Luminex® Performance Assay

Human Adhesion Molecule Multiplex Kit

Catalog Number LKT007

For the simultaneous quantitative determination of multiple human cell adhesion molecules in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Adhesion molecules play a critical role during inflammatory responses by mediating the interactions of leukocytes to endothelial cells and, subsequently, their migration into perivascular tissues. The levels of adhesion molecules such as intercellular cell adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, endothelial (E)-Selectin, and platelet (P)-Selectin are generally low in normal tissues but increase during an inflammatory response. Soluble forms are detected in biological fluids, and their concentrations may reflect their cellular expression levels.

This kit can be used to simultaneously assess the levels of four adhesion molecules in a single sample. For ease of use, the adhesion molecule microparticles are pre-mixed in one vial and the biotinylated detection antibodies are pre-mixed as well.

Analyte	Bead Region
ICAM-1	18
E-Selectin	39
P-Selectin	4
VCAM-1	35

PRINCIPLE OF THE ASSAY

Luminex® Performance Assay multiplex kits are designed for use with the Luminex® 100/200™, or Bio-Rad® Bio-Plex® dual laser, flow-based sorting and detection analyzers.

Analyte-specific antibodies are pre-coated onto color-coded microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibodies, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the captured biotinylated detection antibodies, is added to each well. A final wash removes unbound Streptavidin-PE. The microparticles are resuspended in buffer and read using the Luminex® or Bio-Plex analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples fall outside the dynamic range of the assay, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Luminex® Performance Assay, the possibility of interference cannot be excluded.
- Discrepancies may exist in values obtained for the same analytes utilizing different technologies.
- Luminex® Performance Assays afford the user the benefit of multi-analyte analysis of Biomarkers in a single complex sample. A single multipurpose diluent is used to optimize recovery, linearity, and reproducibility. Such a multipurpose diluent may not optimize any single analyte to the same degree that a unique diluent selected for analysis of that analyte can optimize conditions. Therefore, some performance characteristics may be more variable than those for assays designed specifically for single analyte analysis.
- **Only the analytes listed on the Standard Value Card can be measured with this kit.**

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Protect microparticles and Streptavidin-PE from light at all times to prevent photobleaching.
- For best results, adjust the vacuum strength on the plate washer to between 15 and 40 cm of mercury.

PRECAUTIONS

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED, DILUTED, OR RECONSTITUTED MATERIAL
Human Adhesion Panel Standard Cocktail	893808	2 vials of recombinant human adhesion molecules in a buffered protein base with preservatives; lyophilized.	Use a fresh standard for each assay. Discard after use.
Calibrator Diluent RD6-55	895338	21 mL of a buffered protein base with preservatives. *Use diluted 1:2 in this assay.*	May be stored for up to 1 month at 2-8 °C.* *Once diluted, any unused calibrator diluent must be discarded.*
Human Adhesion Panel Microparticle Cocktail	893806	0.60 mL of a 20X concentrated human adhesion molecule microparticle cocktail with preservatives.	May be stored for up to 1 month at 2-8 °C.* *Once diluted, any unused microparticle cocktail must be discarded.*
Human Adhesion Panel Biotin Ab Cocktail	893807	0.60 mL of a 10X concentrated human adhesion molecule biotin antibody cocktail with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Biotin Antibody Diluent 2	895832	5.5 mL of a buffered protein base with preservative.	
Assay Diluent RD1W	895038	12 mL of a buffered protein base with preservatives.	
Streptavidin-PE	892525	0.07 mL of a 100-fold concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Microplate	640763	1 filter-bottomed 96-well microplate used as a vessel for the assay.	
Plate Sealers	640445	6 adhesive foil strips.	
Standard Value Card	749922	1 card listing the reconstitution volume and working concentrations for this lot of standard.	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Luminex® 100/200™, or Bio-Rad Bio-Plex analyzer with X-Y platform.
- Microplate vacuum manifold (Millipore Multiscreen™ Vacuum Manifold Catalog # MAVM096 or equivalent).
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, manifold dispenser, or automated dispensing unit.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Microcentrifuge.
- 15 mL amber conical tubes (Greiner Bio-One, Catalog # 188280 or equivalent) for the dilution of microparticles, biotin antibody, and Streptavidin-PE.
- **Polypropylene** test tubes for dilution of standards and samples.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifuging for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 30 µL of sample + 270 µL of Calibrator Diluent RD6-55 (1:2). Mix thoroughly.

