ORIGINAL RESEARCH

Protein Biomarkers of Cardiovascular Disease and Mortality in the Community

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***Background*—**The discovery of novel and highly predictive biomarkers of cardiovascular disease (CVD) has the potential to improve risk-stratification methods and may be informative regarding biological pathways contributing to disease.

***Methods and Results*—**We used a discovery proteomic platform that targeted high-value proteins for CVD to ascertain 85 circulating protein biomarkers in 3523 Framingham Heart Study participants (mean age, 62 years; 53% women). Using multivariable-adjusted Cox models to account for clinical variables, we found 8 biomarkers associated with incident atherosclerotic CVD, 18 with incident heart failure, 38 with all-cause mortality, and 35 with CVD death (false discovery rate, $q<0.05$ for all; *P*-value ranges, 9.8×10^{-34} to 3.6×10^{-2}). Notably, a number of regulators of metabolic and adipocyte homeostasis were associated with cardiovascular events, including insulin-like growth factor 1 (IGF1), insulin-like growth factor binding protein 1 (IGFBP1), insulin-like growth factor binding protein 2 (IGFBP2), leptin, and adipsin. In a multimarker approach that accounted for clinical factors, growth differentiation factor 15 (GDF15) was associated with all outcomes. In addition, N-terminal pro-b-type natriuretic peptide, C-reactive protein, and leptin were associated with incident heart failure, and C-type lectin domain family 3 member B (CLEC3B; tetranectin), N-terminal pro-b-type natriuretic peptide, arabinogalactan protein 1 (AGP1), soluble receptor for advanced glycation end products (sRAGE), peripheral myelin protein 2 (PMP2), uncarboxylated matrix Gla protein (UCMGP), kallikrein B1 (KLKB1), IGFBP2, IGF1, leptin receptor, and cystatin-C were associated with all-cause mortality in a multimarker model.

***Conclusions*—**We identified numerous protein biomarkers that predicted cardiovascular outcomes and all-cause mortality, including biomarkers representing regulators of metabolic homeostasis and inflammatory pathways. Further studies are needed to validate our findings and define clinical utility, with the ultimate goal of improving strategies for CVD prevention. **(*J Am Heart Assoc*. 2018;7:e008108. DOI: 10.1161/JAHA.117.008108.)**

Key Words: cardiovascular disease risk factors • epidemiology • proteomics

Cardiovascular disease (CVD) is the leading cause of death in the United States and is also emerging as the leading cause of death in developing countries in light of rapid epidemiological transitions over the past 2 decades.[1] This has brought primary prevention of CVD to the forefront. One of the major challenges in developing preventive strategies is that current CVD risk assessment algorithms have limited predictive value and are subject to miscalibration when applied to different populations.[2] The discovery of novel and highly predictive biomarkers of CVD has the potential to improve risk stratification and enable targeted prevention strategies in the preclinical phase of CVD, when intervention is most likely to be effective. In addition, circulating biomarkers may be informative regarding causal biological

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Accompanying Tables S1 through S4 and Methods S1 are available at http://jaha.ahajournals.org/content/7/14/e008108.full#sec-22

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Clinical Perspective

What Is New?

- Numerous protein biomarkers predict cardiovascular outcomes and all-cause mortality, including biomarkers representing regulators of metabolic homeostasis and inflammatory pathways.

What Are the Clinical Implications?

- Our findings highlight the power of targeted proteomics in refining current understanding of cardiovascular risk.
- Further studies are needed to validate our findings and define clinical utility, with the ultimate goal of improving strategies for cardiovascular disease prevention.

pathways contributing to disease, and hold the promise of future pathway-specific therapies and personalized approaches to treatment.

To that end, the Systems Approach to Biomarker Research in Cardiovascular Disease (SABRe CVD) Initiative was established by the National Heart, Lung, and Blood Institute, to identify biomarker signatures of atherosclerotic CVD and its risk factors.[3] The SABRe CVD initiative included both discovery and targeted proteomics. We hypothesized that a multimarker protein panel can improve CVD risk prediction above and beyond the established clinical risk factors. To that end, we measured 85 high-value candidate protein biomarkers for CVD in participants in the FHS (Framingham Heart Study). Biomarkers were formally nominated and selected based on evidence of association with atherosclerotic CVD (ASCVD) from comprehensive literature review, gene expression profiling (FHS and others), published genome-wide association studies of myocardial infarction or coronary heart disease, and discovery proteomics (FHS and others).

Methods

Study Sample

Analytical methods and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure. Anonymized data have been made publicly available at dbGaP (study accession phs00007.v29.p10). The FHS is a prospective, longitudinal, community-based observational cohort study. The Offspring cohort was recruited beginning in 1971 and the Third Generation cohort started in 2002. We included participants in the Offspring cohort attending the seventh examination cycle (1998–2001, n=3539) and Third Generation participants attending their first examination (2002–2005, n=4095).[4,5] Because deaths and CVD events were exceedingly rare in individuals aged <50 years, we excluded individuals below this age (n=3833). In addition, we excluded individuals who had missing clinical covariates (n=238), no available biomarker measures (n=21), or missing follow-up (n=19), leaving 3523 individuals for analyses examining all-cause mortality. For each analysis, prevalent disease was excluded for the corresponding end point (n=412 for atherosclerotic CVD [ASCVD], n=37 for heart failure [HF]). The study was approved by the institutional review board of Boston University Medical Center, and all participants provided written informed consent.

Clinical Assessment

Participants underwent a comprehensive medical history, anthropometry, and physical examination. Resting, seated blood pressure was obtained by a physician and reported as the average of 2 measurements. Diabetes mellitus was defined as a fasting glucose ≥126 mg/dL or the use of hypoglycemic medications for treating hyperglycemia. Current smoking was defined as smoking >1 cigarette daily on average during the past year.

Biomarker Measures

Blood plasma samples were obtained at the baseline clinical visit, and immediately processed and kept at −80°C until assayed. Candidate biomarkers were selected based on the following criteria: (1) association with ASCVD from a review of published studies; (2) targeting proteins coded for by genes associated with ASCVD in genome-wide association studies; (3) targeting genes associated with ASCVD or its major risk factors in gene expression analyses; and (4) discovery proteomics in the FHS or elsewhere (Table S1). A total of 85 biomarkers were then assayed using a modified ELISA sandwich approach, multiplexed on a Luminex xMAP platform (Sigma-Aldrich, St. Louis, MO; Details in Methods S1). A total of 17 different multiplex panels were generated over 4 years based on several factors, including dilution rate, cross-reactivity, and when the target was added to the assay list. Standard Luminex assays with detailed general methods published previously were used.[6,7] Methods for antibody conjugation and multiplex assay development were conducted by contract lab (Sigma-Aldrich) and used protocols recommended and developed by Luminex. These include critical steps for measuring sensitivity, specificity, precision, accuracy, and linearity. All targets were initially developed as a singleton assay before compatible targets were combined to “multiplex panels” and further tested for cross-reactivity and recovery. Commercial reagents were used for assay development when available, but small-scale protein production procedures were used to generate reference proteins when

needed. Measurements were calibrated with a 7-point calibration curve (in triplicate) and tested for recovery at both ends of the quantitation scale. Both the "Hi" and "Lo" spike control (QC1 and QC2, respectively) were used to QC each marker and provide inter- and intra-assay coefficients of variation of assay performance from the production runs. For further details, please see Methods S1. For low-abundance biomarkers, depletion of high-abundance proteins was performed using ProteoPrep 20 (Sigma-Aldrich), an antibody-based resin designed to deplete 95% of total protein from plasma. Assay performance characteristics including medians, interquartile ranges, and coefficients of variation are summarized in Table S1. As an internal control, we examined traditional biomarkers (N-terminal pro-b-type natriuretic peptide [NT-proBNP] and C-reactive protein [CRP]) in prevalent CVD cases versus noncases and found highest levels of both biomarkers among those with HF, followed by CVD, with much lower levels among noncases (Table S2). In addition, we found strong correlations of CRP (r=0.95, n=3282 Offspring participants, P<0.0001; r=0.86, n=4048 Third Generation, P<0.0001) and NT-proBNP with previous standard assays (r=0.81; n=4006; P<0.0001).

Clinical Outcome Ascertainment

Participants were followed with annual health history updates, and all medical records relevant to CVD outcomes were reviewed. Cardiovascular events were adjudicated by a 3-physician panel. Primary outcomes were (1) ASCVD, a composite end point of nonfatal myocardial infarction, revascularization (percutaneous coronary intervention or bypass surgery), atherothrombotic stroke, and coronary heart disease death, (2) HF,[8] and (3) all-cause mortality, with a secondary end point of CVD-related death.

Statistical Analysis

We recoded 14 biomarkers into binary variables (below/above detection limit) because of low numbers above detection limit, and rank normalized the remaining 71 biomarkers. To relate each biomarker to each outcome, we used multivariable Cox proportional hazards regression models. We ran 2 models accounting for baseline covariates: (1) adjusted for age and sex and (2) also adjusted for systolic blood pressure, antihypertensive drug treatment, diabetes mellitus, body mass index, smoking status, total and high-density lipoprotein cholesterol, and prevalent atrial fibrillation. In HF and mortality analyses, we also adjusted for prevalent myocardial infarction. To account for multiple testing in single biomarker models, we set a false discovery rate (FDR; q-value) <0.05.[9] Next, we constructed multimarker models using a step-wise approach: We considered for entry markers with single-marker P<0.10 and used <0.01 for a marker to enter and stay in the step-wise model; we forced in the clinical covariates. For each outcome, we compared the multimarker model versus the base model (clinical covariates only) using a likelihood ratio test. In each of 1000 bootstrap samples, we chose markers using step-wise selection and we calculated frequency of selection for each marker as evidence of its importance as a predictor.

To assess the incremental benefit for predicting outcome events, we compared C-statistics between covariates-only and multimarker models.[10] We also calculated integrated discrimination improvement and net reclassification improvement metrics.[11] For net reclassification improvement, we used 2 categories with the threshold set at observed event rate. We conducted all analyses using SAS software (v9.4; SAS Institute Inc, Cary, NC).

