MANUAL

AlphaLISA® VCAM-1 Detection Kit

Product number: AL338 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of Intracellular Adhesion Molecule 1 (VCAM-1) in cell culture supernatant and human serum using a homogeneous AlphaLISA assay (no wash steps). The assay shows negligible cross-reactivity with other subtypes and species of VCAM-1.

Sensitivity: Lower Detection Limit (LDL): 0.92 pg/ml

Lower Limit of Quantification (LLOQ): 3.12 pg/ml

EC_{50}: 12.35 ng/ml

Dynamic range: Kit designed to detect [VCAM-1] between: 0.92 – 300 000 pg/ml (Figure 1).

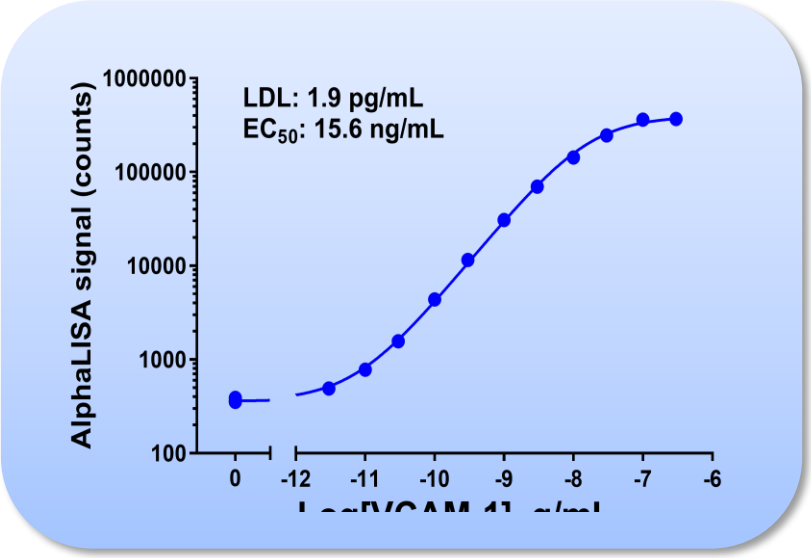


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

Storage: Store kit in the dark at +4˚C. Store reconstituted analyte at -20°C. Limit the number of freeze-thaw cycles.

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.

Analyte of Interest

Vascular Cell Adhesion Molecule-1 (VCAM-1) is a transmembrane glycoprotein with a molecular weight of 110 kDa. VCAM-1 contains six to seven immunoglobulin domains and is expressed on vascular endothelial cells of both small and large blood vessels after stimulation *via* cytokines (i.e. TNFα, IL-4). It mediates immune system response by recruiting additional immune cells to sites of inflammation. After VCAM-1 binds to either Very Late Antigen 4 (VLA-4) or Integrin receptor $\alpha_4\beta_7$ a proteolytic ectodomain shedding process triggers the release of a soluble form of VCAM-1 (sVCAM-1). This soluble form can be utilized as an inflammatory biomarker. Serum levels of sVCAM-1 have been found to be altered in patients with cancer, diabetes, atherosclerosis, rheumatoid arthritis, and other autoimmune diseases. The AlphaLISA kit presented here detects sVCAM-1 in serum, plasma, and cell culture media.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in a no-wash, highly sensitive, quantitative assay. In AlphaLISA 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 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 transfers in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2).

Combining this assay with an AlphaPLEX 645- or AlphaPLEX 545 - based kit will allow the quantification of 2 (or more) analytes in the same well.

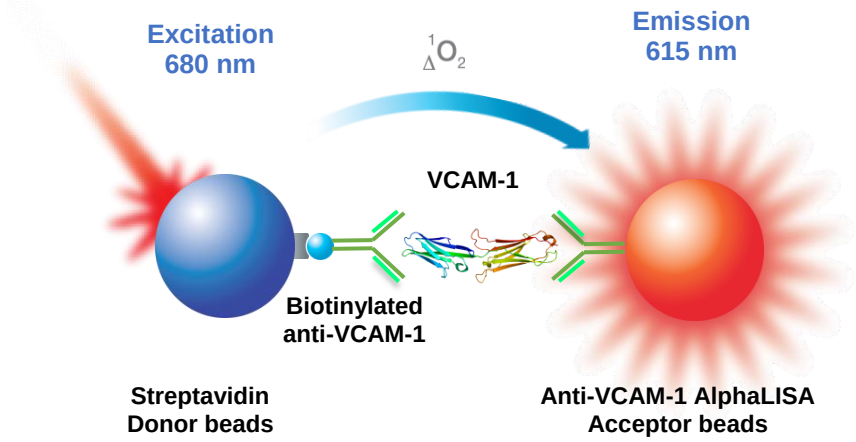


Figure 2. AlphaLISA Assay Principle.

