SUPPLEMENTAL DATA FILE

Development and Validation of Apolipoprotein A-I Associated Lipoprotein Proteome Panel for the Prediction of Cholesterol Efflux Capacity and Coronary Artery Disease

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PART I. DETAILED EXPERIMENTAL METHODS

Affinity Enrichment of ApoA-I Associated Lipoproteins

Using the method described previously, 12 µL of serum was incubated with 24 µL of 0.5 mg/mL ^{15}N-labeled His_6-tagged Apo A-I at 37 ˚C for 20 minutes on a thermocycler (Eppendorf, Hamburg, Germany) before holding at 4˚C. ApoA-I associated lipoprotein particles (AALPs) were then enriched on a Tecan (Männedorf, Switzerland) FreedomEVO automated liquid handler using Phynexus (San Jose, CA) Phytip micro-column tips packed with 5 µL of Ni-NTA HisBind Superflow resin. Samples were diluted to 200 µL with 20 mM sodium phosphate, 150 mM sodium chloride, pH 8.0 and bound to the column using six pipetting cycles at 200 µL/min. Column were subsequently washed with 200 µL of 5 mM imidazole, 20 mM sodium phosphate, 150 mM sodium chloride, pH 8.0, followed by 200 µL of 20 mM imidazole, 20 mM sodium phosphate, 150 mM sodium chloride, pH 8.0. Resin-bound AALPs were then eluted into 120 µL of buffer consisting of 300 mM imidazole, 50 mM Tris-HCl (pH 9.0), and 25% methanol using 6 x 60 µL pipetting cycles. The eluted protein mixtures were thermally denatured at 85 ˚C for 10 minutes. 80 µL of AALP eluent was added to 20 µL of 25 ng/µL of Endoproteinase Lys-C (Waco Chemical, Richmond, VA) and incubated at 37 ˚C for 4 hours. 20 µL of a master mix of $^{13}C_6$, $^{15}N_2$-Lysine-labelled internal standard peptides (New England Peptide, Gardner, MA) were added to 80 µL of peptide digest and served as a single-point calibrator. Peptide mixtures were immediately submitted for LC-MRM analysis or stored at -80^{o}C as required until analysis.

LC-MRM Method for Proteomic Biomarker Discovery

For biomarker discovery experiments, 25 µL of LysC-digested AALP peptides were injected for analysis by liquid chromatography – multiple reaction monitoring mass spectrometry(LC-MRM). The injected sample was loaded and washed on column for 1.25 minutes, and then eluted with a linear

gradient of mobile phase B at 500 µL/min. Peptides were detected using an Agilent 6490 triple quadrupole mass spectrometer operating in dynamic MRM mode, allowing for the targeted detection of peptide targets within a scheduled retention time window. Transitions were selected, optimized and determined to be unique to the peptide targeted within the sample. Two transitions were monitored per peptide, and up to two peptides per protein. A detailed list of peptide targets and their transitions is available in **Supplemental Table 1**. Peptide signal intensities were obtained via integration the chromatographic peak for the quantifier transition using MassHunter Quantitative Analysis software (Agilent). All peaks were manually reviewed using fragment ion ratios and internal standard peaks.

Supplemental Table 1. Peptide Transitions for Biomarker Algorithm Discovery

Protein	Peptide	Internal Standard?	Precursor Ion (m/z)	Product Ion (m/z)	Retention Time (min)	Collision Energy (V)
APOA1	LLDNWDSVTSTFSK.heavy	YES	540.94	577.31	4.8	10
APOA1	LLDNWDSVTSTFSK.15N	NO	543.92	575.28	4.8	10
APOA1	LLDNWDSVTSTFSK.15N	NO	543.92	865.41	4.8	10
APOA1	LLDNWDSVTSTFSK	NO	538.27	569.29	4.8	10
APOA1	LLDNWDSVTSTFSK	NO	538.27	856.44	4.8	10
APOA1	ATEHLSTLSEK.heavy	YES	408.55	576.80	3.4	10
APOA1	ATEHLSTLSEK.15N	NO	410.53	528.25	3.4	10
APOA1	ATEHLSTLSEK.15N	NO	410.53	579.28	3.4	10
APOA1	ATEHLSTLSEK	NO	405.88	522.27	3.4	10
APOA1	ATEHLSTLSEK	NO	405.88	572.80	3.4	10
APOA2	SPELQAEAK.heavy	YES	490.76	667.39	3.3	13
APOA2	SPELQAEAK	NO	486.75	659.37	3.3	13
APOA2	SPELQAEAK	NO	486.75	788.41	3.3	13
APOA2	EQLTPLIK.heavy	YES	475.29	478.35	4.2	8
APOA2	EQLTPLIK	NO	471.29	470.33	4.2	8
APOA2	EQLTPLIK	NO	471.29	571.38	4.2	8
APOA4	VNSFFSTFK.heavy	YES	542.78	784.41	4.7	11
APOA4	VNSFFSTFK	NO	538.77	776.40	4.7	11
APOA4	VNSFFSTFK	NO	538.77	863.43	4.7	11
APOA4	LVPFATELHERLAK.heavy	YES	544.65	710.40	4.4	7
APOA4	LVPFATELHERLAK	NO	541.98	706.39	4.4	7
APOA4	LVPFATELHERLAK	NO	541.98	1096.61	4.4	7
APOC1	QSELSAK.heavy	YES	385.71	313.20	2.87	13
APOC1	QSELSAK	NO	381.70	305.18	2.87	13
APOC1	QSELSAK	NO	381.70	418.27	2.87	13
APOC1	ARELISRIK.heavy	YES	365.24	470.29	3.44	8
APOC1	ARELISRIK	NO	362.56	470.29	3.44	8
APOC1	ARELISRIK	NO	362.56	503.33	3.44	8
APOC2	TYLPAVDEK.heavy	YES	522.28	666.35	3.95	14

