MANUAL

Technology: AlphaLISA™

AlphaLISA Human and Mouse CXCL12 Detection Kit

Part number:	AL3207HV	AL3207C	AL3207F
Assay points:	100	500	5,000

Storage: Store kit in the dark at 4 °C. For reconstituted analyte, aliquot and store at -20 °C. Avoid freeze-thaw cycles.

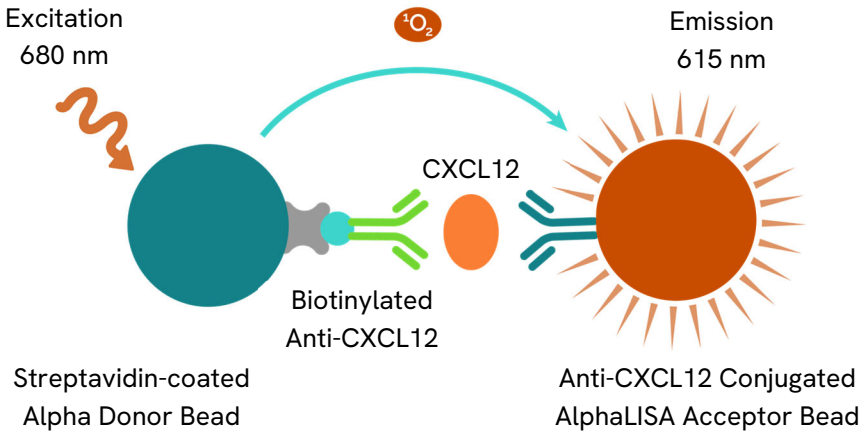
Version: 1 Date: April 2025

ANALYTE OF INTEREST

CXCL12 (SDF-1) is a homeostatic chemokine constitutively expressed by bone marrow stromal cells and is present in many other tissues (skin, thymus, lymph nodes, lung, liver). It plays an essential role in homeostatic regulation of leukocyte traffic, hematopoiesis, organogenesis, cell differentiation and tissue regeneration. CXCL12 binds to and activates the CXCR4 receptor. CXCL12 and CXCR4 are overexpressed in various cancer types, and this aberrant expression strongly promotes proliferation, migration and invasion through multiple signal pathways.

DESCRIPTION OF THE ALPHALISA ASSAY

AlphaLISA technology allows the detection of molecules of interest in DMEM, RPMI and plasma in a highly sensitive, quantitative, reproducible, and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-CXCL12 antibody binds to the streptavidin coated AlphaLISA Donor beads, while an anti-CXCL12 antibody is conjugated to AlphaLISA Acceptor beads. In the presence of CXCL12, 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.



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) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The biotinylated anti-CXCL12 antibody contains sodium azide. Contact with skin or inhalation should be avoided.

KIT CONTENT: REAGENTS AND MATERIALS

Kit components	AL3207HV***	AL3207C****	AL3207F****
AlphaLISA Anti-CXCL12 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	80 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-*CXCL12* 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 human CXCL12*	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)
AlphaLISA BSA Assay Buffer(5X)	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large 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. Refer to the product CoA for stability information on the reconstituted analyte stored at -20°C Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3207S).

** Extra buffer can be ordered separately (cat # AL021C: 10 mL, cat # AL021F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates.

**** The number of assay points is based on an assay volume of 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-CXCL12 antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Additional reagents and materials:

The following materials are recommended but not provided in the kit:

Item	Suggested source
Light gray AlphaPlate™-384	Revvity Inc.
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.
EnVision®-Alpha Reader	Revvity Inc.

RECOMMENDATONS

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 the Donor and Acceptor beads by vortexing before use.
- Use Milli-Q® grade H_2O to dilute buffers 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 Film to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multimode 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. It is recommended to avoid multiple reads of the same well of the assay plate.
- The standard curves shown in this manual are provided for information only. A standard curve must be generated for each experiment.

ASSAY PROCEDURE

- 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 different amounts of samples are tested, the volumes of all reagents must 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.

Format	# Of data points	Volumes for Kit Protocol				Plate recommendation*
		Final	Sample	AlphaLISA Acceptor Beads + biotinylated Ab Mix	SA-Donor beads	
AL3207HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 White ½ AreaPlate-96
AL3207C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96
	500	50 µL	5 µL	20 µL	25 µL	½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate™-384
	1 250	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 ProxiPlate™-384 Plus White OptiPlate-384
	2 500	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536
A3207F	5 000	50 µL	5 µL	20 µL	25 µL	½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate-384
	12 500	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 ProxiPlate-384 Plus White OptiPlate-384
	25 000	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536

*Light gray AlphaPlates were specifically designed for use with AlphaLISA assays and are strongly recommended for best assay performance.