Results

Baseline characteristics of the 3523 participants (mean age, 62 years; 53% women) are displayed in Table 1. During follow-up (median, 14.3 years; Q1=11.4 and Q3=15.2 years), 755 participants died, including 167 from CVD death. Over the follow-up period, there were 392 incident ASCVD events, and 226 participants developed incident HF. Absolute ASCVD event rates were 4% at 5 years and 8% at 10 years. Baseline blood samples were analyzed for a panel of 85 distinct biomarkers, of which 71 had detectable levels for >95% of participant samples, with mean interassay coefficient of variation of 8.9±5.0% and mean intra-assay coefficient of

Table 1. Baseline Characteristics of Participants

Clinical Characteristic	N=3523
Age, y	62 (8)
Women, n (%)	1879 (53)
Systolic blood pressure, mm Hg	128 (18)
Diastolic blood pressure, mm Hg	75 (10)
Body mass index, kg/m^2	28.1 (5.3)
Diabetes mellitus, n (%)	402 (11)
Hypertension treatment, n (%)	1239 (35)
Current smoking, n (%)	434 (12)
Total cholesterol, mg/dL	201 (37)
HDL cholesterol, mg/dL	54 (17)
Prevalent myocardial infarction, n (%)	141 (4)
Prevalent heart failure, n (%)	37 (1)
Prevalent atrial fibrillation, n (%)	132 (4)

Data shown are means and SDs, unless otherwise noted. HDL indicates high-density lipoprotein.

Table 2. Single Biomarker Multivariable-Adjusted Associations With Cardiovascular Outcomes

	Biomarker	HR	95% LCL	95% UCL	*P* Value
Atherosclerotic CVD (N=392)	GDF15	1.38	1.20	1.58	3.6E-06
	TIMP1	1.32	1.17	1.48	5.5E-06
	B2M	1.24	1.10	1.40	3.6E-04
	REG1A	1.19	1.07	1.32	8.5E-04
	Cystatin-C	1.21	1.08	1.36	1.1E-03
	d_Troponin	1.82	1.26	2.63	1.5E-03
	AGP1	1.19	1.06	1.33	2.2E-03
	sICAM1	1.17	1.06	1.30	2.6E-03
Heart failure (N=226)	NT-proBNP	1.98	1.67	2.34	2.4E-15
	GDF15	2.08	1.72	2.53	9.1E-14
	ADM	1.47	1.25	1.73	2.5E-06
	B2M	1.47	1.24	1.73	4.5E-06
	Cystatin-C	1.43	1.22	1.67	6.5E-06
	CRP	1.38	1.19	1.60	2.5E-05
	TIMP1	1.39	1.18	1.63	6.0E-05
	IGFBP1	1.37	1.17	1.61	1.2E-04
	CD14	1.31	1.14	1.51	1.5E-04
	MPO	1.30	1.13	1.48	1.4E-04
	UCMGP	1.32	1.14	1.52	1.8E-04
	EFEMP1	1.34	1.14	1.57	3.2E-04
	GRN	1.29	1.12	1.48	4.2E-04
	Adipsin	1.31	1.12	1.52	5.8E-04
	IGFBP2	1.30	1.11	1.51	8.3E-04
	Resistin	1.25	1.09	1.43	1.1E-03
	A1M	1.21	1.06	1.39	4.5E-03
	CLEC3B	0.82	0.71	0.95	6.3E-03
All-cause mortality (N=755)	GDF15	1.96	1.76	2.17	1.2E-35
	NT-proBNP	1.43	1.31	1.57	2.1E-15
	B2M	1.39	1.27	1.52	1.0E-12
	TIMP1	1.36	1.24	1.48	1.0E-11
	IGFBP2	1.34	1.23	1.46	9.5E-12
	UCMGP	1.32	1.22	1.43	1.8E-11
	ADM	1.33	1.22	1.45	2.5E-10
	EFEMP1	1.28	1.17	1.40	2.6E-08
	CD14	1.24	1.15	1.34	4.2E-08
	CRP	1.24	1.15	1.34	5.9E-08
	CLEC3B	0.82	0.76	0.88	2.9E-07
	sICAM1	1.20	1.12	1.29	1.2E-06
	Cystatin-C	1.23	1.13	1.34	1.9E-06
	d_IL6	1.92	1.45	2.55	4.5E-06
	GRN	1.19	1.10	1.28	5.1E-06

Continued

Table 2. Continued

	Biomarker	HR	95% LCL	95% UCL	*P* Value
	REG1A	1.19	1.10	1.28	7.9E-06
	FGF23	1.19	1.10	1.28	8.0E-06
	AGP1	1.17	1.09	1.27	4.2E-05
	MMP8	1.17	1.08	1.26	4.1E-05
	IGF1	0.87	0.81	0.94	1.9E-04
	IGFBP1	1.18	1.08	1.29	2.0E-04
	CD5L	1.15	1.07	1.24	3.0E-04
	Adipsin	1.16	1.07	1.26	4.1E-04
	SAA1	1.14	1.06	1.23	4.6E-04
	MMP9	1.15	1.06	1.25	4.7E-04
	BCHE	0.87	0.81	0.94	5.6E-04
	PON1	0.88	0.81	0.95	9.4E-04
	MCP1	1.13	1.05	1.22	1.0E-03
	MPO	1.12	1.04	1.21	1.7E-03
	sGP130	1.13	1.05	1.21	1.7E-03
	Ceruloplasmin	1.15	1.05	1.25	2.0E-03
	Resistin	1.12	1.04	1.20	2.8E-03
	A1M	1.12	1.04	1.20	3.2E-03
	COL18A1	1.11	1.03	1.20	4.6E-03
	IGFBP3	0.90	0.84	0.97	5.1E-03
	CNTN1	0.90	0.83	0.97	7.9E-03
	KLKB1	0.90	0.83	0.98	1.3E-02
	PMP2	0.91	0.85	0.98	1.6E-02
CVD death (N=167)	NT-proBNP	2.43	1.99	2.97	1.6E-18
	ADM	1.75	1.45	2.11	4.1E-09
	GDF15	1.96	1.56	2.46	6.9E-09
	B2M	1.72	1.42	2.09	3.0E-08
	EFEMP1	1.60	1.33	1.94	9.5E-07
	Cystatin-C	1.55	1.29	1.87	4.1E-06
	UCMGP	1.43	1.21	1.70	3.6E-05
	Adipsin	1.45	1.21	1.73	4.7E-05
	IGFBP2	1.42	1.19	1.70	1.2E-04
	AGP1	1.38	1.17	1.62	1.1E-04
	REG1A	1.37	1.16	1.61	1.6E-04
	CRP	1.38	1.17	1.63	1.8E-04
	PMP2	0.75	0.64	0.88	4.4E-04
	TIMP1	1.40	1.16	1.69	5.0E-04
	SAA1	1.33	1.13	1.56	6.5E-04
	Ceruloplasmin	1.40	1.15	1.70	7.3E-04
	BCHE	0.76	0.64	0.89	8.3E-04
	NRCAM	0.76	0.65	0.89	8.1E-04

Continued

Table 2. Continued

	Biomarker	HR	95% LCL	95% UCL	*P* Value
	CD14	1.32	1.12	1.56	9.4E-04
	d_IL6	2.46	1.44	4.19	1.0E-03
	CDH13	0.77	0.66	0.91	2.0E-03
	Resistin	1.28	1.09	1.49	2.2E-03
	FGF23	1.28	1.09	1.50	2.6E-03
	sICAM1	1.27	1.09	1.48	2.6E-03
	LDLR	0.78	0.66	0.92	2.6E-03
	MMP9	1.30	1.09	1.54	2.8E-03
	COL18A1	1.27	1.08	1.49	3.2E-03
	ADAM15	0.79	0.68	0.92	3.1E-03
	A1M	1.26	1.08	1.47	3.5E-03
	sGP130	1.25	1.07	1.47	5.3E-03
	d_FLT3	0.64	0.47	0.88	5.4E-03
	IGFBP1	1.31	1.08	1.58	5.6E-03
	FBN	1.25	1.06	1.48	9.4E-03
	d_CSF2RB	0.67	0.49	0.91	1.2E-02
	GRN	1.23	1.05	1.44	1.2E-02

Multivariable-adjusted model, adjusted for age, sex, systolic blood pressure, hypertension treatment, diabetes mellitus, body mass index, smoking, total and HDL cholesterol, and history of atrial fibrillation. In addition, heart failure and mortality analyses were adjusted for prevalent myocardial infarction. CVD indicates cardiovascular disease; d_, dichotomous biomarker; HDL, high-density lipoprotein; HR, hazards ratio per 1-SD change in rank normalized data; LCL, lower 95% confidence interval; UCL, upper 95% confidence interval.

variation 7.8±4.9% (Table S1). The majority of biomarkers were weakly correlated with one another, with correlation coefficients ranging from −0.2 to 0.2 (Table S2). Two percent of pair-wise correlations were modest or strong with $r>0.40$. The strongest pair-wise correlations were observed for cadherin 13 (CDH13) and ADAM metallopeptidase domain-containing protein 15 (ADAM15; $r=0.90$).

Multiple Biomarkers Predict Incident Cardiovascular Events

In multivariable-adjusted single-marker analyses, 8 biomarkers were positively associated with incident ASCVD (FDR-q <0.05 for all; *P*-value range from 3.6×10^{-6} to 2.6×10^{-3}), including growth differentiation factor 15 (GDF15), tissue inhibitor of metalloproteinase-1 (TIMP1), and beta-2-microglobulin (B2M; Table 2; full results presented in Table S3). Similarly, there were 18 biomarkers associated with incident HF (FDR-q <0.05 for all; *P*-value range from 2.4×10^{-15} to 6.3×10^{-3}), including NT-proBNP, GDF15, and adrenomedullin (ADM; Table 2). Of these protein biomarkers, higher concentrations of C-type lectin domain family 3 member B (CLEC3B; tetranectin) were associated with a lower risk of HF (hazard ratio [HR], 0.82 per 1-SD increase in CLEC3B; 95% confidence interval [CI], 0.71–0.95; $P=6.6\times10^{-3}$), whereas all other 17 markers were associated with a higher risk of HF. There were 35 biomarkers associated with CVD death (FDR-q <0.05 for all; *P*values from 1.6×10^{-18} to 3.0×10^{-2}), and 38 biomarkers predicted all-cause mortality (FDR-q <0.05 for all; *P* values from 9.8×10^{-34} to 3.6×10^{-2}; Table 2). Of the 38 biomarkers associated with mortality, CLEC3B, insulin-like growth factor 1 (IGF1), butyrylcholinesterase (BCHE), paraoxonase 1 (PON1), insulin-like growth factor binding protein 1 (IGFBP3), CNTN1, kallikrein B1 (KLKB1), and peripheral myelin protein 2 (PMP2) were associated with lower risk of mortality, whereas all others predicted a higher risk of mortality. Age- and sex-adjusted analyses are presented in Table S4.

