Application Note

Measurement of cardiovascular disease biomarkers in human clinical samples using magnetic bead-based MILLIPLEX® MAP multiplex panels

Introduction

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in the United States. In 2010, healthcare costs for CVD were estimated at $156 billion, more than any other diagnostic group, including cancer. According to the American Heart Association, every 25 seconds, an American suffers some type of coronary event, and every 60 seconds, someone dies from one. CVDs include any disease that affects the heart, arteries, or veins, but more commonly refers to atherosclerotic conditions.

As researchers hope to facilitate early diagnosis and intervention, interest in measurement of circulating CVD biomarkers has increased dramatically in the past decade. Most of CVD biomarker discovery research has focused on arterial plaque-related conditions. This approach is reasonable because arterial plaque development is chronic and progressive, can be measured by soluble markers and encompasses the bulk of CVD cases. Atherosclerotic CVD is a chronic inflammation in the arterial walls that leads to plaque formation and continues along the ischemic cascade. To facilitate research into the progression of atherosclerotic CVD, we have developed magnetic bead-based biomarker assay panels that include characteristic analytes for every cardiac disease stage (Table 1)*. Many analytes are applicable to multiple stages.

*For research use only. Not for use in diagnostic procedures.

CVD Stage	Characteristic Biomarker
Platelet activation	ADAMTS13, L-Selectin, von Willebrand Factor (vWF), sE-Selectin, P-Selectin, sVCAM-1, Thrombomodulin
Inflammation	CXCL6, CLXL16, Endocan-1 (ESM-1), FABP4, Placental Growth Factor (PlGF), GDF-15, Lipocalin-2/NGAL, SAA, α-2-macroglobulin (A2M), C-Reactive Protein (CRP), Fetuin A, α -1-acid glycoprotein (AGP), SAP, Haptoglobin, PF4/CXCL4, Adipsin, PECAM-1, Pentraxin-3 (PTX-3), Oncostatin M (OSM)
Plaque instability/rupture	LIGHT, D Dimer, sICAM-1, Myeloperoxidase (MPO), dPAPP-A
Ischemia	FABP3, Tissue Factor, BNP
Myocardial dysfunction or stress	BNP, NTproBNP, Follistatin, Myoglobin, Myocardial necrosis →CK-MB, Troponin I, Troponin T

Table 1*. Characteristic biomarkers for various stages of cardiovascular disease.

These immunoassays were developed using magnetic bead-based Luminex® xMAP® technology. Magnetic bead-based assays provide several advantages over non-magnetic bead-based assays, including easier automation and high-throughput screening, more flexible plate and plate washer options and elimination of technical obstacles (i.e., clogging of wells) which may result from vacuum manifold/manual washing.

After validating our multiplexed assays for sensitivity, dynamic range and variability, we tested serum and plasma samples from patients with and without diagnosed CVD, and found that many of the CVD biomarkers were significantly elevated in CVD patients, indicating the utility of the assay panels.

Materials and Methods

Samples

Serum and plasma samples were obtained from emergency room patients with and without a cardiovascular disease-related diagnosis. Twenty unmatched samples of each matrix were acquired from CVD negative and CVD positive patients at the same hospital through a commercial vendor. Basic demographic data (age, gender, and ethnicity) were provided for each sample (Table 2). Specific conditions such as chronic heart failure, coronary artery disease, chronic obstructive pulmonary disease, and hypertension were also supplied if available.

	Serum		Plasma	
	CVD	No CVD	CVD	No CVD
N	20	20	20	20
Age in years (median (IQR1))	70.0 (53.3, 86.0)†	29.5 (20.5, 35.8)	80.5 (72.2, 86.0)†	66.0 (52.5, 80.8)
Gender (% male)	55%	30%	25%	45%
Race (% white)	80%	75%	100%	80%
Diagnosis				
% CHF[2]	45%		65%	
% CAD[3]	65%		85%	
% COPD[4]	20%		55%	
% HTN[5]	60%		50%	

Table 2. Population demographics for serum and plasma samples.