APOC2	TYLPAVDEK	NO	518.27	658.34	3.95	14
APOC2	TYLPAVDEK	NO	518.27	771.42	3.95	14
APOC2	ESLSSYWESAK.heavy	YES	647.81	878.41	4.4	20
APOC2	ESLSSYWESAK	NO	643.80	870.40	4.4	20
APOC2	ESLSSYWESAK	NO	643.80	957.43	4.4	20
APOC3	DYWSTVK.heavy	YES	453.73	442.28	4.12	4
APOC3	DYWSTVK	NO	449.72	434.26	4.12	4
APOC3	DYWSTVK	NO	449.72	620.34	4.12	4
APOC4	AWFLESK.heavy	YES	444.74	631.35	4.55	19
APOC4	AWFLESK	NO	440.73	476.27	4.55	19
APOC4	AWFLESK	NO	440.73	623.34	4.55	19
APOD	YLGRWYEIEK.heavy	YES	455.57	544.78	4.35	8
APOD	YLGRWYEIEK	NO	452.90	540.78	4.35	8
APOD	YLGRWYEIEK	NO	452.90	597.32	4.35	8
APOD	NILTSNNIDVK.heavy	YES	619.85	898.47	4.13	20
APOD	NILTSNNIDVK	NO	615.84	890.46	4.13	20
APOD	NILTSNNIDVK	NO	615.84	1003.54	4.13	20
APOE	SELEEQLTPVAEETRARLSK.heavy	YES	765.41	853.99	4.45	23
APOE	SELEEQLTPVAEETRARLSK	NO	762.74	849.98	4.45	23
APOE	SELEEQLTPVAEETRARLSK	NO	762.74	979.02	4.45	23
APOE	LEEQAQQIRLQAEAFQARLK.heavy	YES	793.44	940.04	4.51	34
APOE	LEEQAQQIRLQAEAFQARLK	NO	790.77	900.52	4.51	34
APOE	LEEQAQQIRLQAEAFQARLK	NO	790.77	936.03	4.51	34
APOF	DANISQPETTK.heavy	YES	606.30	798.41	3.35	21
APOF	DANISQPETTK	NO	602.30	575.30	3.35	21
APOF	DANISQPETTK	NO	602.30	790.39	3.35	21
APOL1	WWTQAQAHDLVIK.heavy	NO	535.29	616.35	4.41	20
APOL1	WWTQAQAHDLVIK	NO	532.62	612.34	4.41	20
APOL1	WWTQAQAHDLVIK	NO	532.62	724.44	4.41	20
APOL1	LNILNNNYK.heavy	YES	557.31	773.40	4.15	21
APOL1	LNILNNNYK	NO	553.30	652.30	4.15	18
APOL1	LNILNNNYK	NO	553.30	765.39	4.15	18
APOM	EFPEVHLGQWYFIAGAAPTK.heavy	YES	757.06	552.32	5.15	14
APOM	EFPEVHLGQWYFIAGAAPTK	NO	754.38	544.31	5.15	14
APOM	EFPEVHLGQWYFIAGAAPTK	NO	754.38	615.35	5.15	14
CETP	PALLVLNHETAK.heavy	YES	438.59	820.44	4.06	15
CETP	PALLVLNHETAK	NO	435.92	812.43	4.06	15
CETP	PALLVLNHETAK	NO	435.92	911.49	4.06	15
CETP	LFLSLLDFQITPK.heavy	YES	771.95	856.47	5.52	19
CETP	LFLSLLDFQITPK	NO	767.95	848.45	5.52	19
CETP	LFLSLLDFQITPK	NO	767.95	1161.65	5.52	19
Clusterin	LFDSDPITVTVPVEVSRK.heavy	YES	670.70	822.49	4.65	15
Clusterin	LFDSDPITVTVPVEVSRK	NO	668.03	712.93	4.65	15
Clusterin	LFDSDPITVTVPVEVSRK	NO	668.03	814.48	4.65	15
Clusterin	EIQNAVNGVK.heavy	YES	540.30	709.41	3.55	19
Clusterin	EIQNAVNGVK	NO	536.29	417.25	3.55	19
Clusterin	EIQNAVNGVK	NO	536.29	701.39	3.55	19
Complement C3	TGLQEVEVK.heavy	YES	505.78	739.41	3.85	17
Complement C3	TGLQEVEVK	NO	501.78	731.39	3.85	17
Complement C3	TGLQEVEVK	NO	501.78	844.48	3.85	17
Complement C3	AFSDRNTLIIYLDK.heavy	YES	559.64	729.91	4.75	13
Complement C3	AFSDRNTLIIYLDK	NO	556.97	725.90	4.75	13
Complement C3	AFSDRNTLIIYLDK	NO	556.97	764.46	4.75	13
Haptoglobin	VTSIQDWVQK.heavy	YES	606.33	1011.53	4.32	24
Haptoglobin	VTSIQDWVQK	NO	602.32	916.49	4.32	24
Haptoglobin	VTSIQDWVQK	NO	602.32	1003.52	4.32	24

Haptoglobin	DIAPTLTLYVGK.heavy	YES	649.88	500.30	4.72	11
Haptoglobin	DIAPTLTLYVGK	NO	645.87	496.29	4.72	11
Haptoglobin	DIAPTLTLYVGK	NO	645.87	991.58	4.72	11
LCAT	TYSVEYLDSSK.heavy	YES	650.31	849.41	4.07	23
LCAT	TYSVEYLDSSK	NO	646.31	841.39	4.07	23
LCAT	TYSVEYLDSSK	NO	646.31	1027.49	4.07	23
LCAT	DRFIDGFISLGAPQGGSIK.heavy	YES	682.03	880.48	4.86	16
LCAT	DRFIDGFISLGAPQGGSIK	NO	679.36	744.40	4.86	16
LCAT	DRFIDGFISLGAPQGGSIK	NO	679.36	872.46	4.86	16
PLTP	QEGLRFLEQELETITIPDLRGK.heavy	YES	865.14	693.41	5.36	28
PLTP	QEGLRFLEQELETITIPDLRGK	NO	862.47	473.32	5.36	28
PLTP	QEGLRFLEQELETITIPDLRGK	NO	862.47	685.40	5.36	28
PLTP	GLREVIEK.heavy	YES	476.29	555.32	3.62	22
PLTP	GLREVIEK	NO	472.28	555.32	3.62	22
PLTP	GLREVIEK	NO	472.28	668.41	3.62	18
PON1	YVYIAELLAHK.heavy	YES	443.25	533.31	4.82	11
PON1	YVYIAELLAHK	NO	440.58	529.31	4.82	11
PON1	YVYIAELLAHK	NO	440.58	781.46	4.82	11
PON1	SFNPNSPGK.heavy	YES	478.24	607.33	3.3	10
PON1	SFNPNSPGK	NO	474.23	599.31	3.3	10
PON1	SFNPNSPGK	NO	474.23	713.36	3.3	10
SAA1/2	YFHARGNYDAAK.heavy	YES	474.23	555.78	3.25	8
SAA1/2	YFHARGNYDAAK	NO	471.56	483.24	3.25	8
SAA1/2	YFHARGNYDAAK	NO	471.56	551.77	3.25	8
SAA4	DPDRFRPDGLPK	NO	471.58	543.31	3.77	12
SAA4	DPDRFRPDGLPK	NO	471.58	585.29	3.77	12
SAA4	AEEWGRSGK.heavy	YES	343.17	512.30	3.15	8
SAA4	AEEWGRSGK	NO	340.50	474.73	3.15	8
SAA4	AEEWGRSGK	NO	340.50	504.29	3.15	8
SAA4	DPDRFRPDGLPK.heavy	YES	474.25	585.29	3.77	12

Optimized LC-MRM Method for pCE and pCAD Models

Five proteins (ApoA-I, ApoC-I, ApoC-II, ApoC-III, and ApoC-IV) were quantified using an Agilent 6495 triple quadrupole multiplexed with three Agilent 1260 HPLC systems allowing for sequential collection of data within a selected retention time window. Twenty microliters of a master mix of $^{13}C_6$, $^{15}N_2$-Lysine-labelled internal standard peptides (New England Peptide, Gardner, MA) were added to 80 µL of peptide digest and served as a single-point calibrator. Calibrator levels were assigned based on assigned stock values from triplicate amino acid analysis and mixture analysis by LC-UV-Vis spectroscopy. Final calibrator levels in a given sample consisted nominally of 90.91 nM ApoA-I peptides, 36.36 nM ApoC-I peptides, and 18.18 nM ApoC-II, C-III, and C-IV peptides.

