

# High-Throughput Multiplexed Assays for Biomarkers of Vascular Injury

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## 1 Abstract

A recent FDA expert group report (Kerns, W. et al., *Drug-Induced Vascular Injury - A Search for Biomarkers*, 2001) suggests that the measurement of predictive biomarkers of vascular injury may become part of the drug development process. We have developed two multiplexed panels of electrochemiluminescence-based assays that include nine of the proposed markers, namely Thrombomodulin, Serum Amyloid A, soluble adhesion molecules ICAM-1, ICAM-3, VCAM-1, E-Selectin, and P-Selectin, in MSD 96-well 7-spot plates, and pANCA and cANCA (Anti-Neutrophil Cytoplasmic Antibodies; perinuclear and cytoplasmic) in a second panel. The assay protocol is simple: 25  $\mu$ l of sample and 25  $\mu$ l of detection antibody solution are incubated in the plate for 2 hours, followed by a wash step. Assay ranges are 5 – 100,000 pg/ml for Thrombomodulin, 0.06 – 1,000 ng/ml for SAA, 5 – 50,000 pg/ml for ICAM-1, 8 – 100,000 pg/ml for ICAM-3, 33 – 100,000 pg/ml for VCAM-1, 18 – 100,000 pg/ml for E-Selectin, and 25 – 300,000 pg/ml for P-Selectin. pANCA & cANCA can be detected in up to 100,000-fold diluted positive serum fraction.

## 2 Introduction

	Clinical Ranges	Disease states
ICAM-1	60 – 300 ng/ml normal; elevated up to 700 ng/ml	Vasculitis, inflammation
ICAM-3	70 ng/ml	Initial immune response
VCAM-1	200 – 1,700 ng/ml	Vasculitis, inflammation, infection, autoimmune diseases, cancers
E-Selectin	~ 50 ng/ml	Vascular infection & inflammation, allergic reactions, ocular diseases, septic shock, inflammatory bowel disease, transplantation reaction
P-Selectin	40 – 200 ng/ml	Respiratory distress syndrome, adult respiratory distress syndrome; acute lung injury; ischemia-reperfusion injury; septic shock; thrombotic diseases; rheumatoid arthritis
Thrombomodulin	~20 ng/ml	Thrombocytopenic purpura, disseminated intravascular coagulation, atherosclerosis and inflammatory connective tissues diseases
SAA	1,000 – 5,000 ng/ml normal; elevated up to 1,000 $\mu$ g/ml	Inflammatory diseases
pANCA	No standard	Vasculitis
cANCA	No standard	Wegener's granulomatosis, vasculitis



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## 3 Assay Formats

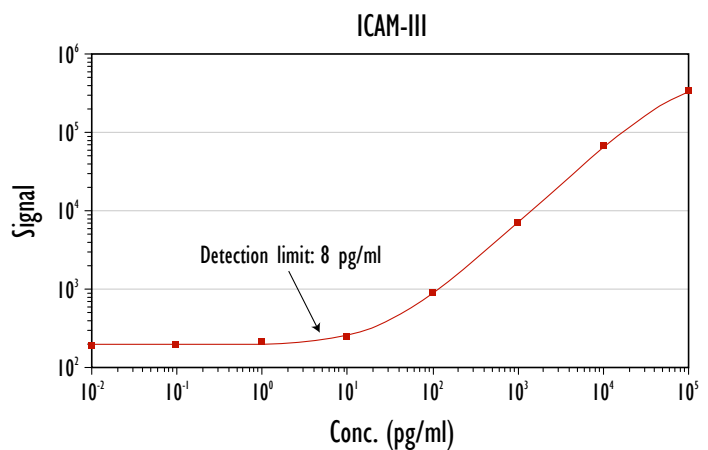
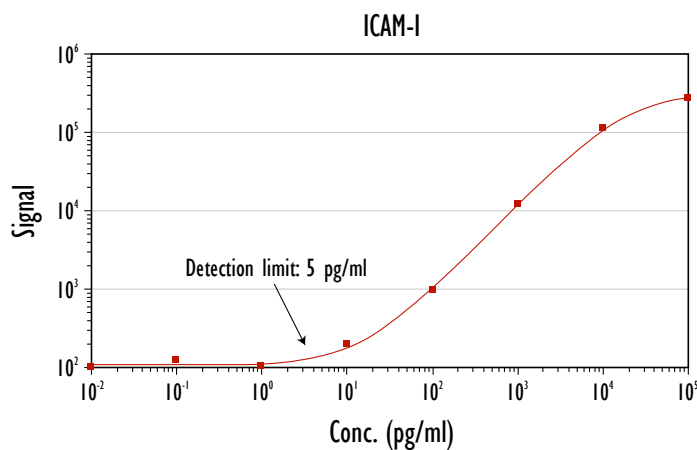
### 7-plex panel (ICAM-I&III, VCAM-I, E&P-Selectin, Thrombomodulin, SAA)