[1]Interquartile range (Q1 value, Q3 value)
[2]Chronic heart failure
[3]Coronary artery disease
[4]Chronic obstructive pulmonary disease
[5]Hypertension
†p<0.01 between CVD and no CVD diagnosis by Mann-Whitney test

CVD biomarker analysis

All 80 samples were analyzed using four MILLIPLEX® MAP Human Cardiovascular Disease Magnetic Bead panels. The multiplex assays were generated using a typical sandwich assay format using analyte-specific, capture antibody-conjugated beads and biotinylated detection antibody. Assays were validated using purified protein standards of known concentration. Each panel simultaneously measured multiple CVD biomarkers as shown below:

- Panel 1 (Catalogue No. HCVD1MAG-67K, for neat serum and plasma samples): BNP, NTproBNP, CK-MB, CXCL6, CXCL16, ESM-1, FABP3, FABP4, PlGF, LIGHT, Oncostatin M, and Troponin I
- Panel 2 (Catalogue No. HCVD2MAG-67K, for 1:100 diluted serum and plasma samples): ADAMTS13, D-Dimer, GDF-15, Myoglobin, sICAM-1, MPO, P-Selectin, Lipocalin-2/NGAL, sVCAM-1, and SAA
- Panel 3 (Catalogue No. HCVD3MAG-67K, for 1:40,000 diluted serum and plasma samples): A2M, CRP, Fetuin A, AGP, Fibrinogen, L-Selectin, SAP, Haptoglobin, PF4/CXCL4, Adipsin, and von Willebrand Factor
- Panel 4 (Catalogue No. HCVD4MAG-67K, neat serum and plasma samples): sE-Selectin, Follistatin, dPAPP-A, PECAM-1, Pentraxin-3, Tissue Factor, Thrombomodulin, and Troponin T

Assays were performed according to the respective protocols. In general, the 96-well assay plate was washed with 200 µL assay buffer per well. To each well was added 25 µL standard/control or buffer, 25 µL matrix (if required) or sample, and 25 µL beads. Plates were incubated overnight with shaking at 4 °C. The assay plate was washed three times with wash buffer. 50 µL detection antibodies were added to each well and incubated 1 h at room temperature (RT). After adding 50 µL streptavidin-phycoerythrin (SAPE) to each well, the plate was incubated at RT for 30 min. The assay plate was then washed three times with wash buffer and beads resuspended in sheath fluid. All plates were analyzed using the Luminex 200™ instrument.

Statistical analysis

Statistical tests were conducted using MiniTab® 16.1.0 software. Due to the small sample sizes and non-normal distribution of the data, the Mann-Whitney U test was used to analyze continuous variables and Fisher's Exact Test was used for categorical variables. P-values less than 0.05 were noted as statistically significant.

Results

Standard curves for each multiplexed assay panel (Figure 1) showed approximately three orders of magnitude in dynamic range. Assay sensitivity for most analytes was in the pg/mL range (Table 3, page 6), and intra-assay and inter-assay coefficients of variation were <10% and <20%, respectively.