Biomarkers Associated Across Different Fatal and Nonfatal Cardiovascular Outcomes

We noted prominent overlap in biomarkers that predicted more than 1 outcome (Figure). In total, 28 biomarkers predicted both CVD death and all-cause mortality, including GDF15, ADM, CRP, cystatin-C, and NT-proBNP, among others. There was overlap in prediction of both fatal and nonfatal cardiovascular outcomes, with GDF15, TIMP1, B2M, and cystatin-C predicting higher risk of atherosclerotic CVD, HF, mortality, and CVD death. Furthermore, all 18 biomarkers that

Figure. Heatmap of biomarkers associated with all-cause mortality (FDR-q <0.05) are also associated with other concomitant fatal and non-fatal cardiovascular outcomes, including atherosclerotic CVD, heart failure, and CVD death. ASCVD indicates atherosclerotic cardiovascular disease; CVD, cardiovascular disease; FDR, false discovery rate; HF, heart failure; HR, hazard ratio.

predicted incident HF also predicted all-cause and/or CVD death.

Multimarker Models Predict Incident Cardiovascular Events and Death

Joint associations of multiple biomarkers with incident events were assessed using step-wise regression, after accounting for clinical covariates (Table 3). When added to a clinical model, only GDF15 was associated with incident ASCVD: (HR, 1.43 per 1-SD increase in GDF15; 95% CI, 1.24–1.65; $P=1.3\times10^{-6}$). Four biomarkers jointly predicted incident HF: NT-proBNP (HR, 1.79; 95% CI, 1.51–2.12; $P=1.9\times10^{-11}$), GDF15 (HR, 1.78; 95% CI, 1.44–2.19; $P=7.5\times10^{-8}$), CRP (HR, 1.29; 95% CI, 1.10–1.50; $P=0.001$), and leptin (HR, 0.73; 95% CI, 0.60–0.89; $P=0.002$).

Twelve biomarkers were significantly associated with all-cause mortality in a multimarker model: GDF15, CLEC3B, NT-proBNP, arabinogalactan protein 1 (AGP1), soluble receptor for advanced glycation end products (sRAGE), PMP2, uncarboxylated matrix Gla protein (UCMGP), KLKB1, insulin-like growth factor binding protein 2 (IGFBP2), IGF1, leptin receptor, and cystatin-C. Six biomarkers were associated with CVD death: NT-proBNP, PMP2, AGP1, matrix metalloproteinase 9 (MMP9), GDF15, and CLEC3B.

We used bootstrap resampling and fitted step-wise multimarker models in 1000 samples to assess frequencies with which biomarkers remained in the multimarker model (Table 3). GDF15 entered the model for all-cause mortality 100% of the time and for HF 96% of the time. Similarly, NT-proBNP entered the model for both HF and CVD death 100% of the time and for all-cause mortality 99% of the time. Other markers were noted to be less robust, including CRP for HF, cystatin-C for mortality, and PMP2 AGP1, GDF15, and CLEC3B for CVD death, each of which entered the specific models <50% of the time.

Performance Metrics of Multimarker Models

Model discrimination was assessed by improvement in the C-statistic with the addition of multimarkers to a clinical model. Specifically, the C-statistic increased from 0.753 to 0.758 for ASCVD, from 0.843 to 0.873 for HF, from 0.783 to 0.818 for all-cause mortality, and from 0.851 to 0.880 for CVD death ($P<0.0001$ for all, comparing the likelihood ratio of the model with and without biomarkers; Table 4). The integrated discrimination improvement was highest for CVD death and HF (0.188 and 0.124, respectively). We examined the 2-category net reclassification improvement, using event rates to define high- versus low-risk categories. The net reclassification improvement for the addition of multiple biomarkers to the base clinical model was <1% for ASCVD, 3.5% for HF, 7.7% for all-cause mortality, and 9.5% for CVD death.

Discussion

Using a proteomic platform that targeted 85 high-value proteins for CVD, we identified 8 individual biomarkers associated with incident ASCVD, 18 with incident HF, 38 with all-cause mortality, and 35 with CVD death after accounting for potential clinical confounders. In a multimarker approach that accounted for clinical factors, GDF15 was associated with all outcomes. In addition, NT-proBNP, CRP, and leptin were associated with incident HF, and CLEC3B (tetranectin), NT-proBNP, AGP1, sRAGE, PMP2, UCMGP, KLKB1, IGFBP2, IGF1, leptin receptor, and cystatin-C were associated with all-cause mortality in a multimarker model. We demonstrate multiple new associations of protein

Table 3. Multimarker Models Associated With Cardiovascular Outcomes

Outcome	Biomarker	HR	95% LCL	95% UCL	*P* Value	Bootstrap (n)*
Atherosclerotic CVD	GDF15	1.43	1.24	1.65	1.3E-06	574
Heart failure	NT-proBNP	1.79	1.51	2.12	1.9E-11	1000
	GDF15	1.78	1.44	2.19	7.5E-08	957
	CRP	1.29	1.10	1.50	1.4E-03	462
	Leptin	0.73	0.60	0.89	1.5E-03	577
Mortality	GDF15	1.71	1.50	1.94	3.7E-16	1000
	CLEC3B	0.80	0.73	0.87	2.4E-07	941
	NT-proBNP	1.30	1.18	1.44	2.7E-07	985
	AGP1	1.24	1.14	1.36	6.8E-07	821
	sRAGE	0.83	0.77	0.91	3.0E-05	884
	PMP2	0.86	0.79	0.93	1.1E-04	517
	UCMGP	1.18	1.08	1.29	2.2E-04	817
	KLKB1	0.84	0.77	0.92	3.0E-04	609
	IGFBP2	1.19	1.08	1.32	4.0E-04	715
	IGF1	0.88	0.81	0.95	9.7E-04	556
	Leptin R	1.14	1.05	1.24	1.9E-03	504
	Cystatin-C	0.87	0.79	0.97	8.6E-03	412
CVD death	NT-proBNP	2.09	1.69	2.58	6.0E-12	1000
	PMP2	0.68	0.57	0.82	2.4E-05	392
	AGP1	1.44	1.20	1.71	6.6E-05	462
	MMP9	1.41	1.16	1.72	4.9E-04	855
	GDF15	1.45	1.12	1.87	5.0E-03	369
	CLEC3B	0.77	0.64	0.92	5.1E-03	366

Multivariable-adjusted model, adjusted for age, sex, systolic blood pressure, hypertension treatment, diabetes mellitus, body mass index, smoking, total and HDL cholesterol, and history of atrial fibrillation. In addition, heart failure and mortality analyses were adjusted for prevalent myocardial infarction. CVD, cardiovascular disease; HDL, high-density lipoprotein; HR, hazards ratio per 1-SD change in rank normalized data; LCL, lower 95% confidence interval; UCL, upper 95% confidence interval.
*Bootstrap analysis: number of times biomarker entered into the model out of 1000 samples.

biomarkers that regulate metabolic and inflammatory pathways with clinical outcomes using a multiplexed high-throughput platform. Previous proteomics approaches among large cohorts have been predominantly focused on CVD case-control designs.[3,12] We now extend previous promising results and test the association of targeted proteomic biomarkers with incident CVD events and death in the community. These findings may have future potential implications with respect to clinical risk prediction.

Whereas GDF15, NT-proBNP, ADM, cystatin-C, fibroblast growth factor 23 (FGF23), and CRP are known to predict incident CVD and other outcomes,[13–16] there are a number of novel associations in our study to highlight. Specifically, a number of regulators of metabolic and adipocyte homeostasis appeared to predict adverse events in our study: IGF1 is involved in adipocyte proliferation and differentiation, and whereas some studies support a protective role with respect to atherosclerosis, others have not shown any association with coronary artery disease.[17,18] In the FHS, lower IGF1 levels were previously reported to be associated with adverse metabolic risk,[19] and we now show an association with mortality in both single- and multimarker models. IGF1, in turn, is regulated by insulin-like growth factor binding protein 1 (IGFBP1), which plays a role in glucose counter-regulation. Higher IGFBP1 levels were previously found to be associated with lower metabolic risk, but greater mortality,[20] and IGFBP1 was reported to predict incident HF among older adults.[3,21] We now extend these observations and show that IGFBP1 predicts incident HF, all-cause mortality, and CVD mortality in our cohort, along with 3 other regulators of metabolic disease and adipocyte function: IGFBP2, adipsin, and leptin. IGFBP2 is the predominant insulin-like growth factor binding protein (IGFBP) produced by white adipose tissue and is thought to protect against obesity and insulin resistance in mice.[8,22] Previous studies, however, did not demonstrate any relation to CVD outcomes.[10,21] Adipsin has been shown to regulate

Table 4. Performance Metrics of Multimarker Models

Performance Metric	Atherosclerotic CVD	Heart Failure	Mortality	CVD Death
C-statistic (95% CI)				
Clinical model	0.753	0.843	0.783	0.851
Clinical+multimarker*	0.758	0.873	0.818	0.880
Change in C-statistics	0.004	0.030	0.036	0.030
P value (LR)	0.00008	<0.0001	<0.0001	<0.0001
IDI (95% CI)	0.012 (0.004, 0.023)	0.124 (0.084, 0.173)	0.083 (0.068, 0.11)	0.188 (0.126, 0.259)
2-category NRI (95% CI)	0.007 (−0.018, 0.065)	0.035 (−0.015, 0.109)	0.077 (0.045, 0.122)	0.094 (0.021, 0.189)

2-category NRI indicates clinical vs clinical+multimarker models, using outcome-specific event rate to define high- vs low-risk categories; CI, confidence interval; CVD, cardiovascular disease; IDI, integrated discrimination improvement; LR, likelihood ratio comparing clinical vs clinical+multimarker models.
*Multimarker models are outcome specific and include markers included in step-wise models described in Table 3.

inflammation in adipose tissue and may improve beta-cell function in diabetes mellitus.[11,23,24] Among women, adipsin is correlated with metabolic parameters, including body mass index, insulin resistance, and carotid atherosclerosis,[3,12,25] but it has not been linked to adverse CVD outcomes previously. Last, resistin is an adipokine that impairs insulin action and is thought to represent a mechanistic link of obesity with diabetes mellitus.[17,18,26] In previous FHS analyses, resistin was reported to be associated with incident HF, and it also predicted adverse outcomes among patients with stable coronary disease in the Heart and Soul study.[19,27,28]