The protocol 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 must be adjusted accordingly.*

1) Preparation of 1X AlphaLISA BSA Assay Buffer:
Add 6 mL of 5X AlphaLISA BSA Assay Buffer to 24 mL Milli-Q® grade H_2O.

2) Preparation of human CXCL12 analyte standard dilutions:

   a. Reconstitute lyophilized human CXCL12 (0.1 µg) in 100 µL Milli-Q® grade H_2O. The remaining reconstituted analyte should be aliquoted immediately and stored at -20 °C for future assays (see page 2 for more details).
   b. A standard curve must be generated for each experiment. The standard curve should be performed in a similar matrix diluent as the samples (e.g. cell culture media for cell supernatant samples, FBS for serum samples). Use of the 1X AlphaLISA BSA Assay Buffer is recommended as a diluent to confirm assay performance.
   c. Prepare standard dilutions as follows (change tip between each standard dilution).

Tube	Vol. of Human CXCL12 (µL)	Vol. of diluent (µL)*	[CXCL12] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted CXCL12	90	1.00E-07	100 000
B	60 µL of tube A	140 µL	3.00E-08	30 000
C	60 µL of tube B	120 µL	1.00E-08	10 000
D	60 µL of tube C	140 µL	3.00E-09	3 000
E	60 µL of tube D	120 µL	1.00E-09	1 000
F	60 µL of tube E	140 µL	3.00E-10	300
G	60 µL of tube F	120 µL	1.00E-10	100
H	60 µL of tube G	140 µL	3.00E-11	30
I	60 µL of tube H	120 µL	1.00E-11	10
J	60 µL of tube I	140 µL	3.00E-12	3
K	60 µL of tube J	120 µL	1.00E-12	1
L	60 µL of tube K	140 µL	3.00E-13	0.3
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 appropriate diluent (e.g. 1X AlphaLISA BSA Assay 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.

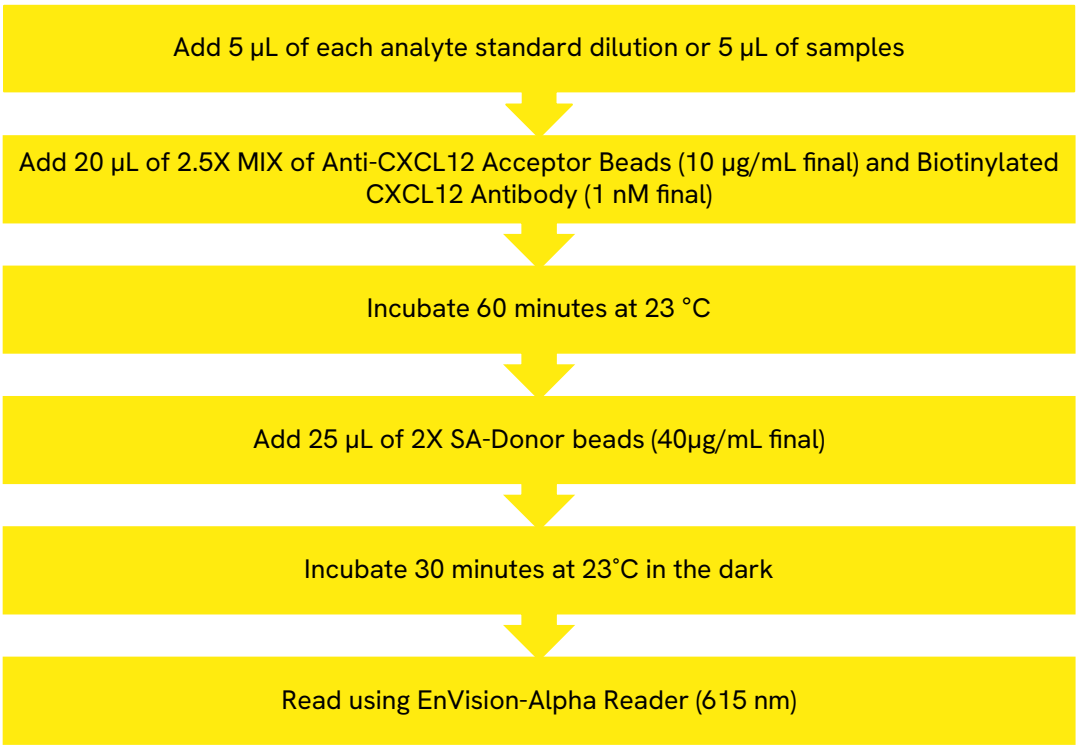
** Four background points in triplicate (12 wells) are used when LDL/LLOQ is calculated. If LDL/LLOQ does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 2.5X MIX AlphaLISA Anti-CXCL12 Acceptor beads (25 µg/mL) + Biotinylated Anti-CXCL12 Antibody (2.5 nM):
   a. Prepare just before use.
   b. Add 50 µL of 5 mg/mL AlphaLISA Anti-CXCL12 Acceptor Bead and 50 µL of 500 nM Biotinylated Anti-CXCL12 Antibody to 9900 µL of 1X AlphaLISA BSA Assay Buffer.