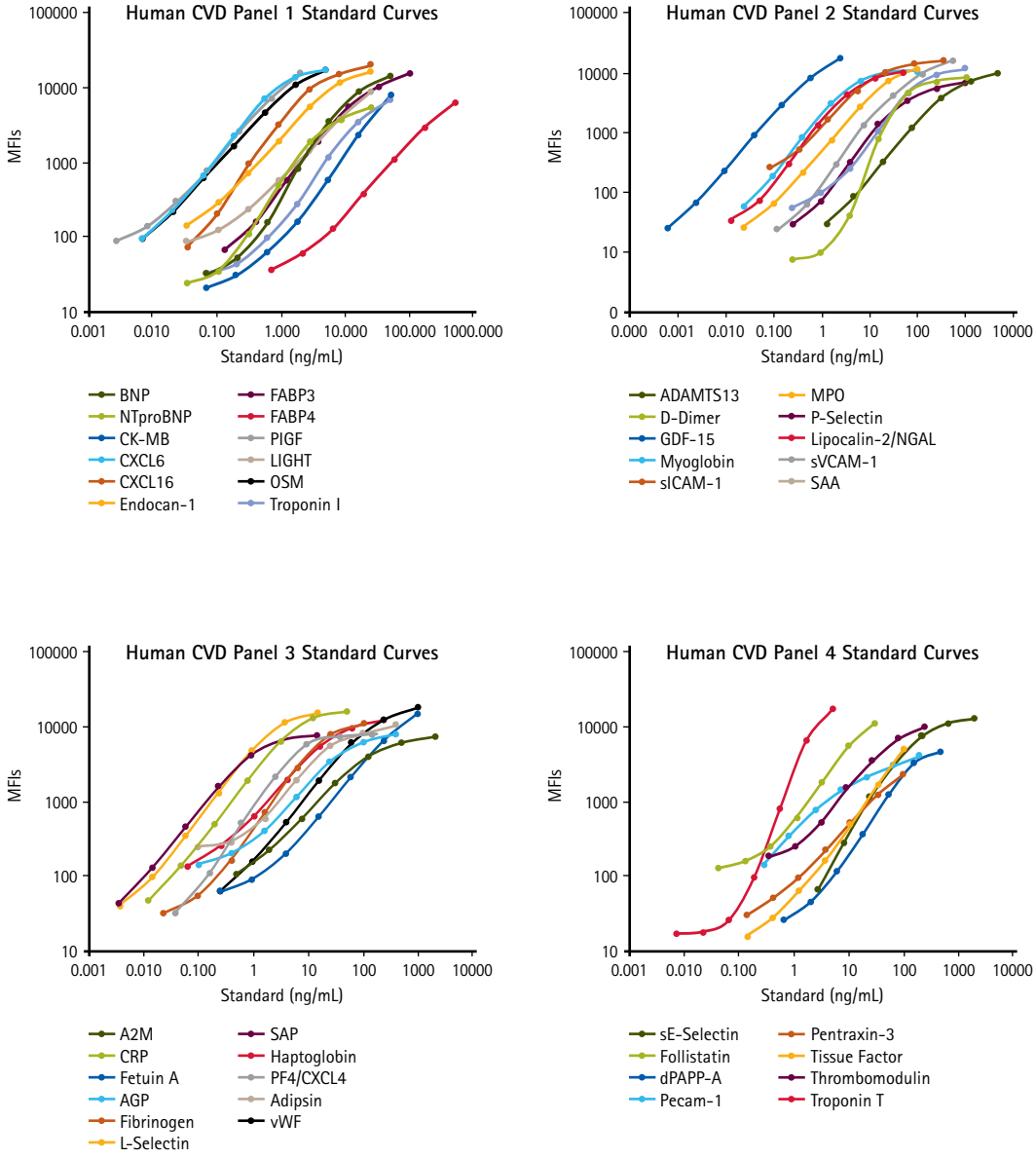

Figure 1.

Standard curves for MILLIPLEX® MAP Human Cardiovascular Disease Magnetic Bead Panels

Table 3.
Assay performance characteristics. The four human CVD panels were validated according to Merck Millipore multiplex assay validation guidelines.