REAGENT PREPARATION

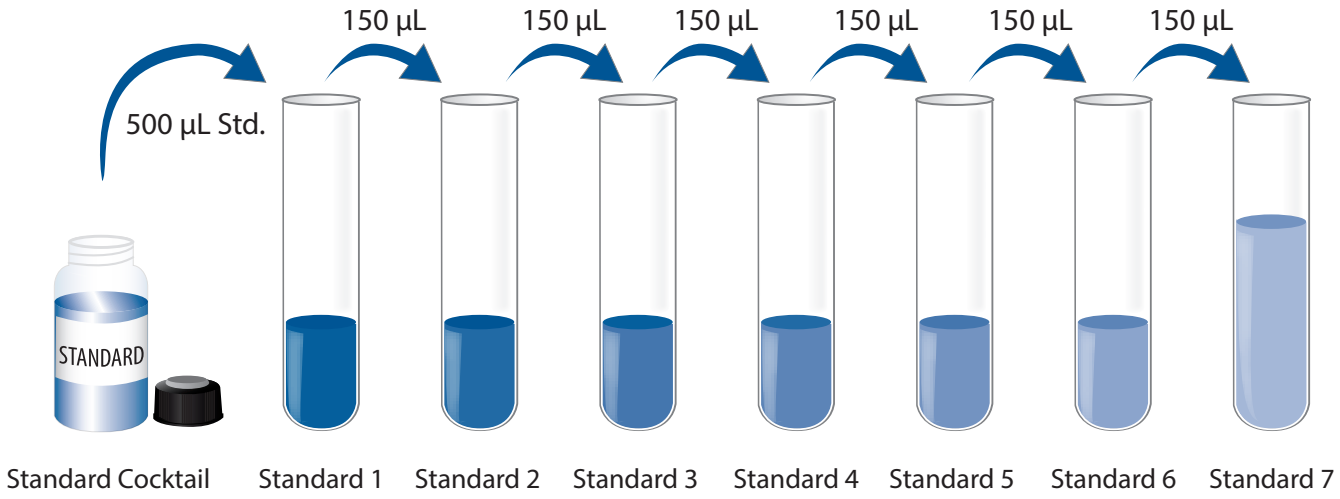
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer. If assaying a partial plate, prepare only as much Wash Buffer as needed.

Calibrator Diluent RD6-55 (diluted 1:2) - Add 20 mL of Calibrator Diluent RD6-55 to 20 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD6-55 (diluted 1:2). If assaying a partial plate, prepare only as much Calibrator Diluent RD6-55 (diluted 1:2) as needed.

Standard - Reconstitute the Standard Cocktail with Calibrator Diluent RD6-55 (diluted 1:2). Refer to the Standard Value Card for the reconstitution volume and assigned values. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 µL of the reconstituted Standard into the Standard 1 tube. Pipette 300 µL of Calibrator Diluent RD6-55 (diluted 1:2) into the remaining tubes. Use Standard 1 to produce a 3-fold dilution series (below). Mix each tube thoroughly before the next transfer. Standard 1 serves as the high standard. Calibrator Diluent RD6-55 (diluted 1:2) serves as the blank.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Centrifuge the Microparticle Cocktail vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial to resuspend the microparticles, taking precautions not to invert the vial.
3. Dilute the Microparticle Cocktail in a polypropylene amber tube or a polypropylene tube wrapped with aluminum foil.