Our results for multiple inflammatory cytokines and regulators are also notable. These include B2M, cluster of differentiation 14 (CD14), soluble intracellular adhesion molecule 1 (sICAM1), granulin (GRN), AGP1, CD5 molecule-like (CD5L), serum amyloid A1 (SAA1), monocyte chemoattractant protein 1 (MCP1), soluble glycoprotein 130 (sGP130), interleukin 6 (IL6), and myeloperoxidase (MPO), which predicted all-cause mortality in our study. B2M is a glomerular filtration marker and acute-phase reactant that has been associated with CVD events and mortality among patients with chronic kidney disease,[20,29] and was also associated with all-cause mortality in the National Health and Nutrition Examination Survey.[30] In addition to predicting all-cause mortality, B2M is also a predictor of incident ASCVD, HF, and CVD mortality in our study. Soluble CD14 is also an acute-phase protein and serves as the receptor for lipopolysaccharide.[31] In clinical studies, soluble CD14 predicted all-cause mortality among older adults.[32] We now extend these findings to a wider age range, and show that soluble CD14 predicts incident HF and all-cause and CVD mortality. sICAM1 is a cell adhesion molecule that previously has been associated with CVD, including myocardial infarction, in case-control studies.[33,34] We now show that sICAM1 predicts not only ASCVD, but also all-cause mortality and CVD mortality. GRN has previously been linked to atherosclerosis through inflammatory activation in experimental studies[35] and predicts incident HF, all-cause, and CVD mortality in our study. AGP1 is another acute-phase reactant, and its protein, orosomucoid, predicted mortality among elderly individuals.[36] In our analyses, AGP1 predicted ASCVD, as well as all-cause and CVD mortality in single- and multimarker models. CD5L inhibits macrophage apoptosis and is thought to regulate infarct size in animal models.[37] SAA1 is an acute-phase reactant that previously has been associated with future coronary disease in various populations.[38] We report an association of SAA1 with all-cause and CVD mortality. MCP1 is a chemokine that regulates monocyte trafficking and previously has been associated with myocardial infarction in FHS and adverse outcomes in patients with acute coronary syndrome.[39,40] sGP130 is a natural inhibitor of IL6 responses and has been associated with adverse outcomes among ischemic HF patients.[41] We now show an association of GP130 with all-cause and CVD mortality. Last, MPO is secreted by activated leukocytes and is thought to mediate oxidative stress and atherogenesis.[42] It has been associated with future coronary disease and HF in case-control studies.[43,44] In our study, MPO predicted incident HF, in addition to all-cause mortality.

Last, our study sought to validate previous genetic associations, with selection of a number of targeted biomarkers from genome-wide association studies. For example, the precursor to GRN is progranulin, and serves as the ligand for sortilin 1 (SORT1),[45] which, in turn, is thought to regulate lipoprotein metabolism and has been linked to risk of myocardial infarction in previous genome-wide association studies studies.[46] We now demonstrate an association of GRN with both CVD and all-cause mortality, supporting the association of the GRN/SORT1 pathway with CVD events.

Numerous studies have examined the utility of multiple biomarkers in CVD risk prediction, with the ascertainment of markers representing different pathophysiological pathways that might add complementary information with respect to

risk prediction. The incremental information provided by multiple protein biomarkers to existing clinical risk factors, however, has remained modest.[14,47,48] When examined in a multimarker approach, we similarly found modest improvements in model discrimination metrics. Interestingly, 12 biomarkers independently predicted all-cause mortality on top of clinical variables. In contrast, in attempting to create a protein multimarker for ASCVD, only GDF15 entered the model. Although biomarkers were targeted on the basis of existing evidence for associations with atherosclerosis, our findings need further validation in external cohorts. However, 45 of 85 biomarkers were selected on the basis of known associations with ASCVD from the literature and 41 on the basis of previous discovery proteomics evidence, highlighting the fact that the majority of biomarkers selected were motivated by existing clinical, genetic, gene expression profiling, or discovery proteomics data. There are other limitations that deserve mention. Ours are observational data, and causal or mechanistic inference cannot be drawn. Furthermore, limitations of the multiple reaction monitoring mass spectrometry platform include bias toward detection of more-abundant proteins.

In conclusion, we identified 8 individual biomarkers that predicted incident ASCVD, 18 that predicted incident HF, and 38 that predicted all-cause mortality. Many biomarkers represent regulators of metabolic and adipocyte homeostasis; others are involved in inflammatory pathways. Using a targeted discovery proteomic platform, we were able to extend previous findings, demonstrate novel biomarker associations with incident CVD events, and validate previous genetic associations. Large-scale mass spectrometry or newer aptamer-based technology[49,50] may allow for profiling of many more proteins in large study samples that may refine current multimarker models. Further studies are needed to validate our findings and define clinical utility, with the ultimate goal of improving targeted strategies for CVD prevention.

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Disclosures

None.

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Solutions and buffers used in Luminex assay

AB-A (1 x Assay Buffer –A)
Final concentration:
PBS (1;10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
1% BSA (optimized. A7906, A7030, A3059 etc)
0.05%Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # E7889, 0.5M)
0.22µ filtrated

Prepare 2 litters
1. Prepare 200mL 10%BSA in dd H2O and filtrate vial 0.2µ syringe filter.
2. Dilute 10 x PBS into 1.5 litters of milli-Q water with a magnetic stirring rod. Add10ml 10% tween-20, 1.6g NaN_3 and 20ml 0.5M EDTA-Na2. Stir to complete dissolve of all components. Adjust pH to 7.4. Adjust total volume to 1.8 litters with dd H2O. Filtrate the buffer through 0.22µ membrane into 2 x 1000 ml bottle.
3. Add 100mL filtered 10%BSA to each bottle.

Store at 4ºC for 2 years.

AB-B (1 x)
1 x PBS (Sigma P7059)
1% BSA (optimized. A7906, A7030, A3059 etc)
0.08% Azide
0.22µ filtrated

AB-C (1 x)
AB-A +
Protease inhibitors (Sigma S8820), 1 tablet/100ml

AB-D (1 x)
AB-A +
Protease inhibitors (Sigma S8820), 1 tablet/100ml
DPP4 inhibitor (1:100)

AB-E
1 x PBS (Sigma# P7059)
0.2%BSA
0.05%Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # 7889, 0.5M)
0.22µ filtrated

AB-F (1 x Assay Buffer –F)
Final concentration:
PBS (1;10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
1% Chicken albumin (Sigma A5503 or A2512)
0.05%Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # 7889, 0.5M)
0.22µ filtrated

AB-G (1 x Assay Buffer –G)
Final concentration:
PBS (1;10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
0.2% Chicken albumin (Sigma A5503 or A2512)
0.05%Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # 7889, 0.5M)
0.22µ filtrated

AB-H (1 x Assay Buffer –H)
Final concentration:
PBS (1;10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
0.05%Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # 7889, 0.5M)
0.22µ filtrated

Beads Diluent A (BD-A)
1 x PBS (Sigma# P7059)
1% BSA (optimized. A7906, A7030, A3059 etc)
0.05%Tween-20 (Sigma # P7949)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # 7889, 0.5M)
IIR (Bioreclaimation, # 6LD1074), 150µg/ml IIR)
0.22µ filtrated

Beads Diluent B (BD-B)
1 x PBS (Sigma# P7059)
0.1 % BSA (optimized. A7906, A7030, A3059 etc)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # 7889, 0.5M)
IIR (Bioreclaimation, # 6LD1074), 100µg/ml IIR)
0.22µ filtrated

Beads Diluent C (BD-C)
1 x PBS (Sigma# P7059)
0.1 % Ovalbumin(Sigma A5503 or A2512)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # 7889, 0.5M)
IIR (Bioreclaimation, # 6LD1074), 100µg/ml IIR)
0.22µ filtrated

Beads Diluent D (BD-D)
1 x PBS (Sigma# P7059)
0.1 % BSA (optimized. A7906, A7030, A3059 etc)
0.08% Azide (Sigma # S8032)
5mM EDTA-Na_2 (Sigma # 7889, 0.5M)
IIR (Bioreclaimation, # 6LD1074), 10µg/ml IIR)
0.22µ filtrated

10 X Wash Buffer (WB), 1000ml
Dissolve 10 bag of PBS Sigma P3563) and 5 g of NaN_3 in a 1000ml bottle. Stir to dissolve completely and filtrate through 0.22µ membrane. Store at RT

1 x WB was diluted from 10 x stock (see below) with water
Final concentration: 10mM PB, pH7.4, 138mM NaCl, 2.7mM KCl, 0.05% Tween-20
0.05% NaN3

Blocking Buffer (BB-A)
Same as AB-B

Blocking Buffer C (BB-C)
1 x PBS (Sigma P7059)
1% Ovalbumin(Sigma A5503 or A2512)
0.08% Azide
0.22µ filtrated

Activation Buffer (100mM NaH_2PO4, pH 6.0), 1000ml
Deionized water 800 ml
Sodium phosphate monobasic (Sigma S3139, NaH_2PO_4), 12g
adjust pH to 6.0 with 5 N NaOH (about 70 drops)
Bring final volume to 1000ml

Coupling Buffer-A (CB-A) (50mM MES, pH5.0), 1000ml
Deionized water 800 ml
MES (Sigma M2933), 9.76g

adjust pH to 5.0 with 5 N NaOH (about 15 drops)
Bring final volume to 1000ml

Coupling Buffer-B(CB-B) (100mM acetic acid, 300mM NaCl, pH 4.0), 250ml
Deionized water 233ml
5M NaCl (Sigma 5150), 15ml
Acetic acid (Sigma #A6283, MW=60.05, d=1.049), 1.5ml
Adjust pH to 4.0 with 5N NaOH (about 30 drops)

PBS, pH 7.4 (1 x)
Purified water 900 ml
PBS (Sigma P7059, 10 X), 100 ml
Final concentration (10mM PB, 155 mM NaCl, pH 7.4)

1 x TBS
Sigma T6664, 50mM Tris pH8.0, 138mm NaCl, 2.7mM KCl

SAPE
Cat#-xx
Assay Buffer + xx µg/ml SAPE
e.g. S866-15 (Invitrogen SAPE Cat#S866, 15µg/mL)

30 x BSA-NaN3
Final concentration:
PBS (1;10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
3% BSA (optimized. A7906, A7030, A3059 etc)
2.4% Azide (Sigma # S8032)
0.22µ filtrated

30 x OA-NaN3
Final concentration:
PBS (1;10 diluted Sigma P7059, 10mM PB pH7.4, 0.9%NaCl)
3% Ovalbumin (Sigma A5503 or A2512)
2.4% Azide (Sigma # S8032)
0.22µ filtrated

Antibody biotinylation (production scale)

Document History
Amicon Ultra-filter tube has been changed from 20mL to 4mL, (MWCO=30 kD), to reduce antibody loss during buffer exchange. Antibody buffer with azide requires buffer exchange

Use

This protocol is to biotinylate antibodies for Luminex assay

Safety

NaN_3 is used in some solutions for this protocol. *EZ-Link Sulfo-NHS-LC-LC-Biotin* may cause eye irritation. Please always wear goggles, gloves and lab coat during experiment. Follow all necessary safety procedures in the lab.