	Analyte	Std Curve Range (ng/mL)	Sensitivity[1] (ng/mL)	Coefficient of Variation Intra-	Coefficient of Variation Inter-	Recovery[2]
Panel 1 (neat samples)	BNP	0.069-50	0.056	4.3%	12.0%	99%
	NTproBNP	0.034-25	0.036	5.9%	10.3%	97%
	CK-MB	0.069-50	0.069	5.7%	13.7%	100%
	CXCL6	0.007-5	0.002	3.8%	10.4%	103%
	CXCL16	0.034-25	0.022	4.4%	9.9%	100%
	ESM-1	0.034-25	0.017	4.3%	10.4%	102%
	FABP3	0.137-100	0.063	3.3%	7.9%	96%
	FABP4	0.686-500	0.502	4.5%	14.2%	100%
	Placental Growth Factor (PlGF)	0.003-2	0.017	3.4%	8.8%	96%
	LIGHT	0.034-25	0.004	4.2%	9.7%	102%
	Oncostatin M (OSM)	0.007-5	0.001	4.4%	9.2%	102%
	Troponin I	0.069-50	0.069	5.2%	5.2%	100%
	Analyte	**Std Curve Range (ng/mL)**	**Sensitivity[1] (ng/mL)**	**Coefficient of Variation Intra-**	**Coefficient of Variation Inter-**	**Recovery[3]**
Panel 2 (1:100 diluted samples)	ADAMTS13	1.221-5000	0.418	2.1%	9.8%	94%
	D-Dimer	0.244-1000	0.629	2.8%	8.6%	83%
	GDF-15	0.0005-2.5	0.0006	3.1%	11.2%	98%
	Myoglobin	0.024-100	0.007	2.7%	8.7%	88%
	sICAM-1	0.085-350	0.039	3.3%	9.4%	83%
	MPO	0.024-100	0.134	5.2%	14.3%	96%
	P-Selectin	0.244-1000	0.119	2.1%	10.1%	90%
	Lipocalin-2/NGAL	0.012-50	0.004	5.6%	9.5%	94%
	sVCAM-1	0.122-500	0.048	1.9%	11.1%	95%
	SAA	0.244-1000	0.250	4.5%	14.6%	96%
	Analyte	**Std Curve Range (ng/mL)**	**Sensitivity[1] (ng/mL)**	**Coefficient of Variation Intra-**	**Coefficient of Variation Inter-**	**Recovery[3]**
Panel 3 (1:40,000 diluted samples)	A2M	0.488-2000	0.182	3.7%	5.6%	108%
	CRP	0.012-50	0.002	2.4%	15.4%	101%
	Fetuin A	0.244-1000	0.199	2.3%	13.5%	110%
	AGP	0.098-400	0.062	3.9%	11.9%	107%
	Fibrinogen	0.024-100	0.008	3.4%	17.7%	109%
	L-Selectin	0.004-15	0.002	1.4%	5.9%	104%
	SAP	0.004-15	0.002	2.6%	5.0%	104%
	Haptoglobin	0.061-250	0.034	3.6%	10.5%	102%
	PF4/CXCL4	0.037-150	0.012	8.3%	10.0%	113%
	Adipsin	0.098-400	0.116	6.6%	12.8%	114%
	von Willebrand Factor (vWF)	0.244-1000	0.066	3.2%	4.3%	118%
	Analyte	**Std Curve Range (ng/mL)**	**Sensitivity[1] (ng/mL)**	**Coefficient of Variation Intra-**	**Coefficient of Variation Inter-**	**Recovery[2]**
Panel 4 (neat samples)	sE-Selectin	2.743-2000	1.681	6.6%	8.4%	109%
	Follistatin	0.041-30	0.040	4.2%	6.3%	100%
	dPAPP-A	0.686-500	0.032	4.8%	11.7%	108%
	PECAM-1	0.274-200	0.128	7.6%	8.6%	106%
	PTX-3	0.137-100	0.106	5.9%	7.1%	104%
	Tissue Factor	0.137-100	0.057	6.3%	7.7%	104%
	Thrombomodulin	0.343-250	0.142	4.9%	6.0%	103%
	Troponin T	0.007-5	0.020	5.2%	15.7%	98%

[1]minDC average+2SD
[2]In serum matrix
[3]In serum samples

Assay comparison to other commercially available assays

The concentrations of selected analytes determined using the MILLIPLEX® MAP CVD panels were compared to concentrations determined using other commercially available assays, such as ELISAs and Luminex® bead-based assays, and plotted in Figure 2. A linear regression line was fit to each data set to assess correlation. In all cases, R values exceeded 0.8, indicating good correlation between these multiplexed assay panels and other commercially available biomarker quantitation assays.