Block plates for one hour at RT or overnight at 4°C and wash (optional)  
Add 25 µl each of detection antibody reagent and sample (recommended: 100x dilution) to each well.  
Incubate for two hours on a shaker  
Wash, add MSD-T Read Buffer, read

### pANCA, cANCA 2-plex

Block plates for one hour at RT or overnight at 4°C and wash (optional)  
Add 25 µl each of sample (recommended: 1000x dilution) to each well.  
Incubate for two hours on a shaker  
Wash; add 25 µl of detection antibody reagent  
Incubate for two hours on a shaker  
Wash, add MSD-T Read Buffer, read

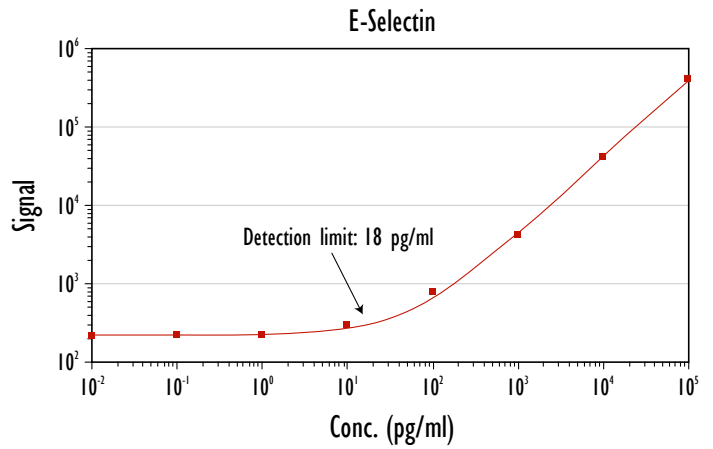
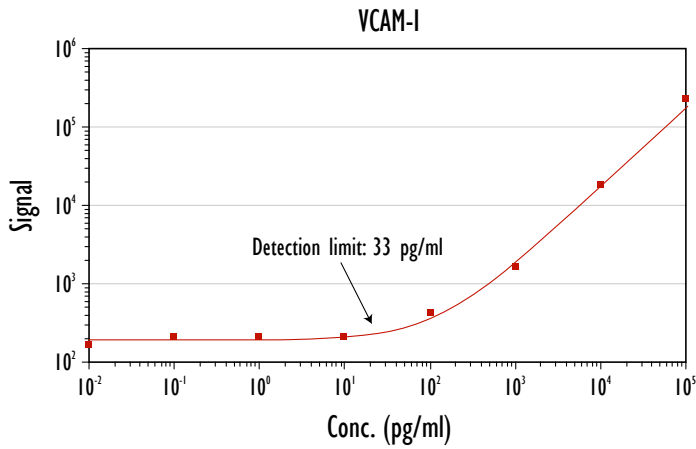
## 4 Calibration Curve: ICAM-I, ICAM-III



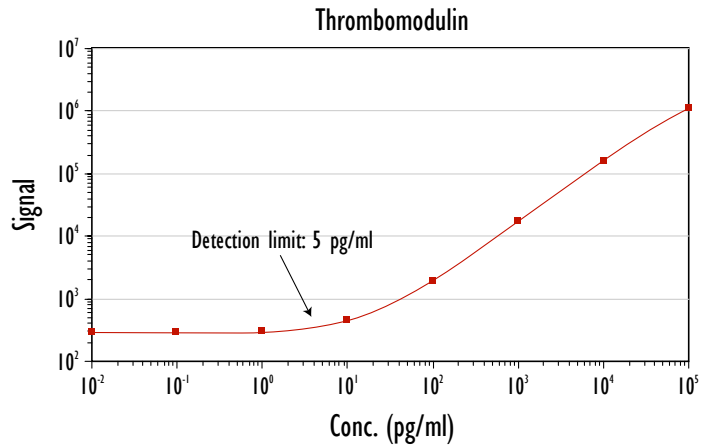
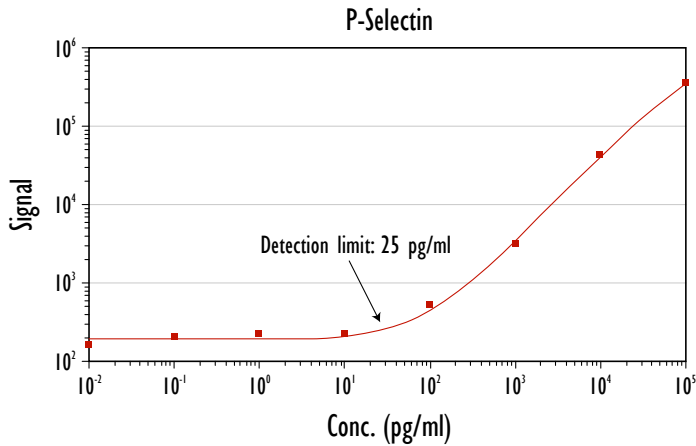
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## 5 Calibration Curve: VCAM-I, E-Selectin



