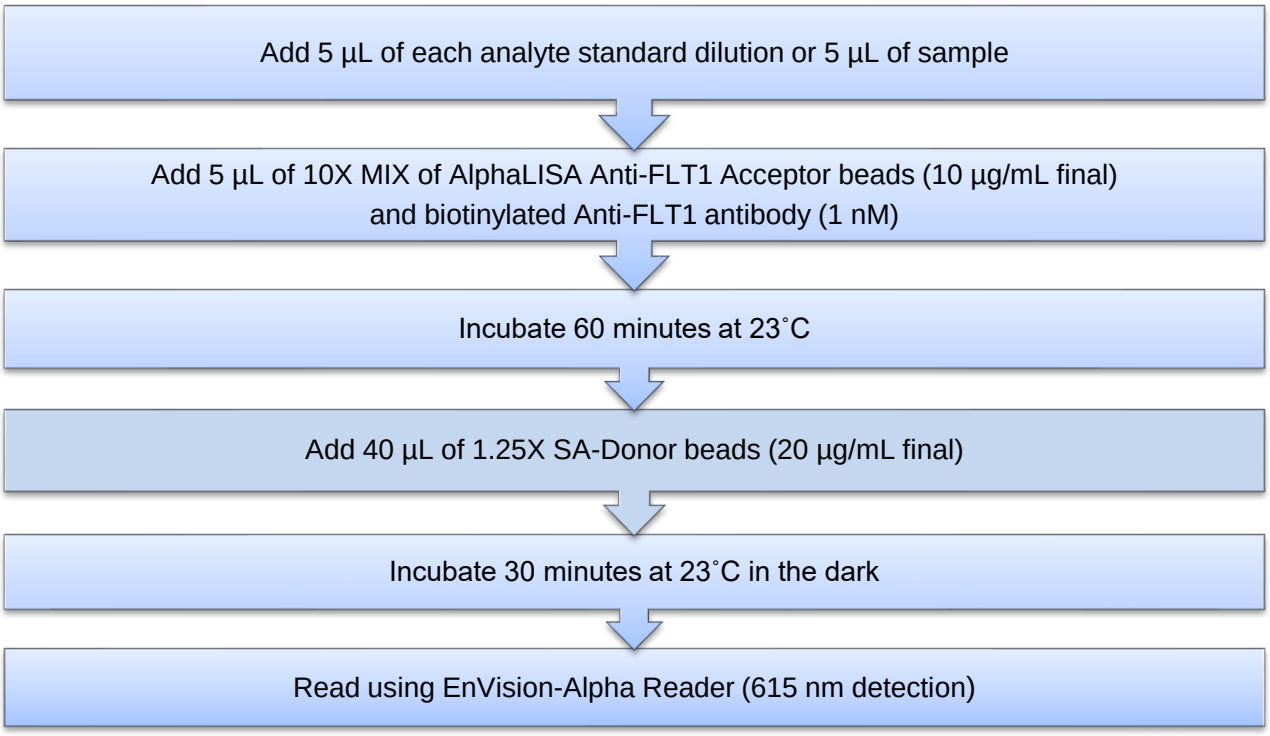
2) Preparation of 10X MIX of AlphaLISA Anti-Human FLT1 Antibody Acceptor beads and Biotinylated Anti-Human FLT1 Antibody (100 µg/mL / 10 nM):

   a. Add 50 µL of 5 mg/mL AlphaLISA Anti-FLT1 Acceptor beads and 50 µL of 500 nM biotinylated Anti-FLT1 antibody to 2400 µl of 1X AlphaLISA Immunoassay Buffer.
   b. Prepare just before use.

3) Preparation of 1.25X Streptavidin (SA) Donor beads (25 µg/mL):

   a. Keep the beads under subdued laboratory lighting.
   b. Add 100 µL of 5 mg/mL SA-Donor beads to 19 900 µL of 1X AlphaLISA Immunoassay Buffer.
   c. Prepare just before use

4) 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 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 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/Medium*	# of experiments
61	AlphaLISA Immunoassay Buffer	6
297	DMEM+ 10% FBS	6
2946	RPMI + 10% FBS	6

* The standard was prepared in these diluents.

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). Since RPMI significantly reduced assay sensitivity we do not recommend using RPMI medium with this detection kit.