4) Preparation of 2X 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 12300 µL of 1X AlphaLISA BSA Assay Buffer.

5) In a light gray AlphaPlate (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).

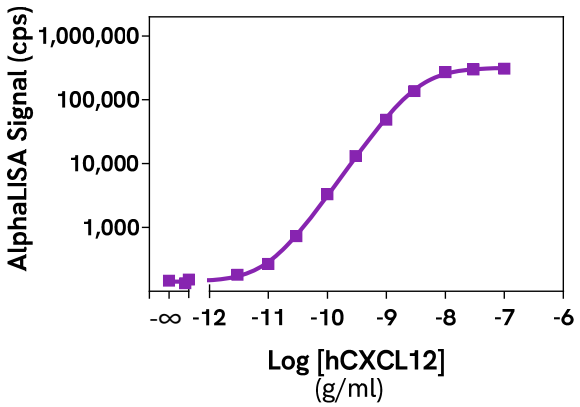
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

ASSAY PERFORMANCE CHARACTERISTICS

AlphaLISA assay performance described below was determined by using the recommended kit protocol using 1X AlphaLISA BSA Assay Buffer as an assay buffer. The analytes (standards) were prepared in different matrix diluents depending on sample type. All other components were prepared in 1X AlphaLISA BSA Assay Buffer.

Standard curve:

A typical sensitivity curve is shown below, using the recommended kit protocol described on page 4, using 1X AlphaLISA BSA Assay Buffer to dilute the standard.



Assay sensitivity:

The LDL was 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 values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL of sample using the recommended assay conditions.

The LLOQ was calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + [10xSD]) on the standard curve. The values correspond to the lowest concentration of analyte that can be accurately quantified in a volume of 5 µL of sample using the recommended assay conditions.

Analyte diluent	LDL (pg/mL)	LLOQ (pg/mL)
AlphaLISA BSA Assay Buffer 1X	8.2	20
DMEM+10% FBS	24.6	51
RPMI+10% FBS	76.2	165
100% FBS	24.2	54.8

Assay precision:

Samples containing different concentrations of analyte were prepared in 1X AlphaLISA BSA Assay Buffer 1X. All other components were prepared in 1X AlphaLISA BSA Assay Buffer. The assays were performed in a 384-well plate format.

Intra-assay precision:

The intra-assay precision was determined using a total of 24 replicates per sample in one assay. Shown as CV% of measured concentration.

CXCL12 CONCENTRATION	CV %
Sample A, 13 324pg/mL	19
Sample B, 1 148 pg/mL	6
Sample C, 107 pg/mL	7

Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with three measurements per sample in each assay. Shown as CV% as measured concentration.

CXCL12	CV %
Sample A, 10 850 pg/mL	18
Sample B, 1 008 pg/mL	13
Sample C, 112 pg/mL	4

Spike and recovery:

Known concentrations of standard (exogenous analyte) were spiked into human poor platelet plasma samples from apparently healthy volunteers containing the endogenous analyte. Non-spiked and spiked samples were measured in the assay. Note that the respective standard curve was prepared by diluting the analyte in the appropriate matrix (FBS). All other assay components were diluted in AlphaLISA BSA Assay Buffer.

Excellent recoveries were achieved for all spiked samples. The results are shown in the table below.