Materials needed

1 x PBS pH 7.4 (diluted from Sigma P7059)
1 M Tris-HCl, pH 7.0 (for small scale biotinylation of Ab <100µg only)
1 x TBS (Sigma T6664, 50mM Tris pH8.0, 138mm NaCl, 2.7mM KCl)
30 x OA-NaN_3 (3% Ovalbumin-2.4%NaN_3) (see protocol for buffer preparation.doc)
30 x BSA-NaN_3 (3% BSA-2.4%NaN_3) (see protocol for buffer preparation.doc)
Assay Buffer-A or -F (AB-A or AB-F) (see protocol for buffer preparation.doc)
Antibody to be biotinylated
EZ-Link Sulfo-NHS-LC-LC-Biotin (PIERCE: #21338), stored at –20ºC in desiccator (each opened vial should be used within three months under proper storage)
Filter tube (Amicon Ultra-4, MWCO=30 kD), need 2 tubes/antibody
1.5 ml screw cap microtube and 15ml tube
Aluminum foil
Plate shaker
Centrifuge (with swing bucket rotor for 50ml Conical tubes)
A print copy of work sheet (Click "***..\Assay Panels\1st year panels\Master work sheet for antibody biotinylation for 1st year targets.xls***")

Protocol

A. Preparatin

1. Bring buffers, *EZ-Link Sulfo-NHS-LC-LC-Biotin* and *antibodies* to RT for at least 30 min.
2. Double check data sheet and vial label to make sure the antibody contains no BSA glycine, or Tris or NaN_3. Azide, Glycine and Tris can be removed by filtration through *Amicon Ultra-4 tube (30 kD)* for three times with 3 x 15 ml PBS at 4,000 RPM centrifugation for 10-15 min (at 25C). BSA can be removed by *NAb Protein A/G Spin Kit* (Pierce # 89950). After buffer exchange or purification, the final concentration of antibody must be measured again.
3. Calculate amount of reagents based on ratio at:
   1mg Antibody/150 µg Biotin (Molecular ration≈1:30)
4. Prepare one filter tube (*Amicon Ultra-15 tube, MWCO 30 kD)* for each antibody to be biotinylated. Add 15 ml TBS to each tube and stay at RT until use at step C.

B. Biotinylation

5. Adjust antibody solution to about 0.1-1mg/ml with PBS

6. Calculate the total volume needed for biotinylation of all antibodies at same time. Prepare appropriate amount of Biotin (1mg/ml, freshly prepared in PBS) at the ratio above. Biotin is light sensitive, please use amber vials or wrap the tube with aluminum foil. The prepared solution should be used as soon as possible.
7. Pipette required volume of Biotin to each antibody tube on the table. (Note: please change tips for each tube) Wrap each tube with aluminum foil before wrapping multiple tubes together. Discard the leftover of Biotin solution.
8. Fix the wrap with rubber belts on the shaker stage. Shake the tubes for 1 hour at RT with maximum intensity of shaker setting.
9. Go next step to stop biotinylation.

C. Stop biotinylation
(light protection is no longer needed from now)

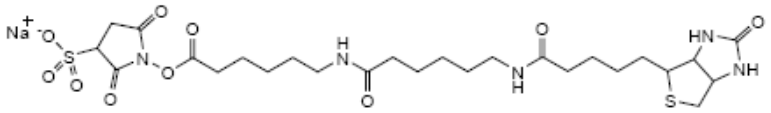
10. Check the filter tubes for leaking. There should not be any TBS flow through to the bottom of collection tubes without centrifugation. Replace with a new filter tube if leaking is observed. Empty all filter tubes.
11. Briefly spin the biotinylation tubes and transfer each labeling solution to the bottom of upper part of filter tubes. Rinse each biotinylation tube with 500µL TBS and pool into filtrate tube. (***Change tip for each antibody to avoid inter-tube contamination***). Balance all tubes for centrifugation at 4,000 RPM at RT for about 5-10 min until the retention volume reach to 100-200 µl. Don't over drain the filter tubes. Collect the filtrate into each microtube and store at 4 C (in case of antibody loss due to membrane leakage).
12. Add 13-15 ml 1 x TBS to each filter tube and centrifuge for 15 min at 4,000 g at 25C (until the final retention volume is about 0.2ml). Discard the filtrate in collection part.
13. Repeat step 12 for two additional times (total three times)
14. Carefully transfer each labeled antibody to a new microtube (Change tip for each antibody). Rinse the filter tube with additional TBS. Keep the total volume smaller than starting volume. Record the volume of antibody.
15. Measure OD_{280} of each biotinylated antibody with NanoDrop spectrophotometer. Record the OD_{280}, dilution factor and concentration to the worksheet (For IgG: $OD_{280}/1.37$ = mg/ml).
16. Add 1/30 of BSA-NaN_3 (30X) or OA-NaN_3 (30 x) for each biotinylated antibody. The final concentration for BSA or OA and NaN_3 is 0.1% and 0.08%, respectively.
17. The stock concentration of biotinylated antibody can be adjusted (500µg/mL for production scale are suggested) with Assay Buffer (AB-A or AB-F)
18. Label each biotinylated antibody with date and antibody name. Store at 4ºC. (e.g. Bt-MPO Mab, Sigma# M3156, clone# 12345, 500µg/mL, 9/23/2008)

Description of the reagent (From product insert)

EZ-Link® Sulfo-NHS-LC-LC-Biotin, 50 mg

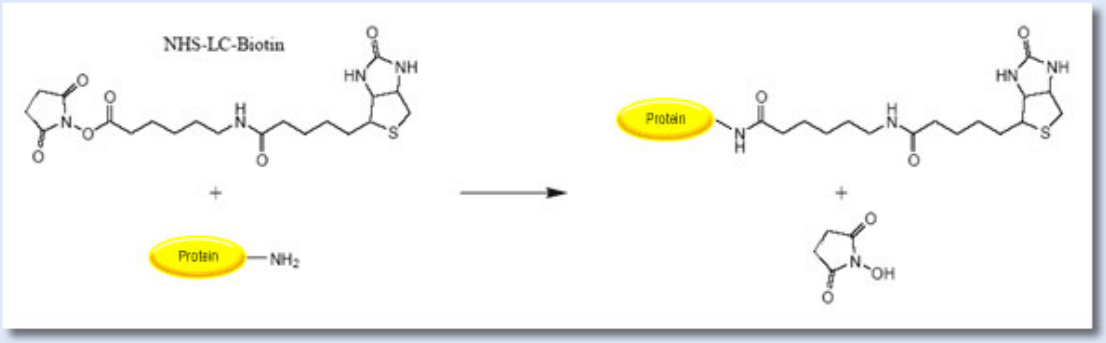
Molecular Weight: 669.75

Spacer Arm: 30.5 Å



Storage: Upon receipt store desiccated at -20°C. Product is shipped at ambient temperature.

N-Hydroxysuccinimide (NHS) esters of biotin are the most popular type of biotinylation reagent. NHS-activated biotins react efficiently with primary amino groups (-NH2) in pH 7-9 buffers to form stable amide bonds. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide that are available as targets for labeling with NHS-activated biotin reagents. Several different NHS esters of biotin are available, with varying properties and spacer arm lengths. Sulfo-NHS ester reagents are water soluble, enabling reactions to be performed in the absence of organic solvents such as DMSO or DMF.



Reaction of NHS-LC-Biotin with a primary amine.

Conjugation of antibodies to Magnetic microsphere (Production scale)

Use

This protocol is to make antibody conjugated beads for Luminex assay in large scale format (1×10^8)

Safety

NaN_3 is used in some solutions for this protocol. *Sulfo-NHS* and *EDC* may cause eye irritation. Please always wear safety goggles, gloves and lab coat during experiment. Follow all necessary safety procedures in the lab.

Materials needed

1 x PBS (diluted from Sigma P7059, 10mM PB, 0.9% NaCl, pH7.4)
Activation buffer (100mM PB, pH 6.0), (see ***Buffer preparation protocol***)
Coupling buffer (50mM MES, pH5.0 or 100mM acetic acid, 300mM NaCl, pH 4.0), (see ***Buffer preparation protocol***)
1 x TBS (Sigma T6664, 50mM Tris pH8.0, 138mm NaCl, 2.7mM KCl)
Blocking buffer-A (BB-A) (see ***Buffer preparation protocol***)
Blocking buffer-C (BB-C) (see ***Buffer preparation protocol***)
Antibody to couple
Luminex microspheres
Sulfo-NHS (N-hydroxysulfosuccinimide), Sigma #56485 or PIERCE: #24510, stored at 4ºC in desiccator.
EDC (N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride) Sigma E7750, stored at –20ºC in desiccator
15mL amber bottle (for conjugation)
50mL amber bottles (for storage)
Aluminum foil
Ultrasonic cleaner
Plate shaker
Magnetic separator or centrifuge (swing rotor with 15mL tube adaptor)
Microscope and hemacytometer

Protocol

D. Preparation

19. Bring buffers, *Sulfo-NHS, EDC* and beads and antibodies to RT for at least 30 min.
20. Double check data sheet and vial label to make sure the antibody contains no BSA glycine, Tris or NaN3. Glycine, Tris and azide can be removed by filtration through *Amicon Ultra-15 tube (30 kD)* for three times with three buffer exchanges (3 x 15 ml PBS at 4,000 RPM centrifugation for 15-20 min at 25C). BSA can be removed by *NAb Protein A/G Spin Kit* (Pierce # 89950). After buffer exchange or purification, the final concentration of antibody must be measured again.
21. Calculate total amount of antibody and beads needed: Without indication, use 200μg antibody for$1x10^8$ beads
22. Calculate volume of Sulfo-NHS (50mg/ml) and EDC (10mg/ml) needed for all coupling work at this time.
23. Record details of antibodies and beads to be used in production document

E. Beads activation

24. Sonicate beads for 20 seconds and then vortex for 15 seconds. Beads are light sensitive and should not be exposed to strong light. Use aluminum foil for light protection.

25. Mark 15mL amber bottle with item # and beads # on cap. Pipette appropriate amount of each bead (1 x 10^8/8mL) into the bottle. Collect beads by magnetic separator or centrifugation
26. Discard supernatant carefully by pipette. Don't touch bead pellet (Change tips for different beads #). Add 5 ml PBS (pH7.4), vortex for 10 seconds and then separate beads as in step 7.
27. Repeat step 8 for two more times (total three times).
28. Resuspend beads (1 x 10^8) in 3 mL activation buffer (pH6.0), vortex for 20 seconds
29. Freshly prepare enough volume of Sulfo-NHS (50mg/ml in activation buffer) and EDC (10mg/ml in activation buffer) in aluminum foil wrapped tubes. Vortex for 20 sec to dissolve all powder.
    Note: Sulfo-NHS and EDC have a short active life. Freshly prepare these stocks before use. If there are more than 5 antibodies for coupling, reduce time during transfer.
30. Add 1mL Sulfo-NHS into each tube, vortex for 10 seconds. Then add 1mL EDC to each tube. Vortex for 30sec.Wrap each tube first with aluminum foil before wrapping together. Shake for 20 min at RT.
31. Separate beads and discard supernatant
32. Wash beads twice with 5 ml coupling buffer as above

C. Conjugation

33. Resuspend the beads in 4mL coupling buffer, sonicate for 30 seconds, then vortex for 30 seconds.
34. Add 200µg antibody for 1 x 10^8 beads. Bring the final volume to 5mL with coupling buffer.
35. Wrap and shake tubes in dark for 2 hr at RT at maximum intensity.

a. <u>Blocking</u>

36. Separate beads and discard supernatant
37. Wash beads once with 5 mL 1 x TBS
38. Resuspend beads in each amber bottle in 5 mL **Blocking buffer (BB-A or BB-C, same for next indications)**. Shake bottles in dark for overnight at 4ºC with moderate intensity on plate shaker (scale 6).
39. Wash beads twice with 5 mL **Blocking Buffer** as above
40. Resuspend beads in 10 mL **Blocking Buffer**. Vortex for 10 seconds and move to 50 mL **amber bottles**. Rinse the original bottle with 3 x 5 mL **Blocking Buffer** and then pool into the same amber bottle. Sonicate for 10 seconds and vortex for 10 seconds.