Number of Wells Used	Microparticle Cocktail	+	Assay Diluent RD1W
96	500 µL	+	10.0 mL
72	375 µL	+	7.5 mL
48	250 µL	+	5.0 mL
24	125 µL	+	2.5 mL

Note: *Protect microparticles from light during handling. Diluted microparticles cannot be stored. Prepare microparticles within 30 minutes of use.*

DILUTED BIOTIN ANTIBODY COCKTAIL PREPARATION

1. Centrifuge the Biotin Antibody vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the Biotin Antibody Cocktail in Biotin Antibody Diluent. Mix gently.

Number of Wells Used	Biotin Antibody Cocktail	+	Biotin Antibody Diluent
96	500 µL	+	5.0 mL
72	375 µL	+	3.75 mL
48	250 µL	+	2.5 mL
24	125 µL	+	1.25 mL

Note: *Diluted biotin antibody cannot be stored. Prepare within 30 minutes of use.*

STREPTAVIDIN-PE PREPARATION

Use a polypropylene amber bottle or a polypropylene tube wrapped with aluminum foil. Protect Streptavidin-PE from light during handling and storage.

1. Centrifuge the Streptavidin-PE vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the 100X Streptavidin-PE to a 1X concentration by adding 55 µL of Streptavidin-PE to 5.5 mL of 1X Wash Buffer.

INSTRUMENT SETTINGS

Adjust the probe height setting on the Luminex® analyzer to avoid puncturing the membrane. Refer to the instrument manual.

a) Assign the bead region for each analyte being measured (see page 1)

b) 50 events/bead

c) Minimum events: 0

d) Flow rate: 60 µL/minute (fast)

e) Sample size: 50 µL

f) Doublet Discriminator gates at approximately 7500 and 15,500

g) Collect Median Fluorescence Intensity (MFI)

Note: *For the Bio-Rad Bio-Plex analyzer, set the gates at 4300 and 10,000. The CAL2 setting for the Bio-Rad Bio-Plex analyzer should be set at the low RP1 target value.*

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Note: *Protect microparticles and Streptavidin-PE from light at all times.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Pre-wet the filter-bottomed microplate by filling each well with 100 µL of Wash Buffer. Remove the liquid through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate.

   Note: *After each final wash cycle and subsequent reagent addition, blot the bottom of the plate with a paper towel to prevent wicking.*
3. Add 100 µL of Standard or sample* per well.
4. Resuspend the diluted Microparticle Cocktail by inversion or vortexing. Add 100 µL of the Microparticle Cocktail to each well. Securely cover with a foil plate sealer. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Using a vacuum manifold device designed to accommodate a microplate, wash by removing the liquid, filling each well with Wash Buffer (100 µL) and removing the liquid again. All of the liquid must be removed through the filter at the bottom of the plate to avoid any loss of microparticles. Complete removal of liquid is essential for good performance. Perform the wash procedure three times.
6. Add 50 µL of diluted Biotin Antibody Cocktail to all wells. Securely cover with a foil plate sealer and incubate for 1 hour at room temperature on the shaker set at 500 ± 50 rpm.
7. Repeat the wash as in step 5.
8. Add 50 µL of diluted Streptavidin-PE to all wells. Securely cover with a foil plate sealer and incubate for 30 minutes at room temperature on the shaker set at 500 ± 50 rpm.
9. Repeat the wash as in step 5.
10. Resuspend the microparticles by adding 100 µL of Wash Buffer to each well. Incubate for 2 minutes on the shaker set at 500 ± 50 rpm.
11. Read within 90 minutes using the Luminex® or BioRad Analyzer.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Use the standard concentrations on the Standard Value Card and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank Median Fluorescence Intensity (MFI).