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 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	AL338HV (100 assay points***)	AL338C (500 assay points***)	AL338F (5000 assay points***)
AlphaLISA Anti-VCAM-1 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 Anti-VCAM-1 Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	15 µL @ 500 nM (1 tube, black cap)	40 µL @ 500 nM (1 tube, black cap)	400 µL µL @ 500 nM (1 tube, black cap)
Human VCAM-1	1 µg, lyophilized * (1 tube, clear cap)	1 µg, lyophilized * (1 tube, clear cap)	1 µg, lyophilized * (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Please note that one VCAM-1 analyte vial contains an amount of VCAM-1 sufficient for performing 10 standard curves. Once reconstituted store analyte at -20°C. Additional vials can be ordered separately (cat # AL338S).

** 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 (AL338HV) 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	Revvity Inc.	6050195
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 H_2O (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer.
- 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.
- AlphaLISA signal is detected using an EnVision Multilabel Reader 2103 equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).
- 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 AlphaLISA Immunoassay buffer.

Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The manual described below is an ***example*** for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations) and 452 samples. The manuals also include testing samples in 384 well plates. 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 reagents 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.

		Volume					
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA beads**	**Biotin Antibody**	**SA-Donor beads**	**Plate recommendation**
AL338HV	100	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL338C	250	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	10 µL	10 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	4 µL	4 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	2 µL	2 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL338F	5 000	50 µL	5 µL	10 µL	10 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	4 µL	4 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	2 µL	2 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

Manual for VCAM-1 AlphaLISA Assay

3 Step Manual – Dilution of standards in 1X AlphaLISA Immunoassay Buffer. The manual described below is for one standard curve (48 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 Immunoassay Buffer:
   Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL H_2O.

2) Preparation of VCAM-1 analyte standard dilutions:
   a. Reconstitute 1 ug lyophilized VCAM-1 with 100 µl of water by gently vortexing, avoiding pipetting solution up and down to avoid bubbles.
   b. Store reconstituted analyte at -20°C. Limit the number of freeze/thaw cycles.
   c. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of VCAM-1 (µL)	Vol. of diluent (µL) *	[VCAM-1] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted VCAM-1	90	1.00E-06	1000000
B	60 µL of tube A	140	3.00E-07	300000
C	60 µL of tube B	120	1.00E-07	100000
D	60 µL of tube C	140	3.00E-08	30000
E	60 µL of tube D	120	1.00E-08	10000
F	60 µL of tube E	140	3.00E-09	3000
G	60 µL of tube F	120	1.00E-09	1000
H	60 µL of tube G	140	3.00E-10	300
I	60 µL of tube H	120	1.00E-10	100
J	60 µL of tube I	140	3.00E-11	30
K	60 µL of tube J	120	1.00E-11	10
L	60 µL of tube K	140	3.00E-12	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 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.