Sample type	[Endogenous CXCL12] in sample (pg/mL)	[Exogenous CXCL12] spiked into sample (pg/mL)	Expected [CXCL12] in spiked sample (pg/mL)	Measured [CXCL12] in spiked sample (pg/mL)	Recovery (%)
Sample 1 Human plasma (poor platelet)	30 362	20 000	50 362	48 986	97
Sample 2 Human plasma (poor platelet)	1 505	20 000	21 505	22 822	106

Dilution linearity:

A sample of mouse recombinant CXC12 was serially diluted in 1X AlphaLISA BSA Assay Buffer. The assay was performed on neat and serially diluted samples, along with a standard curve prepared in the same diluent. Concentrations of CXCL12 in samples were determined by interpolating from the standard curve. The other components of the assays (anti-CXCL12 Acceptor beads, biotinylated anti-CXCL12 antibody, and SA-Donor beads) were prepared in 1X AlphaLISA BSA Assay Buffer.

Excellent dilution linearity ($R^2 > 0.99$, slope = 0.97) and dilution recovery were achieved in the diluted samples (in the range of 2- down to 256-fold dilution). The results are shown in the table below.

Sample	Sample dilution factor	Expected [CXCL12] (pg/mL)	Measured [CXCL12] (pg/mL)	Dilution Recovery (%)
Mouse recombinant CXCL12 diluted in AlphaLISA BSA Assay Buffer	neat	-	12935	-
	1/2	6468	7198	111
	1/4	3599	3109	86
	1/8	1555	1442	92
	1/16	721	729	101
	1/32	364	362	99
	1/64	181	170	93
	1/128	85	82	96
	1/256	41	38	92

Specificity:

Cross-reactivity of the AlphaLISA CXCL12 Detection Kit was tested using recombinant CXCL12 from Mouse as analytes in an assay using the protocol as described above. The cross reactivities were established using the CXCL12 related proteins below at a test concentration of 10 000 pg/mL. Percentage recovery was computed by comparing the measured Interpolated concentration versus the theoretical one. Selectivity between isoforms was measured by testing Human CXCL12 isoform α, Human CXCL12 isoform β, Human CXCL12 isoform γ.

Proteins	Cross reactivity (%)
Mouse CXCL12	100
Human CXCL12 isoform **α**	100
Human CXCL12 isoform **β**	0
Human CXCL12 isoform **γ**	0

Cell experiments:

Cell culture conditions

BJ, human fibroblast cells, were plated in a 96-well culture plate at 200k and 4000k cells/well in MEM medium with 10% FBS for 24H at 37°C. NIH-3T3 mouse fibroblast cells were plated in a 96-well culture plate at 200k and 400k cells/well in DMEM medium with 10% FBS for 24H at 37°C. Then, 5 µL of supernatants were transferred into an AlphaPlate-384 to measure the concentration of secreted CXCL12. The standard curve was prepared in MEM medium + 10% FBS. All other assay components were diluted in AlphaLISA BSA Assay Buffer and added into the wells using the kit protocol. AlphaLISA counts were measured and the CXCL12 concentration in each supernatant sample was interpolated from the standard curve.

Plasma experiments:

CXCL12 binds with high affinity to the CXCR4 receptor present on platelets. Therefore, it is recommended to work on platelet poor or platelet free plasma to avoid variability results. The presence of any free platelets in plasma samples can lead to inconsistency and accuracy of the measurement of CXCL12 using this assay.

Plasma experiments were done on Platelet Poor Plasma prepared from EDTA collected blood of healthy donors. For this, an additional centrifugation step of the separated plasma at 10 000g for 10 minutes at 2-8°C was added to remove residual platelets.

Normal platelet poor plasma samples from healthy volunteers were tested for endogenous level of CXCL12. 5 µL of plasma was transferred into an AlphaPlate-384 to measure the concentration of endogenous CXCL12. The standard curve was prepared in FBS. All other assay components were diluted in AlphaLISA BSA Assay Buffer and added into the wells using the kit protocol. AlphaLISA counts were measured and the CXCL12 concentration in each supernatant sample was interpolated from the standard curve.

Sample	Mean [CXCL12] pg/mL	Range (pg/mL)
Human platelet poor plasma (N=20)	4828	736- 11497

TROUBLESHOOTING

To find detailed recommendations for common situations you might encounter with your AlphaLISA assay kit, please visit our website at www.revvity.com.

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