A 5 µL injection of peptide sample was separated on a Kinetex 2.6 µm C18 100Å 50 x 3 mm HPLC column (Phenomenex). The analytical column was equilibrated in 98% mobile phase A (0.1% formic acid in water) at the flow rate of 0.5 mL/min followed by a 3.5 min gradient from 2 - 36% mobile phase B (0.1% formic acid in acetonitrile). After a brief wash at 95% mobile phase B the column was re-equilibrated. Chromatography conditions were optimized so that all proteotypic peptides eluted in a 3.3-minute acquisition window. The mass spectrometer was operated in positive mode using dynamic MRM mode. Two unique peptides (when possible) were measured for each protein. Two transitions for each peptide was monitored as fragment ion pairs. Details for transitions and representative chromatograms are provided (**Supplemental Table 2, Figure 2A**). Data inspection and quantitative analysis was performed in MassHunter Quantitative Analysis Software (Agilent).

Supplement Table 2. Peptide Transitions for Validated pCE/pCAD Assay

Protein	Peptide	Used for Algorithm?	Quantifier Transition (m/z)	Qualifier Transition (m/z)	Collision Energy (V)	Retention Time (min)
ApoA-I	ATEHLSTLSEK	Yes	405.9 → 572.8	405.9 → 522.3	10	3.3
	ATEHLSTLSEK.15N	Yes	410.5 → 579.3	410.5 → 528.3	10	3.3
	ATEHLSTLSEK.heavy	Yes	408.6 → 576.8	408.6 → 526.3	10	3.3
	LLDNWDSVTSTFSK	No	538.3 → 670.3	538.3 →569.3	10	4.6
	LLDNWDSVTSTFSK.15N	No	543.9 → 677.3	543.9 → 575.3	10	4.6
	LLDNWDSVTSTFSK.heavy	No	540.9 → 678.3	540.9 → 577.3	10	4.6
ApoC-I	ARELISRIK	Yes	362.6 → 470.3	362.6 → 503.3	8	3.4
	ARELISRIK.heavy	Yes	365.2 → 470.3	365.2 → 511.3	8	3.4
	QSELSAK	No	381.7 → 305.2	381.7 → 418.3	13	2.3
	QSELSAK.heavy	No	385.7 → 313.2	385.7 → 426.3	13	2.3
ApoC-II	TYLPAVDEK	Yes	518.3 → 658.3	518.3 → 771.4	14	3.8
	TYLPAVDEK.heavy	Yes	522.3 → 666.4	522.3 → 779.4	14	3.8
	ESLSSYWESAK	No	643.8 → 870.4	643.8 → 957.4	20	4.3
	ESLSSYWESAK.heavy	No	647.8 → 878.4	647.8 → 965.5	20	4.3
ApoC-III	DYWSTVK	Yes	449.7 → 620.3	449.7 → 434.3	12	4.0
	DYWSTVK.heavy	Yes	453.7→628.4	453.7 → 442.3	12	4.0
ApoC-IV	AWFLESK	Yes	440.7 → 623.3	440.7 → 476.3	11	4.4
	AWFLESK.heavy	Yes	444.7 → 631.4	444.7 → 484.3	11	4.4

Method Validation

At completion of data collection, figures-of-merit represent total imprecision, bias, limit of analytical sensitivity, linearity, matrix suppression effect, inferences, pre-analytical stability, and in-process

stability. Testing materials included three QC pools with high, medium, low pCAD score, and twenty-five individual patient specimens. Assay precision and accuracy were evaluated by running three QC pools, and two individual specimens with four replicate preparations of each sample on 15 different days on each of three individual HPLC streams. Sample stability at -80 ˚C, -20 ˚C, 4 ˚C, and ambient temperature were assessed using nine individual serum specimens and three pooled sera from 1 to 21 days of storage. These serum samples were subjected to six freeze/thaw cycles each to determine sample stability. Three pairs of serum specimens with low- and high-estimated pCAD score were mixed in ratios of 1:0, 1:3, 1:1, 3:1, 0:1 to determine linearity of the response with respect to changing composition. Three serum specimens were spiked at varying levels with unconjugated bilirubin, hemoglobin, and intralipid to evaluate the impact of common endogenous interferents on the assay result. Details are found in the supplemental results section.

Twenty-nine apparently healthy, non-smoking, and ethnically diverse volunteers (fourteen men and fifteen women; age range of 30 – 60 years) were recruited for a prospective short-term biological variation study. Subjects using any lipid modifying drugs or those with diagnosed diabetes were excluded. Venous blood was collected from each subject once a week for eight weeks using a serum separator tube. After centrifugation, serum was stored at -70 °C until analysis.

Specimens for CEC Model Development

Serum samples for efflux correlation model development were taken from de-identified remnant specimens at CHL collected in two batches, 6 weeks apart (to minimize bias from selecting samples at a single time point), for provision of training and tests sets respectively. Quantitative analyses of LDL-c, HDL-c, ApoA, ApoB, Triglycerides, and high sensitivity C-reactive protein (hsCRP) were used to guide selection of candidate samples for each set, ultimately yielding a well-matched set of

specimens (**Supplemental Table 3**). Quantitative ApoA-I associated proteome analyses and CEC measurements were collected for 105 specimens along with highly-characterized serum pools as quality control material.

Supplemental Table 3. Description of CEC Model Development Specimen Set.

	Training Set	Validation Set
Number of Samples	70	35
hsCRP (mg/L)	2.9 (3.2)	3.4 (3.1)
ApoA1 (mg/dL)	159 (36)	159 (39)
ApoB (mg/dL)	91 (22)	107 (31)
ApoB/ApoA1 ratio	0.61 (0.23)	0.72 (0.29)
Total Cholesterol (mg/dL)	192 (33)	200 (48)
LDL-C (mg/dL)	109 (31)	115 (41)
HDL-C (mg/dL)	59 (21)	55 (22)
non-HDL-C (mg/dL)	133 (35)	150 (56)
Triglycerides (mg/dL)	123 (70)	148 (60)

Note: Values represent mean (standard deviation)

Specimens for Algorithm Validation in Fairbanks Institute CAD Cohort

Specimens were selected from the Fairbanks Institute for Healthy Communities biobank which consists of serum samples from 1500 men and women aged 22 to 87; 750 with documented diagnosis of CAD via coronary angiography (≥50% occlusion) and 750 control subjects with no positive findings for CAD, positive stress test, diabetes, hypertension, or abnormal lipids (LDL-C ≥130 mg/dL, HDL-C≤40 mg/dL, total cholesterol ≥240 mg/dL or triglycerides ≥200 mg/dL). Fasting blood samples were collected according to the study SOP and subsequently stored at -80 ˚C. Subjects with diagnosed CAD were evaluated to establish two groups, cases, and cases with events. All CAD patients were filtered for ICD-9 code codes for major adverse cardiovascular events (MACE);

myocardial infarction (410), coronary bypass graft or angioplasty (36.1, 45.82), or stroke (434.91). For confirmation of myocardial infarct from ICD9 screening, records were reviewed to select patients with two of three of the following - history of ischemic pain, abnormal ECG, abnormal troponin. In total, 74 CAD subjects without events and 83 CAD subjects with MACE events were selected (**Supplemental Table 4**). A set of 74 matched controls were also selected.