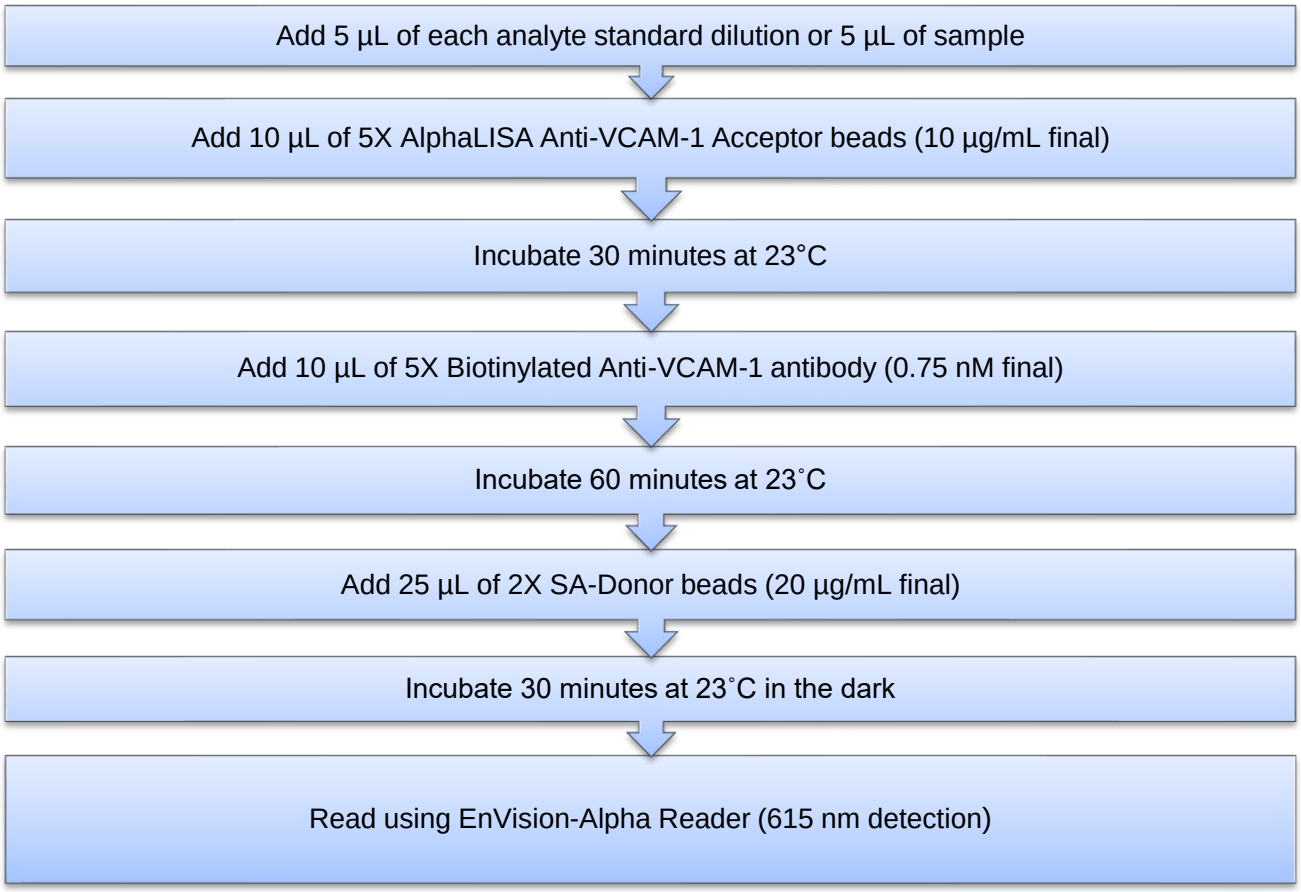
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X Anti-VCAM-1 AlphaLISA Acceptor beads (50 µg/mL):
   a. Prepare just before use.
   b. Add 50 µL of 5 mg/mL AlphaLISA Anti-VCAM-1 Acceptor beads to 4950 µl of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 5X biotinylated Anti VCAM-1 Antibody (3.75 nM):
   a. Prepare just before use.
   b. Add 37.5 µL of 500nM biotinylated Anti VCAM-1 Antibody to 4962.5 µl of 1X AlphaLISA Immunoassay Buffer.

5) Preparation of 2X Streptavidin (SA) Donor beads (40 µg/mL):
    a. Prepare just before use.
    b. Keep the beads under subdued laboratory lighting.
    c. Add 100 µL of 5 mg/mL SA-Donor beads to 12400 µL of 1X AlphaLISA Immunoassay Buffer.

6) In a white Optiplate (384 wells):



Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader 2103 equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

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 manual.

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)	LLOQ (pg/mL)	Buffer/Cell culture media	# of experiments
0.94	3.12	AlphaLISA Immunoassay Buffer	10
2.00	6.81	DMEM+ 10% FBS	6
15	65.9	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 Cell Culture media. All of other components were prepared in Immunoassay Buffer.