E. Beads counts

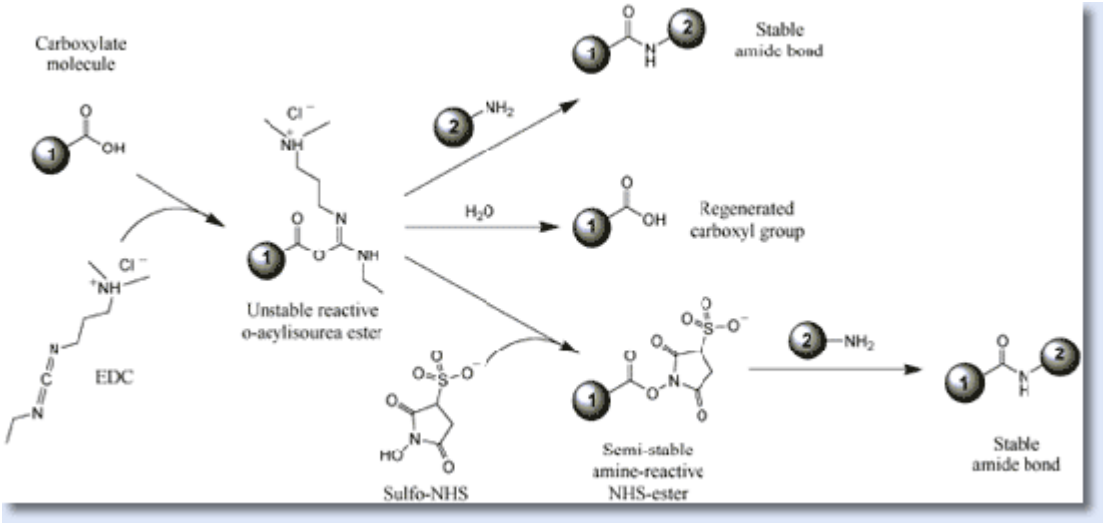
41. In a 0.5 ml microtube, make dilution of each conjugated bead at 1:5 in PBS pH7.4 (20 µl beads + 80µl PBS). Vortex briefly and Charge both sides of a

hemacytometer with 10 µl of the sample. Count the number of beads in the center square on both sides of the hemacytometer.

# of beads/ml = $\frac{\text{(total \# of beads counted)}}{2} \times 10^4 \times 5\text{(dilution)}$

42. Adjust the beads density with **Blocking Buffer** to 2.4 x 10^6/ml (30 X Beads stock). Label vial for each conjugated beads and store at 4ºC. **Don't freeze beads**.
43. Record all information into production document.

Principle of Crosslinking mediated by EDC



EDC reacts with a carboxyl group on molecule #1, forming an amine-reactive *O*-acylisourea intermediate. This intermediate may react with an amine on molecule #2, yielding a conjugate of the two molecules joined by a stable amide bond. However, the intermediate is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution. The addition of Sulfo-NHS (5 mM) stabilizes the amine-reactive intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions[1,2] . The amine-reactive Sulfo-NHS ester intermediate has sufficient stability to permit two-step crosslinking procedures, which allows the carboxyl groups on protein #2 to remain unaltered.

REAGENTS

Product	Source	Catalog Number
EDC (1-ethyl-3-[3dimethylaminopropyl] carbodiimide hydrochloride)	Pierce (Thermo Scientific)	77149
Sulfo-NHS (N-hydroxysulfosuccinimide)	Pierce (Thermo Scientific)	24510 24520
Streptavidin-Alexa Fluor® 532	Molecular Probes (Invitrogen)	S-11224
Streptavidin-R-phycoerythrin (1 mg/ml)	Molecular Probes (Invitrogen)	S-866
PhycoLink® Streptavidin-R-phycoerythrin	Prozyme	PJ31S
PhycoLink® Streptavidin-R-phycoerythrin	Prozyme	PJRS34 (ROU)
Streptavidin-PE conjugate with BSA	MOSS Inc.	SAPE-001
Streptavidin-PE conjugate without BSA	MOSS Inc.	SAPE001NB
Flogen® Streptavidin-phycoerythrin	Far East Bio-Tec	1SA
Streptavidin R-Phycoerythrin LumiGrade	Roche Applied Science	05065925103
Streptavidin R-Phycoerythrin LumiGrade Ultrasensitive	Roche Applied Science	05351693103
2-Propanol	VWR	MK304306
MES (2-[N-Morpholino]ethanesulfonic acid hydrate)	Sigma	M2933
TWEEN® 20 (Polyoxyethylenesorbitan monolaurate)	Sigma	P9416
SDS (Sodium lauryl sulfate), 10% solution	Sigma	L4522
Sarkosyl (N-Lauroylsarcosine sodium salt), 20% solution	Sigma	L7414
TE (Tris-EDTA) Buffer, pH 8.0, 100X	Sigma	T9285
5M TMAC (Tetramethylammonium chloride solution)	Sigma	T3411
1M Tris-HCL, pH 8.0	Sigma	T3038
0.5 M EDTA, pH 8.0	Invitrogen	15575-020
Sodium Chloride	Sigma	S6191
Triton® X-100	Sigma	T8787

Quick Protocol for P3-CVD Multiplex Assay

P3-CVD is a multiplex panel of Luminex assay to quantify the concentration of five high abundant protein targets (Clusterin, Apo-A1, Apo-B100, LP(a) and Hs-RP) from human plasma in a 96-well microplate format. Samples are diluted at 1/10,000 and incubated with capture antibody coated beads for 18-20 hours at 4℃. After washing to remove unbound proteins, beads are mixed with a cocktail of five Biotin-labeled detection antibodies. The bead mounted antibody-protein complex is then detected with Streptavidin-Phycoerythrin by BioPlex-200 reader. Bio-Plex Manager V 6.0 software is applied to analyze raw data with standard curves and calculate the concentration of five proteins from unknown samples.

Special notice

1. All FHS samples are classified to be biohazard level 2 materials. Please follow all BSL-2 lab guidelines and wear proper Personal Protection Equipments (PPE) during work.
2. Always keep tools and hands free of possible contaminates from samples or waste during assay. Change new tips and gloves whenever is suspicious.

Kit Components and Storage

Cat# P3-CVD **Lot# P3-0611**

P3-CVD Reagent Bag	**Cat#**	**Lot#**	**Quantity**	**Storage**
Assay Buffer-A	AB-A	AB-A-0311	150mL	2nd FL cold room (#2321 in Lab2317)
Bead Mix	P3-CAP	P3-0511	9mL	
Detection Mix	P3-DET	P3-0511	9mL	
SAPE	SAPE-15	P3-0511	10mL	
P3-CVD Accessories Bag				
Assay plate (Bio-Rad)	171025001	N/A	3	Lab2305 (RT, storage box for P3-CVD)
Deep well block (0.5mL)	Costar 3957	N/A	1	
Dilution plate (360µL)	Costar 3363	N/A	1	
Sample Diluent Tube (15mL)	Corning 430052	N/A	1	
Strip tubes	N/A	8/strip	2 strips	
Plate sealer (Sealplate®)	100SEALPLT		6 sheets	
Premade labels	N/A	N/A	6	
Frozen reagents				
Standard Mix	P3-STD	P3-0511	0.8mL	Lab2305 (-80℃ freezer, 13FR-0166, shelf 2, rack A)
Quality Control 1	P3-QC1	P3-0511	0.8mL	
Quality Control 2	P3-QC2	P3-0511	0.8mL	
Sample Diluent (10X)	P3-SMD	P3-0511	1mL	

Daily pre-assay check list:

1. Pipettes and tips:
Adjustable multichannel pipettes:10µL, 20µL, 50µL, 300µL and 1mL
Adjustable single channel pipettes: 100µL, 200µL, 1mL, 5mL
2. 50mL Reagent Reservoir (Cornings, Can be get from basement supply room)
3. Aluminum foil
4. Ice tray
5. Rack for 1mL strip tubes (for preparation of standard and QCs)
6. Plate centrifuge. Rotina 38R, change rotor for plate (#1760)
7. Sonicator (Brookstone)

Day 1

1. Preparations

1). Take one **P3-CVD Reagent Bag** from cold room #2321 to Lab2305 fridge and one **P3-CVD Accessories Bag** and 3 **Assay Plates (Bio-Rad Cat#171025001**) from stock box onto bench**.** Take Assay Buffer bottle out onto the bench only. Mark the initial, date and mother plate ID on reagent bottles and bags**.**

2). Label 3 assay plates with premade labels (Plate # A, B and C) on the front side of plate. Mark A, B or C to 3 circular labels for labeling wraps later on. Label the 15mL Corning tube with "Sample Diluent Tube". Put the STD and QCs labels to the front side of each strip tubes.

3). Dispense 200µL 1 x Wash Buffer to each well of assay plate by Microplate dispenser (Statmatic I) or Bio-Rad wash station (program "DISP"). **Put plates on a safe place to avoid contamination during sample dilution**.

4). Transfer 9mL AB-A into the Sample Diluent Tube. Then transfer 1mL (10X) P3-SMD into the tube. Rinse the stock tube once. Vortex the 1X sample diluent tube briefly

5). Take one vial of each: P3-STD (0.8mL), P3-QC(0.8mL), P3-QC2(0.8mL) and P3-SMD (10X) from Lab2305 -80C freezer (13FR-0166) to bench. **Once standard and QCs are completely thawed up, move them into ice tray or 4℃ until use**.

2. Prepare samples

Please wear gloves and use BSL-2 hood for dilution of FHS samples. Use ice tray during sample dilution.