Supplemental Table 4. Description of Fairbanks Specimen Set

	Control		CAD		CAD w/ Event	
Gender	Male	Female	Male	Female	Male	Female
	n= 35 (47%)	n= 39 (53%)	n= 47 (64%)	n= 27 (36%)	n= 50 (60%)	n= 33 (40%)
Age	55 (8)		58(8)		59(11)	
BMI	26.9 (4.8)		30.5 (6.7)		31.3 (7.1)	
White Ethnicity	69 (93%)		71 (96%)		75 (90%)	
hsCRP (mg/L)	3.0 (3.7)		4.8 (8.6)		5.0 (4.6)	
ApoA1 (mg/dL)	194 (35)		170 (34)		159 (28)	
ApoB (mg/dL)	112 (25)		100 (26)		109 (36)	
ApoB/ApoA1 ratio	0.60 (0.17)		0.60 (0.18)		0.71 (0.26)	
Total Cholesterol (mg/dL)	227 (39)		190 (39)		195 (49)	
LDL-C (mg/dL)	127 (32)		98 (31)		104 (40)	
HDL-C (mg/dL)	69 (19)		55 (19)		48 (13)	
non-HDL-C (mg/dL)	158 (41)		135 (37)		147(49)	
Triglycerides (mg/dL)	152 (88)		187 (124)		223 (112)	
Number Taking Lipid Modifying Rx	0 (0%)		70 (95%)		80 (96%)	
Events						
Revascularization	-		-		29 (35%)	
Myocardial Infarction	-		-		38 (46%)	
Stroke	-		-		16 (19%)	

Specimens to Assess Validated Method Robustness

To assess the characteristics of the assay in a diverse population, 241 de-identified remnant specimens meeting selected criteria were collected (LDL-C<130 mg/dL, HDL>40 mg/dL, total

cholesterol <240 mg/dL, triglycerides <200 mg/dL), HbA1c<5.7%, hsCRP≤1.0 mg/L) and pCE and pCAD was determined.

UKCTOCS Biobank Specimens for Validated Method Evaluation

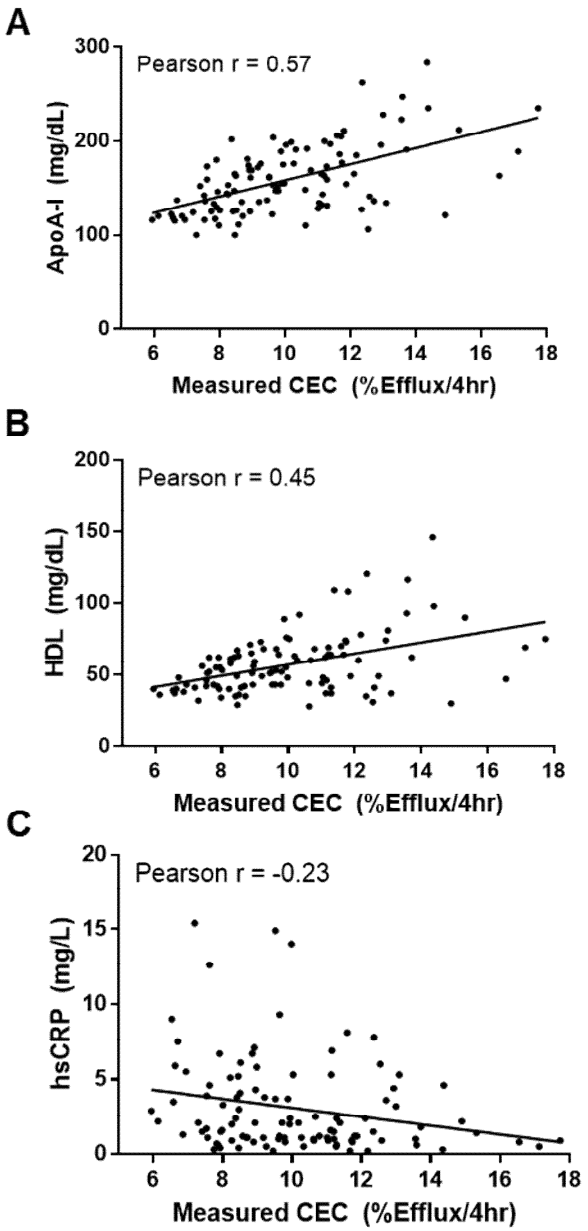
We examined a subset of serum samples collected during the course of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), a 13-center randomized controlled trial investigating the impact of ovarian cancer screening on disease mortality. The trial design is detailed previously (Menon et al., 2008). Briefly, UKCTOCS participants (n=202,638) were all post-menopausal women aged 50-74 with no active malignancy and no history of ovarian cancer at recruitment between 2001 and 2005. Participants were randomly assigned (2:1:1 ratio) to routine care (control; n=101,359) or annual screening using serum cancer antigen 125 (CA125) (multimodal screening, n=50 640) or transvaginal ultrasound (n=50,639). All participants were linked using their National Health Service number to national cancer and death registry electronic health records as well as Hospital Episode Statistics (those resident in England) and the Myocardial Ischaemia National Audit Project (MINAP). In addition, women were sent two follow-up questionnaires, the most recent in 2014. All women provided a blood sample at recruitment with women randomized to the multimodal screening group (n=50,640) continuing to donate serum annually for up to 11 years from randomization. Sample collection stopped at the end of screening in December 2011 (Menon et al JCO 2015).