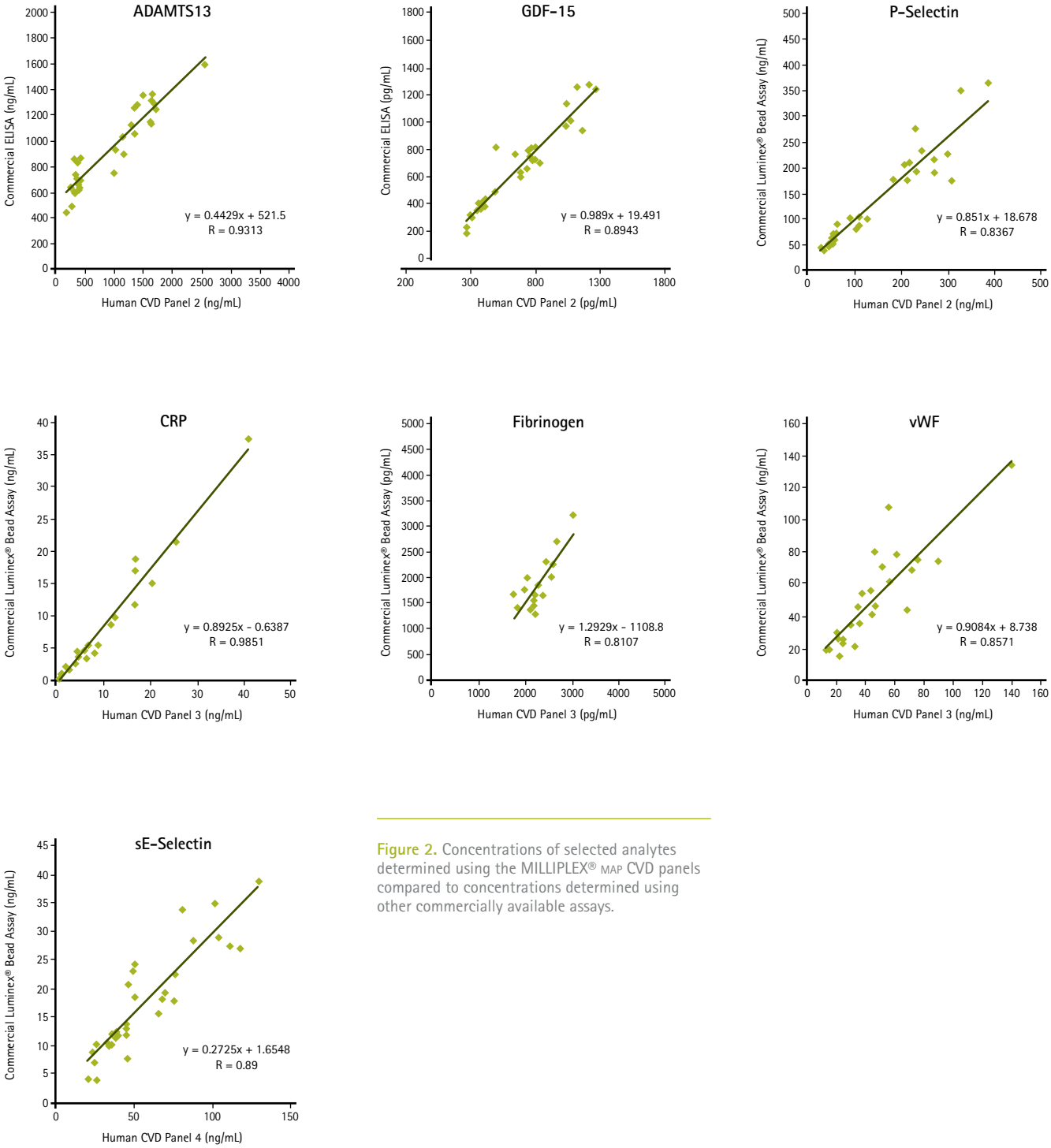

Figure 2. Concentrations of selected analytes determined using the MILLIPLEX® MAP CVD panels compared to concentrations determined using other commercially available assays.

For biological validation, the four human CVD panels were used to measure biomarkers in serum and plasma samples collected from subjects with and without CVD diagnosis (Table 4). Since serum and plasma were not collected from same patients, the analyte concentrations in serum and plasma were not expected to be perfectly matched.

Table 4*.
Median (interquartile range[1]) analyte concentrations. All analyte concentrations are in ng/mL units except where noted.