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 medium, or RPMI medium with 10% FBS. 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 AlphaLISA Immunoassay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using 3 independent experiments for a total of 16 independent determinations in triplicate. CV% were calculated for each individual experiment then averaged. Shown is the average intra-experimental CV%.

VCAM-1	IAB	DMEM	RPMI
CV%	8	9	12

- Inter-assay precision:

The inter-assay precision was determined using the data across 3 independent experiments with 16 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%.

VCAM-1	IAB	DMEM	RPMI
CV%	15	19	17

- Spike Recovery:

Three known concentrations of VCAM-1 were spiked into AlphaLISA Immunoassay Buffer (IAB), DMEM medium, RPMI medium, with 10% FBS. All samples, including non-spiked Immunoassay Buffers were measured in the assay. The average recovery was reported from 4 independent experiments each with 3 measurements in triplicate and compared to an IAB standard.

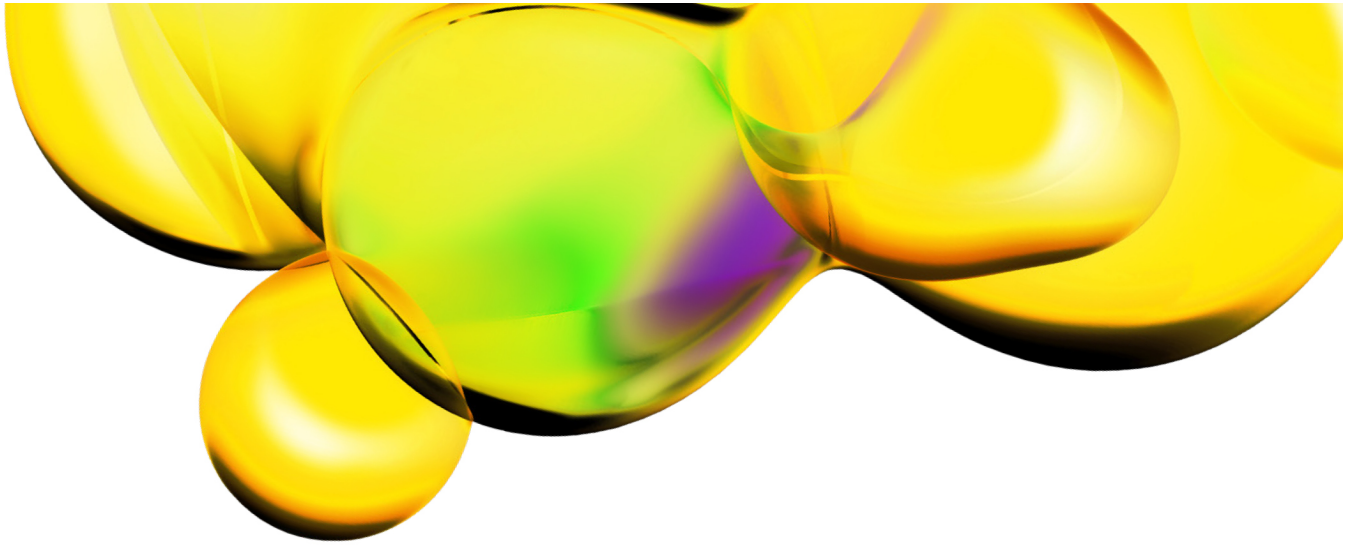
Spiked VCAM-1 (ng/mL)	% Recovery		
	IAB	DMEM	RPMI
3	114	100	100
0.3	103	91	107
0.03	110	94	97

Human Serum Experiments

Human Serum (HS) was purchased and AlphaLISA Immunoassay Buffer (IAB) was used as the diluent**.** VCAM-1 was detected in the normal Human Serum (data not shown). VCAM-1 is expected to be present at detectable levels in HS from normal healthy subjects.

Serial dilutions of Human Serum supplemented with 50 ng/mL of VCAM-1 were prepared and compared to an IAB standard curve, the average of three separate experiments was taken to give the data below.

Dilution Factor	% Recovery
32	100
64	90
128	79
256	82
512	84
1024	89
2048	83
4096	96
8192	89
16384	95



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