In our study evaluation of pCE and pCAD biomarker algorithms utilized 69 cases and 68 matched controls (one failed control sample analysis) from the biobank. Cases were defined as volunteers diagnosed with CVD (acute coronary syndrome with positive or negative troponin result or Acute ST-segment elevation myocardial infarction) 1 to 2 years after blood sample collection based on the MINAP

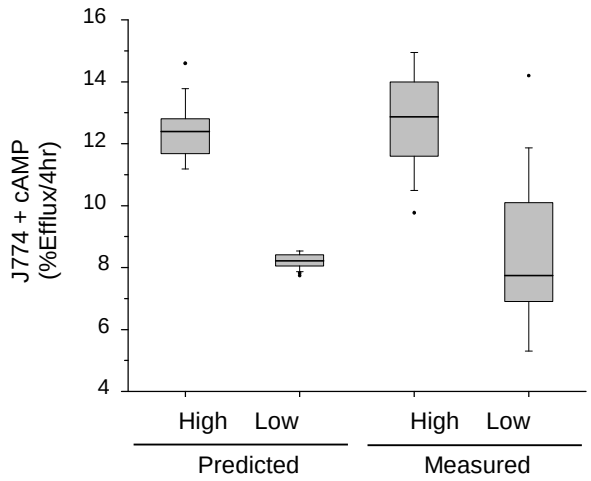
database. Controls consisted of UKCTOCS volunteers not identified as a case and not known to have a diagnosis of diabetes. Controls and Cases were matched based on age (±1 year), time from sample collection to spin (±4 hours), BMI category and Blood Pressure (high and low). Samples were licensed for use via Abcodia Ltd, a company focused on the development of early detection tests for chronic disease

Ethical approval

UKCTOCS was approved by the UK North West Multicentre Research Ethics Committee (North West MREC 00/8/34) with site specific approval from the local regional ethics committees and the Caldicott guardians (data controllers) of the primary care trusts. All women gave informed written consent for use of samples and data in ethically approved secondary studies undertaken in collaboration with academia and/or industry. The subset of samples used for the present study has been approved by the London Bromley Research Ethics Committee (REC Ref 16/LO/2228).



Supplemental Figure 1. Plotted association of cell-based assay of CEC with other clinical metrics, including A) Apo-A-I, B) HDL-C and C) high sensitivity C-reactive protein (hsCRP).

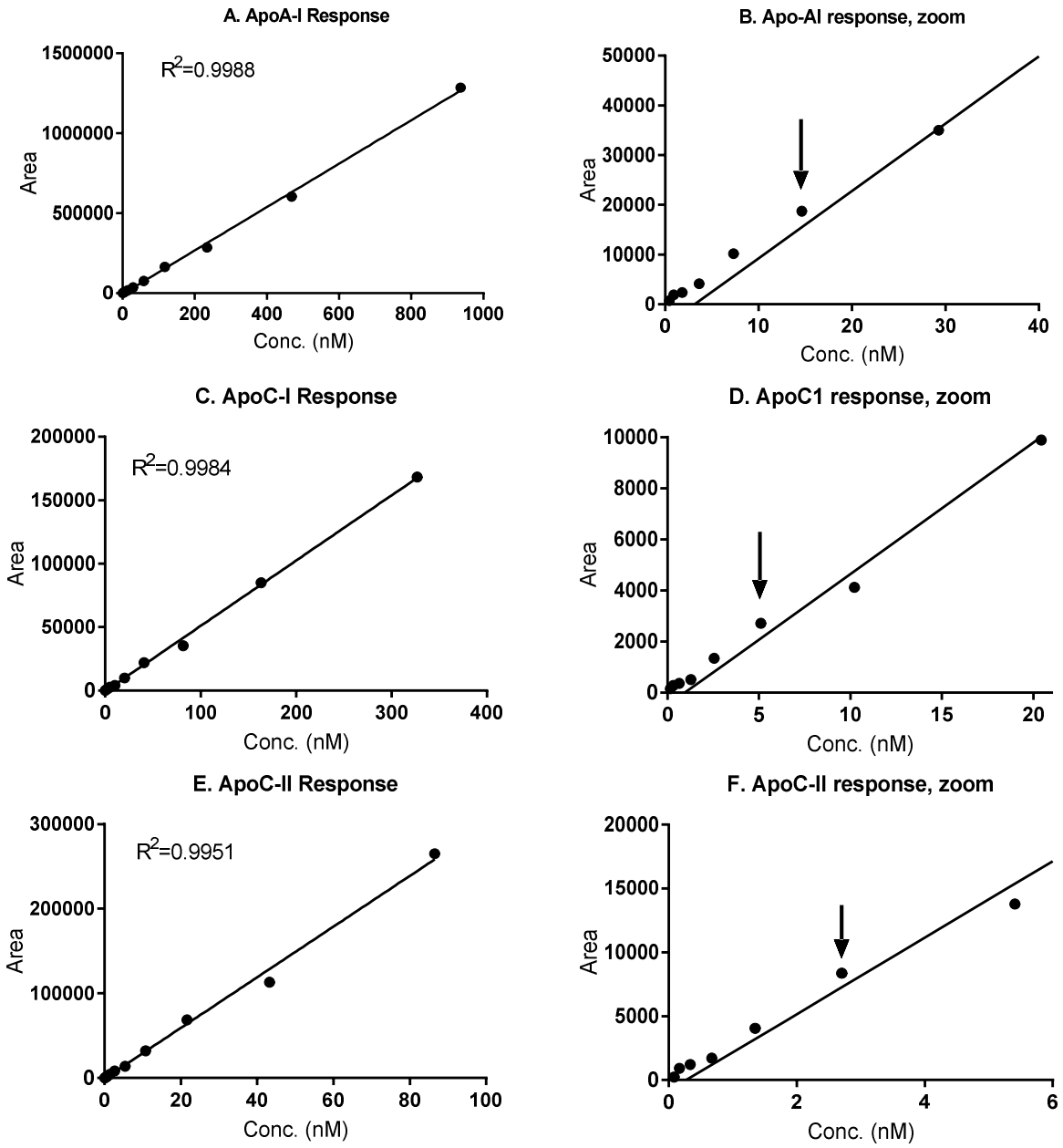


Supplemental Figure 2. Comparison of predicted cholesterol efflux from LC-MRM analysis of 30 samples (n=15 High pCE, n=15 Low pCE) with the measured cellular cholesterol efflux of the same sample set from cAMP-stimulated J774 macrophages.