For 10µL aliquot samples:

Tools	µL of STD	µL of AB-A	Dilution fold
Source plate	10	190	1/20
deep well plate	10	490	1/50
360µL plate	20	180	1/10
Final dilution			1/10,000

For 20µL aliquot samples:

Tools	µL of STD	µL of AB-A	Dilution fold
Source plate	20	180	1/10
deep well plate	10	490	1/50
360µL plate	10	190	1/20
Final dilution			1/10,000

1). Take one daughter plate of FHS samples (10 or 20µL aliquot) to the hood. Use the computer for Luminex#04 to open the file "**A copy to FHS ID for production.xls** "containing FHS sample ID information from **C:\Mysessions\P03-CVD-prod.** Scan plate ID into the Plate ID sheet and input user information.

2). When samples are completely thawed up, pellet samples by centrifugation with plate rotor (Rotina 38R) at 1,000 RPM for 30 sec. Move the plate into ice tray or 4C until use.

3). Carefully remove the plate sealer to avoid sample spillover cross wells. Pipette indicated volume of diluent (AB-A) to the 1st row of wells and then mix them by pipetting up and down for 5 times. Change tips for next row of samples and finish the 1st dilution.

4). Transfer 10µL 1st dilutions to the deep well plate with 490µL AB-A. Mix them row by row with multichannel pipette (200µL volume) for 10 times. This is the 2nd dilution

5). Dispense indicated volume of AB-A to each well of the 96-well dilution plate and make the final dilution **(1/10,000)** with indicated volume of 2nd dilutions. Move the plate to ice tray until use. The final diluted samples can be mixed now with plate shaker (covered with a new plate sealer) for 1-3 min or mixed by pipette before loading to assay plates (see step 4.3). **Store the daughter plate to -80℃ freezer(13FR-0166) with initial, date and dilution information on the aluminum plate sealer**. Store all other diluted sample plates at 4℃ until all three plates are set up at step 4.5.

3. Prepare Standards and QCs

Change a new pair of gloves before making standard dilutions.

Dilution table (5 fold serial dilution)

Standard	µL of STD	µL of AB-A	Dilution fold
STD1	800	0	Starting tube
STD2	100	400	5
STD3	100	400	5
STD4	100	400	5
STD5	100	400	5
STD6	100	400	5
STD7	100	400	5
Background	0	400	Background

1). Make sure frozen standard and QCs are completely thawed up at RT (about 15-30min). Mix each vial by brief vortex and move to ice tray until use. Put the STD and QC strip tubes onto an empty rack with label side towards front. **For the convenience of using multiple plates and reducing variations, an 8-channel pipette will be used to transfer standard and QCs from strip tubes to 3 assay plates**.

2). Pipette 400µL of AB-A to the bottom 7 wells of Standard strip. Transfer all standard solutions from the original tube into the 1st well of Standard strip. Transfer 100µL standard from well 1 to well 2, mix by pipetting up and down for 15 times. Change tips and make remaining serial dilutions until well 7. The well 8 has buffer only.

3). QC1 and QC2 are ready to use. No further dilution is required. Directly dispense 200µL of QC1 to each top 4 wells of QC strip and 200µL QC2 to each bottom 4 wells.

4. Set up assay plate

Dump and blot plates that were prewet with Wash Buffer. **The following dispense steps should go column by column.**

Platemap

1). Use multichannel pipette (with 8 channels) to dispense 25µL AB-A to standard wells and 25µL

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	STD1	QC1	S1	S1	S9	S9	S17	S17	S25	S25
B	STD2	STD2	STD2	QC1	S2	S2	S10	S10	S18	S18	S26	S26
C	STD3	STD3	STD3	QC1	S3	S3	S11	S11	S19	S19	S27	S27
D	STD4	STD4	STD4	QC1	S4	S4	S12	S12	S20	S20	S28	S28
E	STD5	STD5	STD5	QC2	S5	S5	S13	S13	S21	S21	S29	S29
F	STD6	STD6	STD6	QC2	S6	S6	S14	S14	S22	S22	S30	S30
G	STD7	STD7	STD7	QC2	S7	S7	S15	S15	S23	S23	S31	S31
H	BKG	BKG	BKG	QC2	S8	S8	S16	S16	S24	S24	S32	S32

1 X sample diluent (P3-SMD) to all other wells of each plate (see platemap above).

2). Pipette 25µL of standard dilutions, QCs into each plate.

3). Before pipetting samples, **mix them by pipetting up and down for 10 times** and then transfer 25µL to sample wells of each plate. Change tips for each column of samples

4). Sonicate Bead Mix for 30 sec and vortex for 15 sec. Pipette 25µL of Beads Mix into each well.

5). Seal the plate with plate sealer and wrap the plate with aluminum foil. **Mark the plate# and starting time of shaking and projected end time (18-20hr)**. Move plates to shaker at 4C and set shaking speed at 6. Shake plate for 18-20 hours. Put remaining reagents back to the bag.

Day 2

5. Preparation

1). Take P3-CVD reagents (Detection Mix, SAPE) and instrument calibration kits to bench

2). Turn on the plate shaker on bench, set temperature to 25C, speed to 600 RPM

3). Turn on 3 Bio-Plex 200 readers, check sheath fluid and waste bottles. Refill as necessary. Warm up instrument (require 30min). Calibrate each instrument at the beginning of each day (1st user only). If validation recommendation is prompt (every month), run validation.

4). Turn on Bio-Rad wash station. Prime washers (channel=1, volume=30mL) with wash buffer. Check fluid bottles and refill buffer if needed.

6. Run assay

1). Take assay plates out from shaker and wash each plate twice with Bio-Rad washer (Program "MAG 2X").

2). Pipette 25µL Detection Mix to each well of plates. **Use a new plate sealer for each plate**. Wrap and shake plates on 25℃ shaker for 1 hour at 600 RPM.

3). During this period, run start up, calibration or validation for three Bioplex-200 instruments (1st user only) after warm-up is finished (30min). Then:

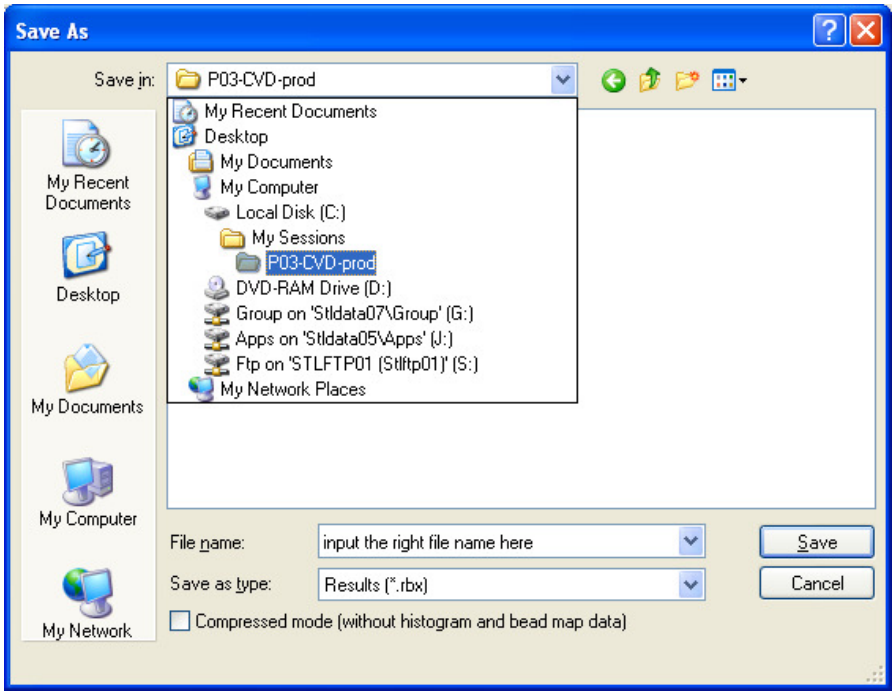
a. From the file menu, open the assay protocol "**P03-CVD prod**" from **C:\Mysessions\P03-CVD-prod** folder. ***Do not change any default settings***.

b. Identify and double check the sample ID related to the mother plate from the "**FHS ID for production.xls**" which is located in **G:\R&D\PEP\Luminex**. Copy the 1st 32 sample IDs (yellow highlight) to plate# A profile, 2nd 32 sample IDs(orange) to plate# B and the 3rd 32 sample IDs(green) to plate# C.

c. Click "Run protocol" and Set file destination to **C:\Mysessions\P03-CVD-prod** folder as below

d. Name the raw data file as **"P03-plate ID-A, B or C-YYYYMMDD"**).

e). For wells with no samples: Copy the well positions and sample IDs from sample ID sheet to the description page to notify data analysis



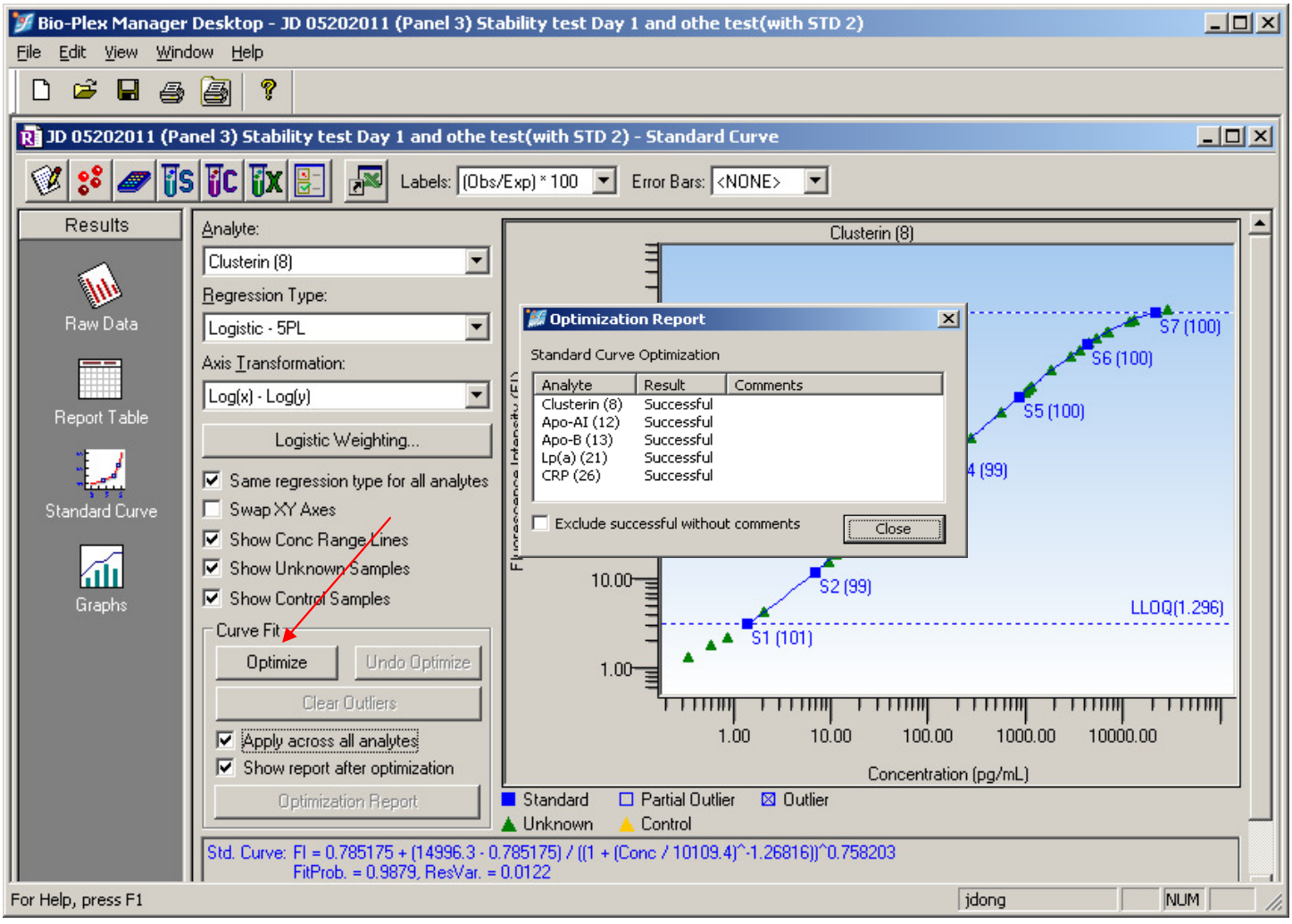
4). Wash plates twice. Pipette 25µL SAPE-15 into each well of plates. Seal, wrap and shake plates or 30min at 25℃. Put all remaining reagents back to the bags.