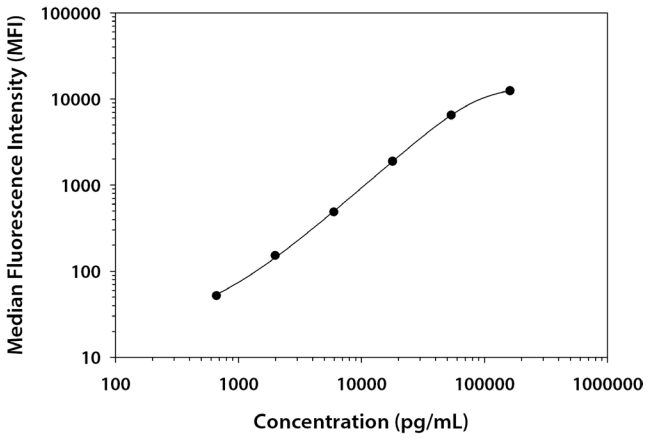
Create a standard curve for each analyte by reducing the data using computer software capable of generating a five parameter logistic (5-PL) curve-fit.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

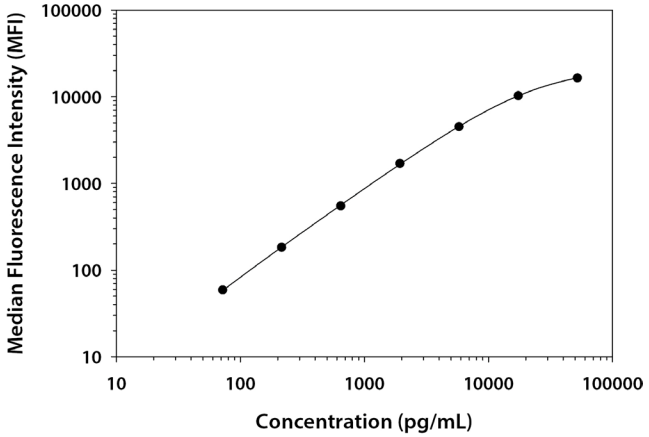
These standard curves are provided only for demonstration. Standard curves must be generated each time an assay is run, utilizing values from the Standard Value Card.

ICAM-1



Standard	(pg/mL)	MFI	Average	Corrected
Blank	0	42 43	43	—
Standard 1	161,200	12,245 12,762	12,504	12,461
Standard 2	53,733	6258 6802	6530	6487
Standard 3	17,911	1894 1967	1931	1888
Standard 4	5970	503 557	530	487
Standard 5	1990	182 208	195	152
Standard 6	663	93 97	95	52

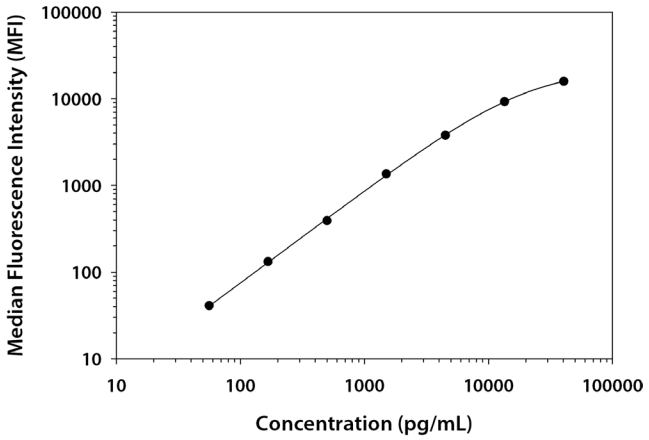
E-Selectin



Standard	(pg/mL)	MFI	Average	Corrected
Blank	0	3 4	3.5	—
Standard 1	52150	16,214 16,688	16,451	16,447
Standard 2	17383	9928 10,567	10,247	10,244
Standard 3	5794	4229 4805	4517	4513
Standard 4	1931	1624 1773	1699	1695
Standard 5	644	528 577	553	549
Standard 6	215	185 187	186	183
Standard 7	72	62 62	62	59