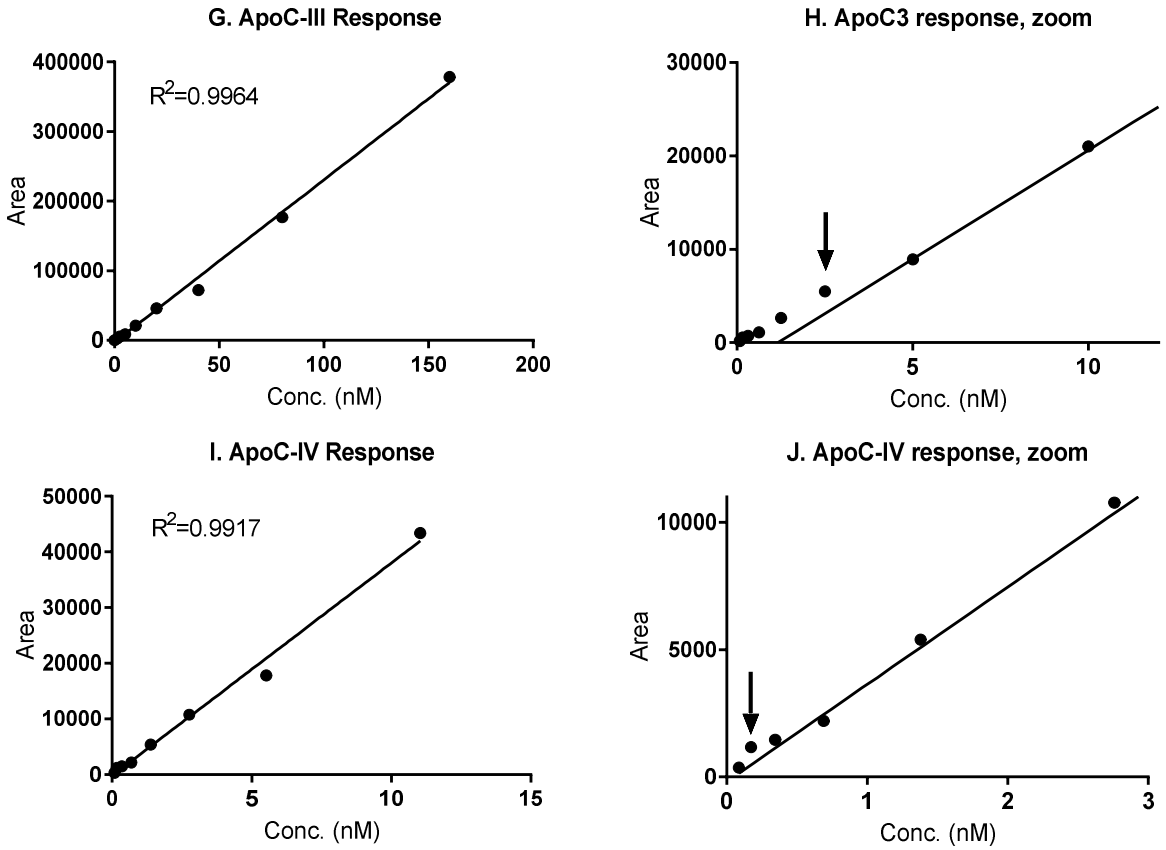
METHOD VALIDATION RESULTS

Calibrator Performance and Limits of Quantitation

This assay is internally calibrated via a mixture single point calibrator peptides. To demonstrate that assay response is linear and that typical measurements of unknowns are within this linear range, calibrator stocks were diluted in matrix and evaluated for linearity. Unweighted regression was used to calculate linear response and dilution plots (**Supplemental Figure 3**) were inspected to identify the range at which experimental observations regularly deviated from ideal linear response. The departure from linearity for each peptide established the lower limit of quantitation for this assay. The linear ranges are established as follows: ApoA-I, 15-935 nM; ApoC-I, 10-325 nM; ApoC-II, 5-85 nM; ApoC-III, 5-160 nM; ApoC-IV, 0.2-11 nM. In 233 random patient samples, all observed protein concentration ranges lie well within the linear range of response for each analyte.



Supplement Figure 3A-F. Limit of analytical sensitivity



Supplement Figure 3G-J. Limit of analytical sensitivity

Total Imprecision

Each test material was analyzed with four experimental replicates for 15 days, across three HPLC systems (300 observations). Estimates of total imprecision were determined at the peptide, pCE and pCAD score levels. At the peptide level, the coefficients of variation (CV) for ApoA-I, ApoC-I, ApoC-II, and ApoC-III ranged of 5 - 8% for these five specimens (**Table 2** and **Supplement Table 5**). The CV of lowest abundance protein ApoC-IV ranged from 7.7 to 14%, which is well below the acceptance criteria of 20%. Mean pCE values and 95% confidence intervals for the five specimen types are: High

pool, 7.8±0.1; Medium pool, 13.1±0.5; Low pool, 12.9±0.6; Patient 1, 11.8±0.6; Patient 2, 9.3±0.3. Mean pCAD values and 95% confidence intervals for the five specimen types are: Low pool, -1.91±0.64; Medium pool, 0.03±0.62; High pool, 2.8±0.28; Patient 1, -1.67±0.80; Patient 2, -0.63±0.39.

Supplement Table 5. Assigned protein concentrations and total imprecision at peptide level

		15N-ApoA1	ApoA1	ApoC1	ApoC2	ApoC3	ApoC4
Low QC	Imprecision	5.2%	5.3%	6.5%	5.3%	5.5%	9.6%
	Conc. (nM)	718.1	368.5	47.7	11.6	33.8	0.90
Med QC	Imprecision	7.0%	7.3%	7.0%	7.4%	7.2%	8.5%
	Conc. (nM)	759.1	322.9	45.3	12.3	35.5	1.0
High QC	Imprecision	5.2%	5.2%	6.6%	6.4%	7.9%	14%
	Conc. (nM)	847.6	178.9	13.3	3.3	4.6	0.29
Patient 1	Imprecision	5.8%	5.6%	6.5%	6.5%	7.4%	7.7%
	Conc. (nM)	724.1	325.3	57.5	14.3	28.6	1.1
Patient 2	Imprecision	6.0%	6.4%	6.7%	6.7%	8.1%	13.6%
	Conc. (nM)	749.5	280.3	23.3	2.9	14.0	0.29
Range	Imprecision	≤ 7.0%	≤ 7.3%	≤ 7.0%	≤ 7.4%	≤ 8.1%	≤ 14.0%

Bias

In the absence of reference material, bias is evaluated as a difference from assigned values. Prior to start of validation, test materials were extensively characterized, and values were assigned for each of the 5 proteins and the calculated pCE value. As shown in **Supplement Table 6**, no protein other than ApoC-IV in the high QC pool demonstrated a bias >±20%. The more modest performance of ApoC-IV is likely due to the fact that it is the least abundant protein in the panel, nearly 4 orders of magnitude lower in abundance than ApoA-I. In the case where ApoC-IV exceeded The bias of pCE score were in the range of -4.4 to 4.6% CV for Low, Medium, and High pool. Ranges of bias for the pCAD score

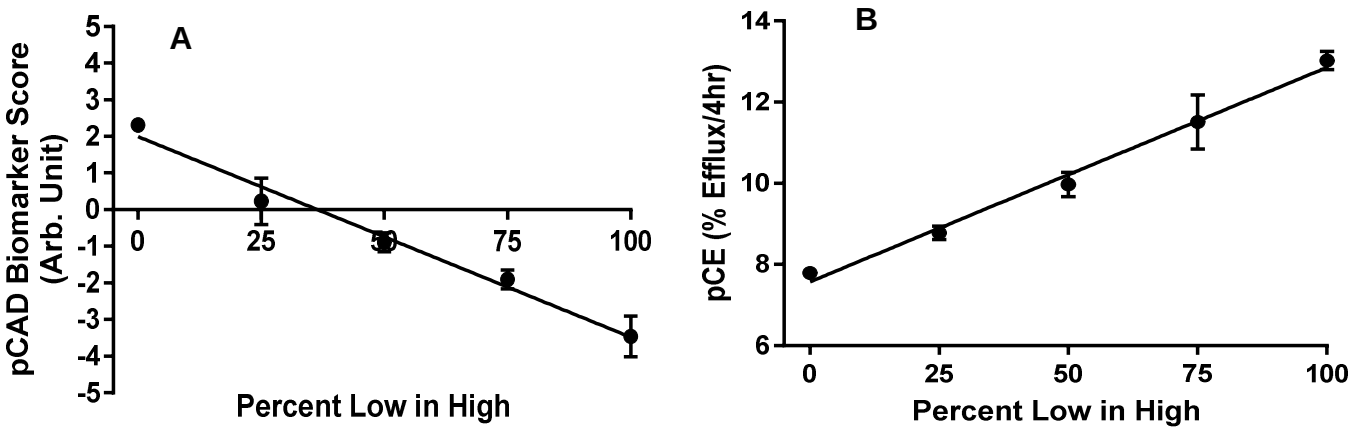
are; Low pool, -18.2 to 27.6%; High pool, -10.2 to 6.6%. Since the mean pCAD value for Medium pool is approaching zero, the bias was not calculated.