		Serum (ng/mL)		Plasma (ng/mL)	
		CVD	No CVD	CVD	No CVD
Panel 1	BNP	0	0	0.2†	0
		(0.0, 0.0)	(0.0, 0.0)	(0.1, 0.6)	(0.00, 0.08)
	NTproBNP	0.1	0.008	1.30†	0.11
		(0.0, 0.4)	(0.000, 0.222)	(0.5, 1.6)	(0.08, 0.15)
	CK-MB	4.2	3	3.3	3.3
		(2.6, 7.7)	(2.0, 4.5)	(1.7, 4.6)	(1.8, 4.9)
	CXCL6	0.2	0.2	0.14†	0.21
		(0.1, 0.3)	(0.1, 0.3)	(0.10, 0.19)	(0.16, 0.28)
	CXCL16	0.6*	0.4	0.6†	0.4
		(0.5, 0.7)	(0.3, 0.6)	(0.5, 0.8)	(0.3, 0.5)
	Endocan-1	1.1†	0.7	2.8†	0.9
		(0.8, 1.5)	(0.5, 1.0)	(1.6, 4.4)	(0.7, 1.3)
	FABP3	7.7†	1.9	7.9†	3.3
		(4.1, 19.5)	(1.1, 2.7)	(4.6, 9.4)	(2.5, 4.1)
	FABP4	13.3†	2.5	10.3†	4.5
		(2.9, 29.3)	(1.1, 6.8)	(6.7, 35.3)	(3.3, 10.2)
	PlGF	0.009	0.013	0.019†	0.007
		(0.004, 0.043)	(0.005, 0.023)	(0.008, 0.037)	(0.006, 0.015)
	LIGHT	0.01	0.007	0.012*	0.006
		(0.005, 0.017)	(0.003, 0.015)	(0.010, 0.016)	(0.003, 0.011)
	OSM	0	0	0.3	0.5
		(0.000, 0.011)	(0.00, 0.05)	(0.2, 0.6)	(0.3, 0.7)
	Troponin I	0	0	0.20†	0.05
		(0.0, 1.5)	(0.0, 0.3)	(0.08, 1.01)	(0.00, 0.16)
Panel 2	ADAMTS13	299.0†	1034	607.0†	280.5
		(153.0, 784.0)	(898.5, 1155.0)	(281.5, 827.8)	(224.5, 305.5)
	D-Dimer	1486.0†	230	872	1055.5
		(1062.0, 1562.0)	(188.0, 376.0)	(217.0, 1094.0)	(849.3, 1068.8)
	GDF-15	2.3*	0.7	3.3†	1.4
		(1.7, 3.0)	(0.3, 6.2)	(1.8, 3.9)	(0.7, 1.7)
	Myoglobin	71.8†	32.3	80.8*	52.2
		(56.7, 170.8)	(22.1, 46.1)	(44.3, 248.8)	(42.4, 68.7)
	sICAM-1	79.1	63	95.8*	76.2
		(65.1, 103.3)	(42.9, 85.0)	(77.5, 131.5)	(50.6, 91.5)
	MPO	226	275	305	270
		(150.3, 335.5)	(146.5, 392.8)	(182.0, 754.0)	(208.3, 397.3)
	P-Selectin	81.2	66.1	111	89.5
		(56.6, 112.0)	(48.6, 96.7)	(94.8, 147.5)	(72.1, 105.0)
	Lipocalin-2/NGAL	187.0†	123	332	317.5
		(140.0, 450.0)	(72.7, 154.8)	(185.0, 505.0)	(192.8, 412.3)
	sVCAM-1	1150.5†	622	1117.5*	907.5
		(861.3, 1289.3)	(550.3, 752.3)	(918.8, 1329.8)	(760.0, 1082.0)
	SAA	15326	9020	20465.0†	7906
		(6586.0, 50775.0)	(2456.0, 18847.0)	(9000.0, 31148.0)	(3623.0, 13066.0)

		Serum (ng/mL)		Plasma (ng/mL)	
		CVD	No CVD	CVD	No CVD
Panel 3	A2M (pg/mL)	1248.1	1305	1392	1261.3
		(1120.7, 1604.3)	(1025.0, 1640.0)	(1143.0, 1848.0)	(1134.8, 1641.6)
	CRP (pg/mL)	9.1	3.9	50.5†	5.2
		(2.9, 29.0)	(0.8, 14.2)	(7.0, 135.4)	(1.3, 10.2)
	Fetuin A (pg/mL)	231.6	226.8	207.8	257.4
		(180.5, 255.9)	(179.5, 285.0)	(176.3, 273.5)	(211.7, 291.4)
	AGP (pg/mL)	1931.0*	1388	2259	1811
		(1505.0, 3079.0)	(1197.0, 2012.0)	(1769.0, 2764.0)	(1633.0, 2409.0)
	Fibrinogen (pg/mL)	799.0†	1	7.0†	1518.2
		(5.0, 1223.0)	(0.6, 2.3)	(2.0, 1397.0)	(1355.0, 1692.1)
	L-Selectin (pg/mL)	0.4	0.6	0.4	0.5
		(0.2, 0.6)	(0.3, 0.7)	(0.3, 0.5)	(0.3, 0.6)
	SAP (pg/mL)	4.8	4.7	5.1	5.7
		(3.7, 6.4)	(3.9, 5.6)	(4.0, 6.5)	(3.9, 5.6)
	Haptoglobin (pg/mL)	962	901.8	1444	1337
		(648.0, 1292.0)	(423.9, 1175.4)	(881.0, 2230.0)	(527.0, 2039.0)
	PF4/CXCL4 (pg/mL)	5.4†	9.7	8.2	7.5
		(2.2, 7.3)	(7.9, 13.1)	(6.7, 11.9)	(5.6, 7.8)
	Adipsin (pg/mL)	6.3†	3.1	7.9†	4.7
		(5.0, 9.6)	(2.6, 3.6)	(5.5, 11.9)	(3.1, 5.6)
	vWF (pg/mL)	28.7*	18.2	27.6†	11.9
		(18.1, 47.6)	(13.7, 26.5)	(21.2, 44.0)	(9.2, 19.5)
Panel 4	sE-Selectin	60.5	61.4	34.3	32.2
		(44.4, 111.0)	(45.2, 74.5)	(23.6, 63.1)	(22.7, 42.4)
	Follistatin	0.6	0.7	1.1†	0.5
		(0.3, 1.0)	(0.1, 1.0)	(0.7, 1.4)	(0.3, 0.7)
	dPAPP-A	0.6	0.2	1.3†	0.6
		(0.0, 0.9)	(0.0, 0.5)	(0.7, 2.4)	(0.3, 1.3)
	PECAM-1	2.2	1.9	1.8†	1.3
		(1.4, 3.1)	(1.3, 2.5)	(1.3, 2.5)	(1.0, 1.5)
	PTX-3	1.5	1.3	8.2†	2.8
		(1.1, 2.4)	(0.7, 1.4)	(3.3, 19.6)	(1.9, 3.8)
	Tissue Factor	0.11†	0	0.55†	0.4
		(0.06, 0.23)	(0.00, 0.10)	(0.35, 0.81)	(0.28, 0.60)
	Thrombomodulin	6.4	4	9.1	7
		(3.1, 9.2)	(2.4, 6.4)	(5.9, 14.0)	(5.0, 9.3)
	Troponin T	0	0	0.02	0.03
		(0.00, 0.00)	(0.00, 0.00)	(0.02, 0.07)	(0.00, 0.08)