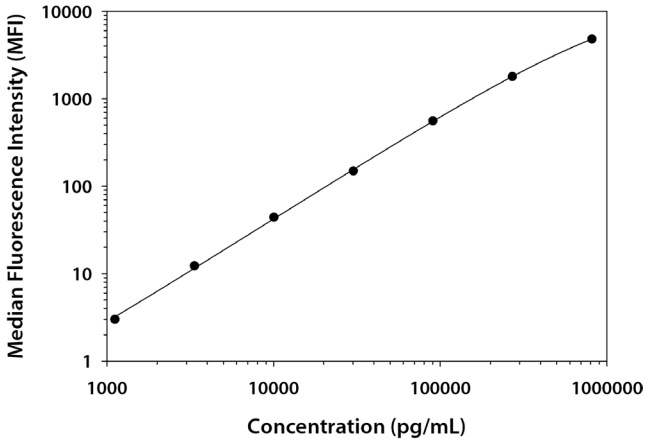
TYPICAL DATA *CONTINUED*

P-Selectin



Standard	(pg/mL)	MFI	Average	Corrected
Blank	0	21 23	22	—
Standard 1	40,500	15,726 16,023	15875	15,853
Standard 2	13,500	9013 9556	9284	9262
Standard 3	4500	3781 3818	3800	3778
Standard 4	1500	1356 1406	1381	1359
Standard 5	500	411 415	413	391
Standard 6	167	148 160	154	132
Standard 7	56	60 66	63	41

VCAM-1



Standard	(pg/mL)	MFI	Average	Corrected
Blank	0	1 2	1.5	—
Standard 1	813,000	4683 4942	4812	4811
Standard 2	271,000	1720 1866	1793	1791
Standard 3	90,333	541 579	560	558
Standard 4	30,111	139 162	151	149
Standard 5	10,037	40 50	45	44
Standard 6	3346	13.5 14.0	13.8	12.3
Standard 7	1115	4 5	4.5	3.0

CALIBRATION

This assay is calibrated against highly purified recombinant human adhesion molecules produced at R&D Systems®.

SENSITIVITY

Twenty-nine assays were run and the minimum detectable dose (MDD) was determined by adding two standard deviations to the MFI of twenty zero standard replicates and calculating the corresponding concentration.

Analyte	Mean (pg/mL)	Range (pg/mL)
ICAM-1	130	64-303
E-Selectin	2.1	0.9-7.4
P-Selectin	6.4	3.0-12.2
VCAM-1	252	122-529

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in eighty-seven separate assays to assess inter-assay precision.

ICAM-1

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	87	87	87
Mean (pg/mL)	5120	42,130	1704	5622	53,471	1921
Standard deviation	297	1498	141	813	8683	324
CV (%)	5.8	3.6	8.3	14.5	16.2	16.9

E-Selectin

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	87	87	87
Mean (pg/mL)	996	8693	611	948	8491	561
Standard deviation	28	509	20	113	798	65
CV (%)	2.8	5.9	3.3	11.9	9.4	11.6

P-Selectin

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	87	87	87
Mean (pg/mL)	2168	17,455	512	1999	18,652	447
Standard deviation	100	1029	24	190	1640	54
CV (%)	4.6	5.9	4.7	9.5	8.8	12.1

VCAM-1

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	87	87	87
Mean (pg/mL)	25,870	223,400	8608	23,543	221,030	8283
Standard deviation	1344	6754	347	2213	14,061	956
CV (%)	5.2	3.0	4.0	9.4	6.4	11.5

RECOVERY

The recovery of each analyte spiked to levels throughout the range of the assay in various matrices was evaluated.

ICAM-1

Sample Type	Average % Recovery	Range
Cell culture supernates (n=8)	89	79-101%
Serum (n=8)	105	96-119%
EDTA plasma (n=8)	99	82-122%
Heparin plasma (n=8)	98	84-120%

E-Selectin

Sample Type	Average % Recovery	Range
Cell culture supernates (n=8)	95	91-101%
Serum (n=8)	97	88-106%
EDTA plasma (n=8)	93	84-109%
Heparin plasma (n=8)	92	88-102%

P-Selectin

Sample Type	Average % Recovery	Range
Cell culture supernates (n=8)	95	91-99%
Serum (n=8)	99	86-116%
EDTA plasma (n=8)	91	86-109%
Heparin plasma (n=8)	94	79-112%

VCAM-1

Sample Type	Average % Recovery	Range
Cell culture supernates (n=8)	90	82-106%
Serum (n=8)	97	86-124%
EDTA plasma (n=8)	92	82-115%
Heparin plasma (n=8)	92	81-117%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of each analyte were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Serum and plasma samples were diluted prior to assay as directed in the Sample Preparation section.