Supplement Table 6. Total bias at peptide level and assigned protein concentrations

		15N-ApoA1	ApoA1	ApoC1	ApoC2	ApoC3	ApoC4
Low QC	Bias	-5.2% - 3.1%	-4.5% - -3.5%	-8.4% - 10.1%	-5.2% - 6.3%	-6.0% - 6.4%	-10.7% - 12.1%
	Conc. (nM)	718.1	368.5	47.7	11.6	33.8	0.90
Med QC	Bias	-12.3% - 11.1%	-10.2% - 13.0%	-8.0% - 14.2%	-6.4% - 12.7%	-7.9% - 11.8%	-13.3% - 8.5%
	Conc. (nM)	759.1	322.9	45.3	12.3	35.5	1.0
High QC	Bias	-8.1% - 5.2%	-6.1% - 6.0%	-9.0% - 9.5%	-12.7% - 9.2%	-12.7% - 12.9%	-20.6% - 21.5%
	Conc. (nM)	847.6	178.9	13.3	3.3	4.6	0.29
Patient 1	Bias	-7.7% - 5.2%	-5.5% - 7.8%	-8.2% - 10.9%	-9.2% - 8.5%	-8.2% - 16.3%	-7.9% - 8.7%
	Conc. (nM)	724.1	325.3	57.5	14.3	28.6	1.1
Patient 2	Bias	-7.1% - 11.4%	-7.1% - 11.5%	-8.7% - 12.7%	-11.4% - 9.7%	-8.9% - 16.7%	-16.9% - 16.4%
	Conc. (nM)	749.5	280.3	23.3	2.9	14.0	0.29
Range	Bias	-12.3% - 11.4%	-10.2% - 13.0%	-8.7% - 14.2%	-12.7% - 12.7%	-12.7% - 16.7%	-20.6% - 21.5%

Linearity

A five-point mixing experiment was performed to evaluate the linearity of pCAD and pCE output values. Six patient samples were specially selected to generate three high/low pCAD pairs. Each pair of specimen were mixed in 3:1, 1:1, and 1:3 volume ratio along with two initial samples. The pCAD and pCE values were plotted as a function of titration level. Representative results are shown in **Supplement Figure 4** for one high/low pair. Because of difference in the coefficient of multivariate models, a specimen with high pCAD score has low pCE score. The response of pCAD and pCE values were linear as a function of dilution for all pairs. The r^2 values were > 0.94 for pCE mixing results and r^2 > 0.93 for pCAD values of three high/low pairs.

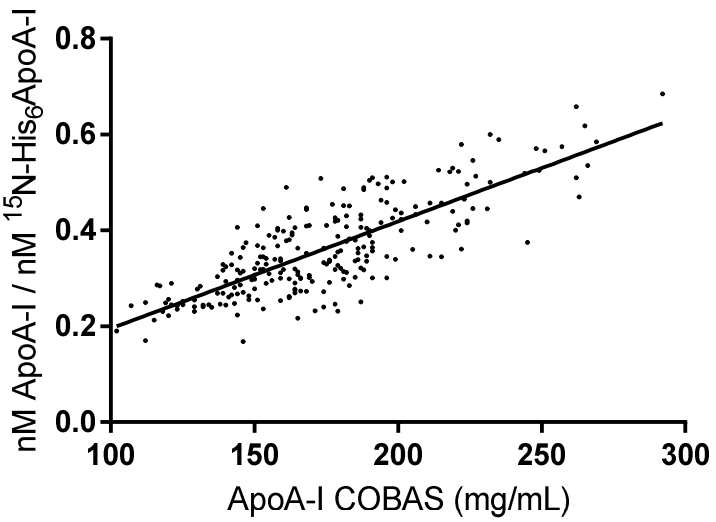


Supplement Figure 4. Titration of pCAD biomarker score (A) and pCE values (B) for one pair of specimen with high/low pCAD score. A specimen with high pCAD score has low pCE score.

Endogenous ApoA-I Recovery

While it is impossible to assess spike recovery in this biological system. We examined the relationship between ApoA-I levels in serum measured by automated turbidimetric immunoassay (COBAS, Roche

Diagnostics) and in AALP isolation measured by LC-MS/MS method from a set of 233 random patient samples. Recovery of ApoA-I in the AALP fraction from serum was highly correlated to the serum measurement (Pearson r=0.80**, Supplemental Figure 5**).