- 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 AlphaLISA Immunoassay Buffer (IAB), 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 AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

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

hFLT1	IAB	DMEM +10% FBS	RPMI +10% FBS
CV(%)	7	7	7

- 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 are CV%.

hFLT1 (30 ng/ml)	IAB	DMEM +10% FBS	RPMI +10% FBS
CV (%)	5	8	7

- Spike Recovery:

One known concentration of analyte was spiked in buffer or culture media respectively. The average recovery from three independent measurements is reported.

Spiked hFLT1 (ng/mL)	% Recovery		
	IAB	DMEM+10% FBS	RPMI+10% FBS
300	106	131	113
30	98	85	92
3	103	105	125

- Specificity:

Cross-reactivity of the AlphaLISA hFLT1 Kit was tested using the following proteins at 100 ng/mL in IAB. Reactivity to hFLT1 is 100%.

Protein	% Cross-reactivity
mFLT1 / VEGF R1	0
rbFLT1 / VEGF R1	0
hVEGF R2 / FLK1	0
hVEGF R3 / FLT4	0

Lysate Experiments

- Overexpression lysate of FLT1

Lysates from native cells which transiently overexpress FLT-1 (Novus) were diluted with lysis buffer. FLT1 was detected in cell lysates and compared to a standard curve prepared in AlphaLISA immunoassay buffer. AlphaLISA lysis buffer can also be used in replacement of RIPA lysis buffer.

Dilution Factor	Detected FLT1 concentration (g/mL)	Calculated FLT1 concentration in Lysate (ng/mL)
2	1.5E-8	30
4	7.5E-9	30
8	3.5E-9	28

- Spike Recovery:

A 300 ng/mL concentration of FLT1 was spiked into Human HeLa Cell Lysate (unstimulated, Millipore) and AlphaLISA Immunoassay Buffer (IAB) was used as the diluent. FLT1 was not detectable in non-spiked HeLa Cell Lysate (data not shown). The average recovery was reported from 3 wells and compared to FLT1 standard in IAB. We recommend diluting cell lysates between 10-100 fold for proper recovery.

Dilution Factor	% Recovery
1	38
10	77
100	105

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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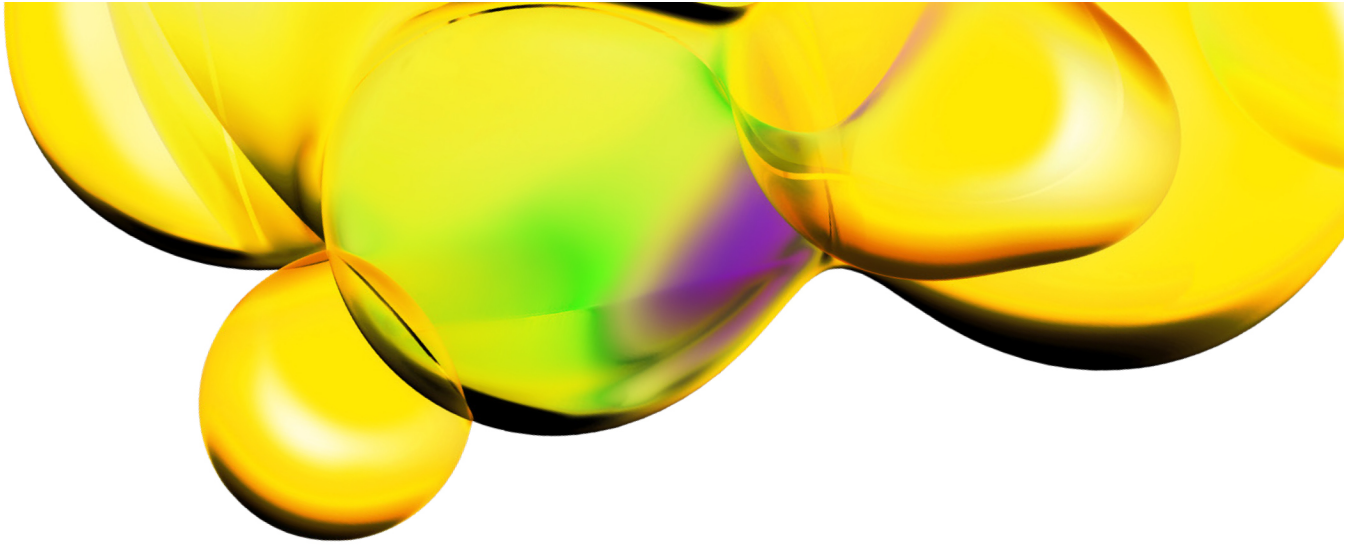

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