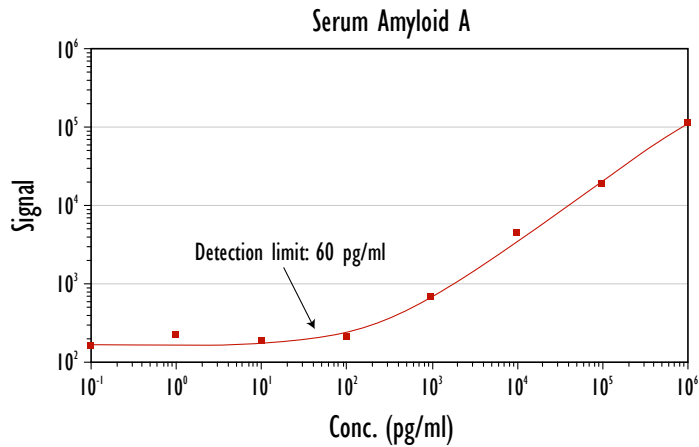
## 6 Calibration Curve: P-Selectin, Thrombomodulin



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## 7 Calibration Curve: Serum Amyloid A



## 8 7-Spot Multiplex: % Cross-reactivity

	ICAM-1	ICAM-3	VCAM-1	E-Selectin	P-Selectin	Thrombomodulin	SAA
ICAM-1 at 100,000 pg/ml	X	0.3%	<0.1%	<0.1%	<0.1%	0.1%	0.2%
ICAM-3 at 100,000 pg/ml	0.1%	x	<0.1%	<0.1%	<0.1%	0.3%	0.1%
VCAM-1 at 100,000 pg/ml	0.1%	0.5%	x	0.1%	0.1%	0.1%	2%
E-Selectin at 140,000 pg/ml	<0.1%	0.1%	<0.1%	x	<0.1%	0.1%	0.1%
P-Selectin at 140,000 pg/ml	0.1%	0.3%	<0.1%	<0.1%	x	0.1%	0.1%
Thrombomodulin at 140,000 pg/ml	<0.1%	<0.1%	<0.1%	0.1%	<0.1%	x	<0.1%
SAA at 1.4 ug/ml	<0.1%	0.1%	<0.1%	<0.1%	<0.1%	0.1%	x

% Cross-reactivity: (Signal at high cross-reactant concentration - signal without cross-reactant)/cross-reactant signal



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## 9 pANCA & cANCA Multiplexed

cANCA positive sample: Dilution factor	pANCA ECL signal	cANCA ECL signal
10x	3017	61276
100x	1998	45376
1,000x	509	10167
10,000x	97	1275
100,000x	41	209
1,000,000x	50	67
diluent	49	71
diluent	33	48

pANCA cross-reactivity at 100x diluted cANCA sample: 4%

pANCA positive sample: Dilution factor	pANCA ECL signal	cANCA ECL signal
10x	210344	3557
100x	166408	1511
1,000x	26866	503
10,000x	3326	207
100,000x	354	70
1,000,000x	73	65
diluent	50	75
diluent	48	69

cANCA cross-reactivity at 100x diluted pANCA sample: 0.9%

## 10 Dynamic Range (Multiplexed, undiluted sample)

Analyte	Detection Limit	Upper end of assay	Dynamic range
ICAM-1	5 pg/ml	50,000 pg/ml	10,000
ICAM-3	8 pg/ml	100,000 pg/ml	12,000
VCAM-1	33 pg/ml	>100,000 pg/ml	3,000
E-Selectin	18 pg/ml	>100,000 pg/ml	5,000
P-Selectin	25 pg/ml	>100,000 pg/ml	4,000
Thrombomodulin	5 pg/ml	100,000 pg/ml	20,000
SAA	60 pg/ml	>1,000,000 pg/ml	20,000
pANCA	500,000x	200x	2,500
cANCA	150,000x	200x	750

Note: recommended sample dilution is 100x for 7-plex panel and 1000x for pANCA/cANCA panel.



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## II Conclusion

A multiplex assay for 7 vascular injury markers was developed (96-well 7-spot).

Detection limits are in the 5 – <100 pg/ml range.

Dynamic range for the 7 analytes span 3 – 4 logs.

Cross-reactivity is low (~ 0.1%).

The assay format is simple: sample and detection antibody reagent are added, and after two hours the plate is washed, read buffer is added and the plate is read (1 min per plate).

A multiplex assay for cANCA and pANCA was developed.

Two-step format: sample; 2 hours; wash; detection antibody, 2 hours; wash; read.

The assay is highly sensitive (detection limit 150,000x & 500,000x diluted positive sample).

Cross-reactivity is low (4% & 0.9% for cANCA and pANCA respectively).

Dynamic range: approximately 3 logs.



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