5). Wash plates twice. Pipette 100µL sheath fluid into each well of plates. Seal, wrap and shake plate for 5min at 25℃.

6). Carefully remove plate sealer to avoid spillover cross wells. Move the plate to BioPlex-200 reader. **Input user name and plate IDs plus A, B or C for corresponding instrument.**

7). Monitor the first 2-5 well readings to make sure beads regions are correct. After reading plate. Bioplex reader will automatically save the raw data (.rbx) to previously indicated folder at **C:\Mysessions\P03-CVD-prod.** Then:

a). Within the pop-up window of “Report table”, click the icon “Standard Curve” on left side of window, check the options as show below. Click “Optimize” then close the pop-up window “Optimization Report”.



b).Switch to “Report Table” on the left side of window. Select the “Export Table” from “File” menu and choose “Multiple Analyte Layout” (see figure below). Click “OK” to generate a report file (Excel format).

For wells with no samples:

Overwirte the correspondent wells in the spreadsheet "Concentrataion in range" of report table with letter "E" before.

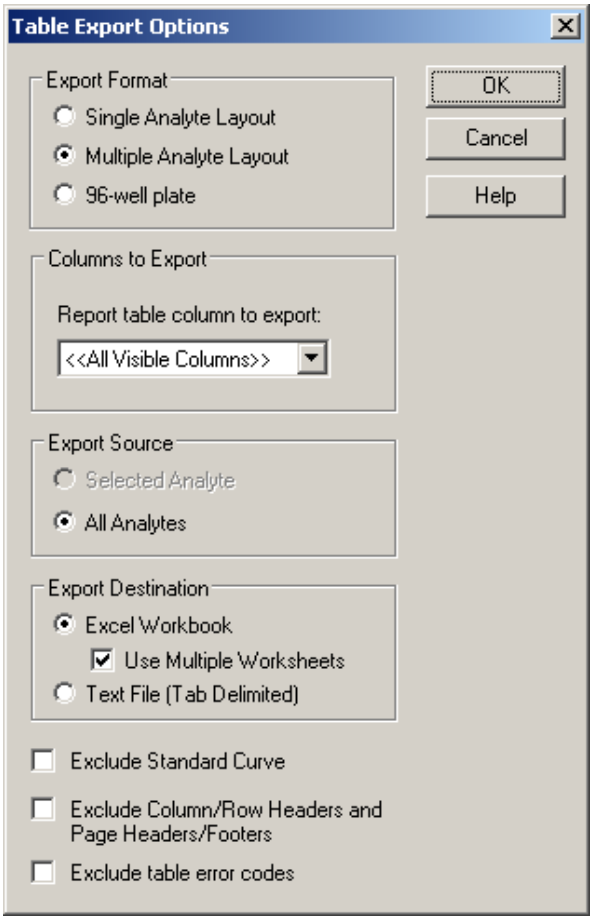
Save the report file into **C:\Mysessions\P03-CVD-prod** folder.

c). Click “Save” from File menu to save the updated raw data.

d). Copy both the raw data file (.rbx) and report file (.xls) from local PC to G: drive at **G:\R&D\PEP\Luminex\CVD Biomarkers. NIH projects\Screening Data for Framingham Sample\P03-CVD**

8). Remove plates from readers and save them at 4℃ until verification of the raw data is done.

9). Clean the instrument as instructions. Turn off the instruments at the end of day (last user only)



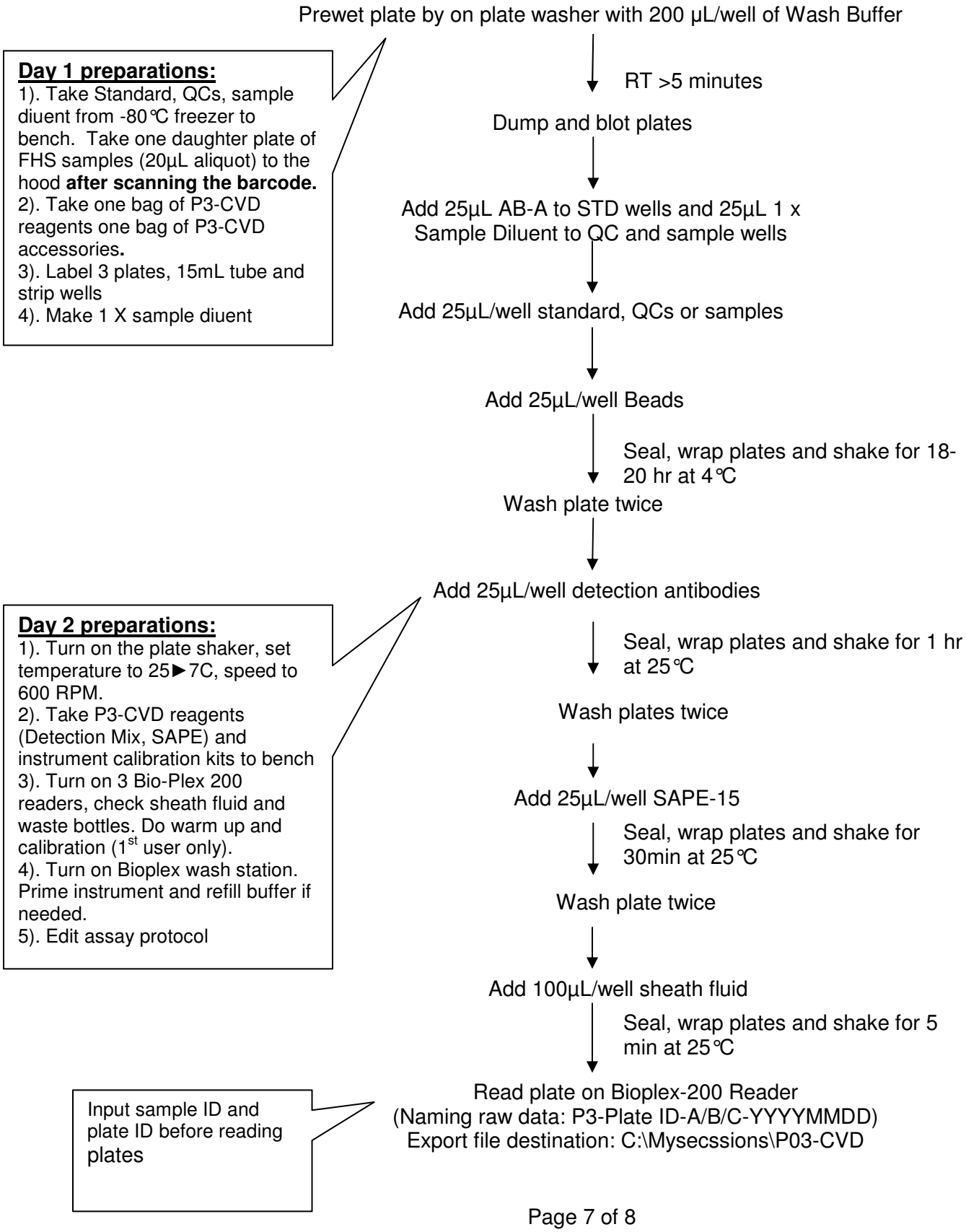
7. Data verification (for reviewer use only)

1). Verify the agreement of plate ID appeared in raw data file name(.rbx) and report file (Excel format) with “**FHS ID for production.xls**.

2).Input the QC concentrations (mean) from Report file of each assay plate to the file “**P03-CVD QC track.xls**” which is located in **G:\R&D\PEP\Luminex\CVD Biomarkers. NIH projects\Screening Data for Framingham Sample\P03-CVD**. Sign the sheet.

3). Check the mark of wells with no sample

Attachment 1: Luminex Assay Flow Chart



Attachment 2: Settings for Standard and QCs

(Assay Protocol: P03-CVD-prod)

Cat# P3-STD Lot# P3-0511 Exp. 06/01/2012

	Clusterin(008)	Apo-AI(012)	Apo-B100(013)	Lp(a)(021)	Hs-CRP(026)
Standard	**pg/mL**	**pg/mL**	**pg/mL**	**pg/mL**	**pg/mL**
STD1	26693.00	328084.00	3889609.00	5414013.00	3476.00
STD2	5338.60	65616.80	777921.80	1082802.60	695.20
STD3	1067.72	13123.36	155584.36	216560.52	139.04
STD4	213.54	2624.67	31116.87	43312.10	27.81
STD5	42.71	524.93	6223.37	8662.42	5.56
STD6	8.54	104.99	1244.67	1732.48	1.11
STD7	1.71	21.00	248.93	346.50	0.22

Panel: P03-CVD, Lot P3-0611

		Clusterin (8)	Apo-AI (12)	Apo-B (13)	Lp(a) (21)	CRP (26)
QC1	**Mean**	86.41	9550.34	9736.44	18339.01	46.34
	SD	14.25	2133.32	1489.95	2837.23	6.77
QC2	**Mean**	1038.38	72690.23	114961.15	226307.82	542.01
	SD	131.37	15616.83	13297.23	31719.07	53.65