[1]Interquartile range (Q1 value, Q3 value)
*p<0.05 between CVD and no CVD diagnosis by Mann-Whitney test
†p<0.01 between CVD and no CVD diagnosis by Mann-Whitney test

We selected analytes from each panel that showed significant elevation in CVD patients and used individual value dot plots to visualize the range of analyte concentrations in the individual samples for both CVD and no-CVD groups. In panel 1, biomarkers ESM-1, FABP3, PIGF and Troponin I were elevated in CVD patients compared to no-CVD patients (Figure 3). Among the biomarkers in panel 2, ADAMTS13 and GDF-15 showed the most noticeable elevation in the majority of CVD patients (Figure 4). C-Related Protein, von Willebrand Factor and Adipsin were significantly elevated in CVD plasma samples analyzed using panel 3 (Figure 5), and Folllistatin, dPAPP-A, PECAM-1 and PTX3 were all elevated in CVD plasma samples analyzed using panel 4 (Figure 6).

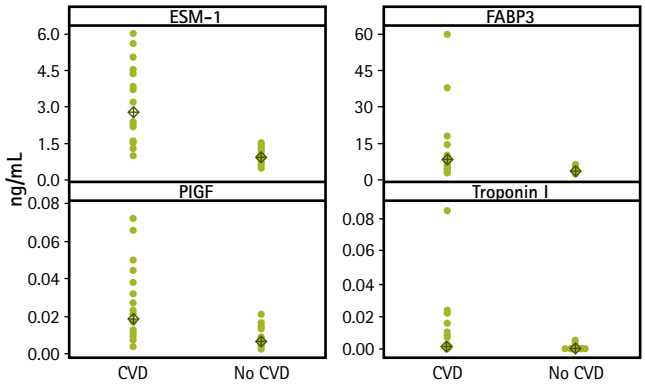

Figure 3. Individual value plots for selected plasma analytes using MILLIPLEX® MAP Human CVD Magnetic Bead Panel 1; p<0.05 for all analytes

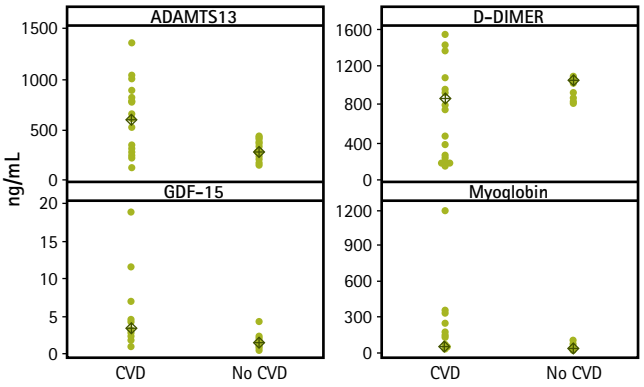

Figure 4. Individual value plots for selected plasma analytes using MILLIPLEX® MAP Human CVD Magnetic Bead Panel 2; p<0.05 for all analytes except for D-dimer.