ICAM-1		Cell culture supernates	Serum	EDTA plasma	Heparin plasma
1:2	Average % of Expected	102	107	105	105
	Range (%)	89-118	92-117	91-113	94-120
1:4	Average % of Expected	106	107	106	106
	Range (%)	103-109	84-122	90-122	92-129
1:8	Average % of Expected	113	102	95	98
	Range (%)	109-118	80-118	74-108	85-114

E-Selectin		Cell culture supernates	Serum	EDTA plasma	Heparin plasma
1:2	Average % of Expected	101	96	98	100
	Range (%)	90-114	86-107	89-107	85-112
1:4	Average % of Expected	102	101	100	101
	Range (%)	92-111	88-113	88-114	92-111
1:8	Average % of Expected	105	107	105	102
	Range (%)	97-114	94-116	88-119	86-122

P-Selectin		Cell culture supernates	Serum	EDTA plasma	Heparin plasma
1:2	Average % of Expected	99	104	102	103
	Range (%)	95-103	96-111	90-112	97-110
1:4	Average % of Expected	96	106	106	108
	Range (%)	78-108	88-117	89-115	100-116
1:8	Average % of Expected	111	113	109	112
	Range (%)	107-114	100-124	92-125	98-124

VCAM-1		Cell culture supernates	Serum	EDTA plasma	Heparin plasma
1:2	Average % of Expected	108	97	92	97
	Range (%)	96-116	83-108	83-105	87-104
1:4	Average % of Expected	117	96	89	93
	Range (%)	113-122	82-107	81-99	85-108
1:8	Average % of Expected	108	99	91	92
	Range (%)	105-111	82-111	75-109	81-100

SPECIFICITY

This assay recognizes natural and recombinant human ICAM-1, E-Selectin, P-Selectin, and VCAM-1.

Cross-reactivity - The factors listed below were prepared at 500 ng/mL in calibrator diluent, assayed, and measured less than 0.8% cross-reactivity.

Recombinant human:

ALCAM
BCAM
Cadherin-8
Cadherin-11
CEACAM-1
CHL-1
CNTN-2
CNTN-4
Contactin-1
DC-SIGN R
Desmoglein-1
Desmoglein-2
DNAM-1
E-Cadherin
E-Calectin
Endocan
EpCAM
Galectin-1
Galectin-2
Galectin-3
Galectin-4
Galectin-7
Galectin-8
ICAM-2
ICAM-3
ICAM-5
JAM-1
JAM-2
JAM-3
LOX-1
L-Selectin
M-Cadherin
MCAM
N-Cadherin
NCAM-L1
NrCAM
P-Cadherin
PECAM
Siglec-2
Siglec-3
Siglec-5
Siglec-6
Siglec-7
Siglec-9
Siglec-10
TROP-2
VE-Cadherin

Recombinant mouse:

ALCAM
CHL-1
DCC
E-Cadherin
Endocan
E-Selectin
Galectin-1
Galectin-3
Galectin-7
ICAM-1
ICAM-2
ICAM-5
JAM-1
JAM-2
JAM-5
LOX-1
L-Selectin
MAdCAM-1
P-Cadherin
Siglec-2
Siglec-F
VCAM-1
VE-Cadherin

Recombinant rat:

E-Selectin
ICAM-1
L-Selectin
MAG

ICAM-1 Multiplex Partners:

E-Selectin
P-Selectin
VCAM-1

E-Selectin Multiplex Partners:

ICAM-1
P-Selectin
VCAM-1

P-Selectin Multiplex Partners:

ICAM-1
E-Selectin
VCAM-1

VCAM-1 Multiplex Partners:

ICAM-1
E-Selectin
P-Selectin

Interference - Preparations of the factors listed above were prepared at 500 ng/mL in a mid-range recombinant human adhesion molecule control and assayed for interference. None of the factors interfered in the assay.