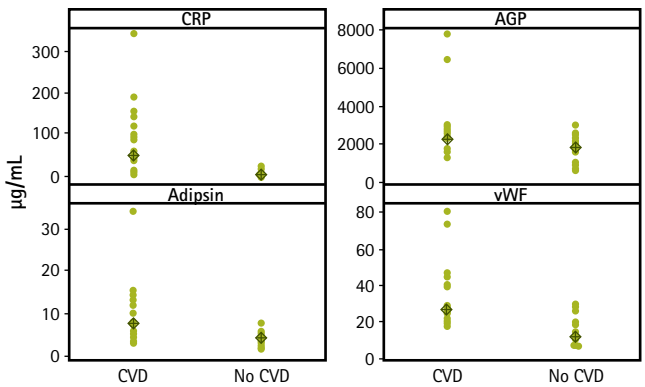

Figure 5. Individual value plots for selected plasma analytes using MILLIPLEX® MAP Human CVD Magnetic Bead Panel 3; p<0.05 for all analytes except AGP.

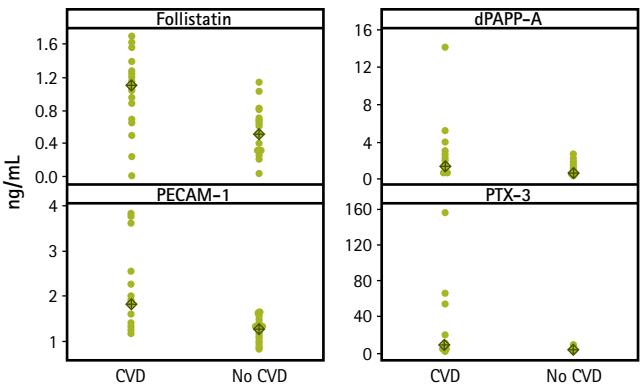

Figure 6. Individual value plots for selected plasma analytes using MILLIPLEX® MAP Human CVD Magnetic Bead Panel 4; p<0.05 for all analytes

Conclusions

Because CVDs are such complex diseases involving multiple organs and systems, multiplexed biomarker analysis is crucial for research into CVD diagnosis and treatment. These four magnetic bead-based multiplex assay panels enable accurate, sensitive, reproducible, simultaneous measurement of 41 CVD biomarkers in serum, plasma and tissue culture samples. In comparison with other commercial assay kits, these new multiplex panels show good correlations. Using these new human CVD biomarker assay panels, we found that, compared to samples from no-CVD subjects, CVD patient samples showed significantly elevated levels of many CVD biomarkers, such as ESM-1, FABP3, PIGF, Troponin I, ADAMTS13, GDF-15, Myoglobin, CRP, vWF, dPAPP-A, PECAM-1, PTX3, and others.

Because of the increased ease of use, throughput and hands-free operation of magnetic bead-based assays compared to traditional methods of biomarker quantitation, these panels have the potential to increase the efficiency of biomarker discovery for clinical CVD research.

Featured Products

Description	Catalogue No.
MILLIPLEX® MAP Human CVD Magnetic Bead Panel 1	HCVD1MAG-67K
MILLIPLEX® MAP Human CVD Magnetic Bead Panel 2	HCVD2MAG-67K
MILLIPLEX® MAP Human CVD Magnetic Bead Panel 3	HCVD3MAG-67K
MILLIPLEX® MAP Human CVD Magnetic Bead Panel 4	HCVD4MAG-67K

Related Instruments & Software

Description	Catalogue No.
MILLIPLEX® Analyst 5.1 (1 Seat License)	40-086
MAGPIX® System	40-072
MAGPIX® System with MILLIPLEX® Analyst Software	40-073
Luminex 200™ xPONENT® System	40-012
Luminex 200™ System with MILLIPLEX® Analyst 5.1	40-013
FLEXMAP 3D® System	40-014
FLEXMAP 3D® System with MILLIPLEX® Analyst	40-022
BioTek® Magnetic 96-well Washer	40-094
BioTek® Magnetic/Vacuum Filtratoin 96-well Washer	40-095
BioTek® Magnetic 96-well Washer with Touch Screen and Ultrasonic Cleaning	40-096
BioTek® Magnetic/Vacuum Filtration 96-well Washer with Touch Screen and Ultrasonic Cleaning	40-097
Handheld Magnetic Separator Block for 96 Well Flat Bottom Plates or 96 Well Conical Well Plates	40-285

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