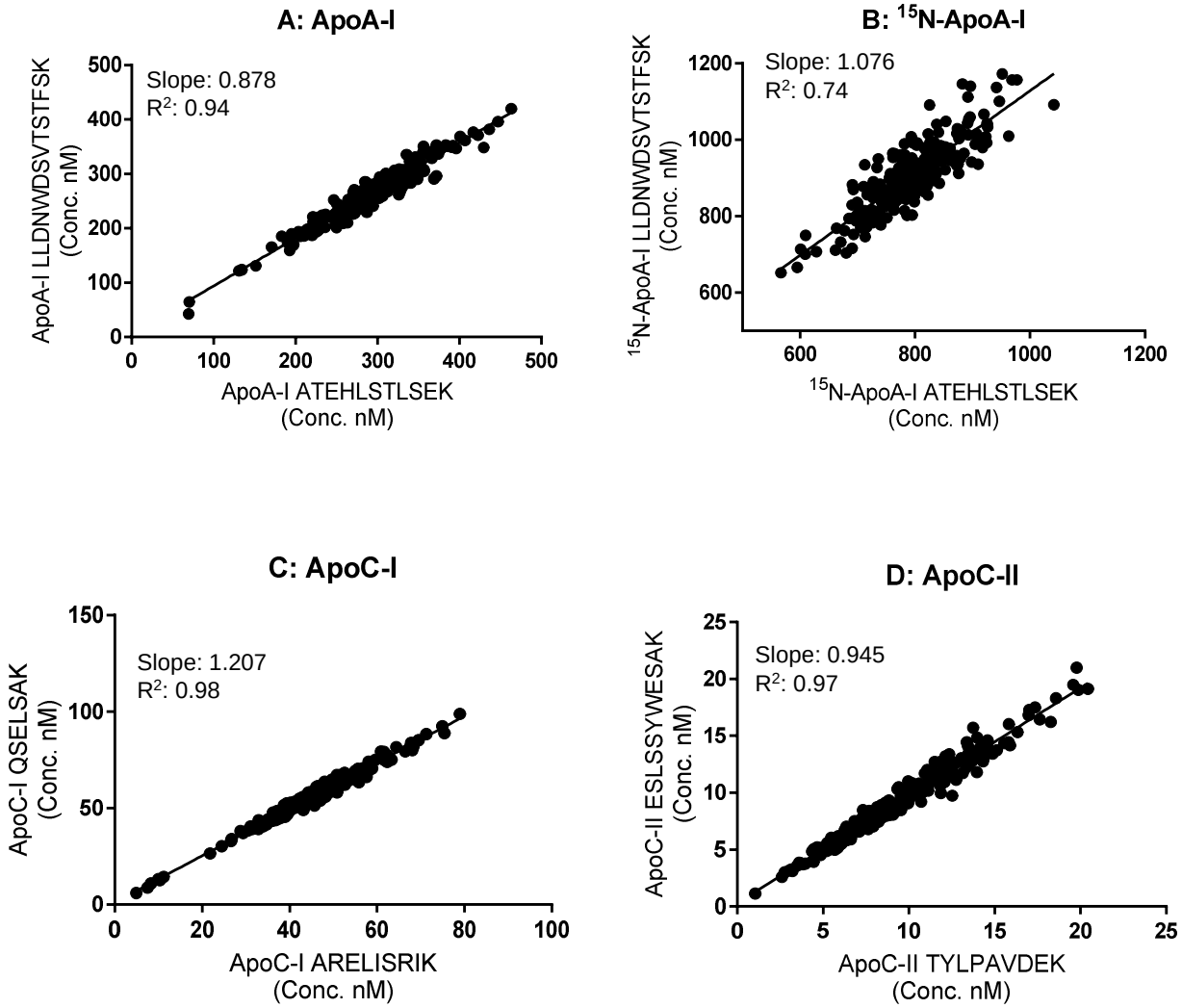


Supplement Figure 5. ApoA-I recovery from serum specimens. The ApoA-I concentration in serum was determined by automated turbidimetric immunoassay (COBAS). Level of ApoA-I in AALP fraction was measured by LC-MS/MS and normalized to ^{15}N-His$_6$ApoA-I to control for variable recovery during lipoprotein enrichment.

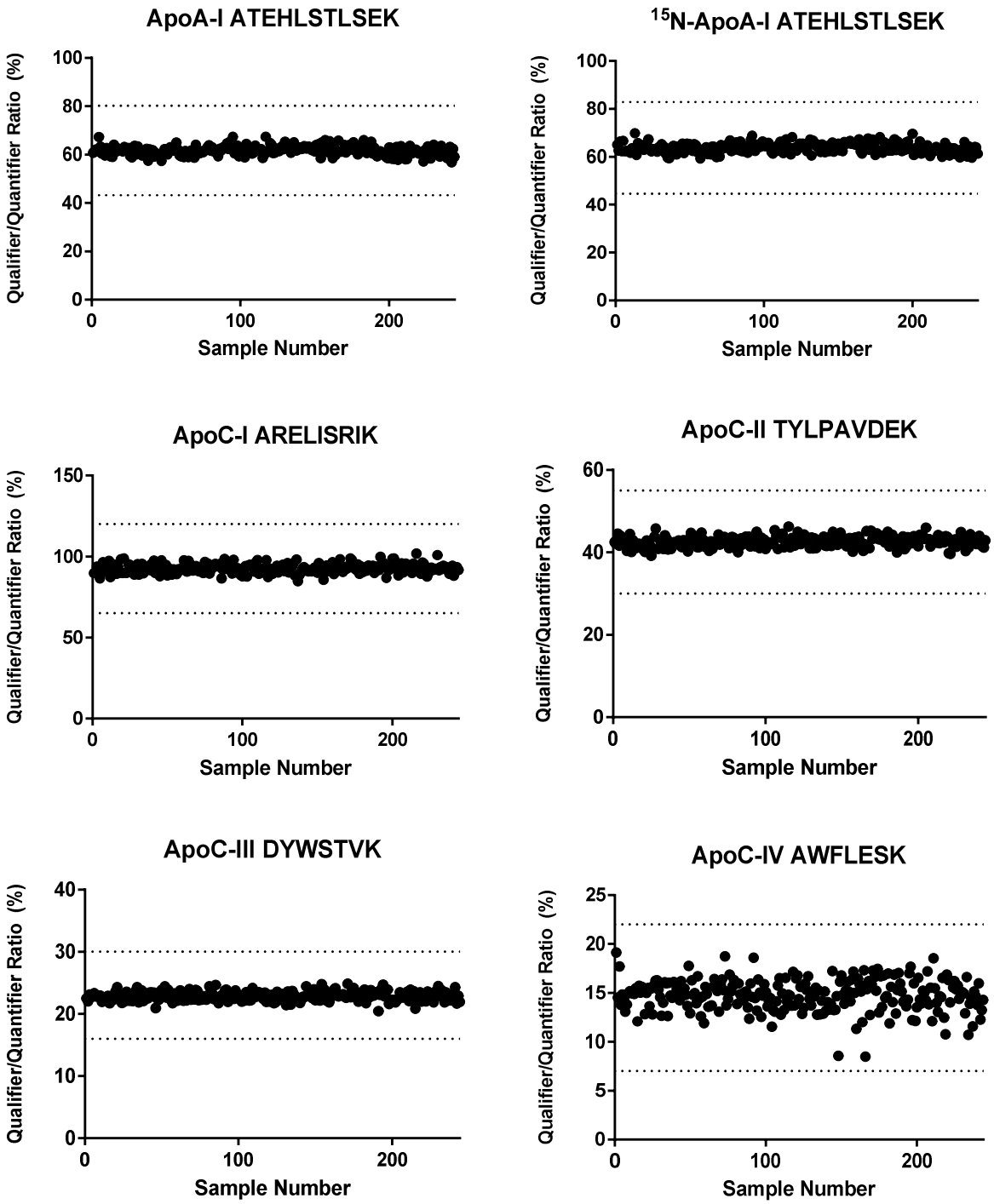
Mass spectrometry quality control

Best practice for protein LC-MS/MS assays is: 1) assess the agreement between two or more peptides from each protein (when possible); 2) evaluate adherence to fragment ion intensity ratios established from characterization of synthetic internal standard peptides. ApoA-I, ^{15}N-ApoA-I, ApoC-I, ApoC-II protein have peptide pairs monitored during data acquisition, while ApoC-III and ApoC-IV are small proteins and only a single peptide was quantified. For each protein with two peptides available, stable linear relationships were observed across all measurements of the 233 random patient samples (**Supplement Figure 6**). Fragment ion intensity ratios of signature peptides are used in monitoring the specificity of a mass spectrometric measurement. Qualifier ions with intensity over 50% of the quantifier

ions should agree with the theoretical ratio within ±20%. For qualifiers with lower intensity, the tolerance is expanded to ±30%. No fragment ion ratio outliers were observed for the data set (**Supplement Figure 7**).



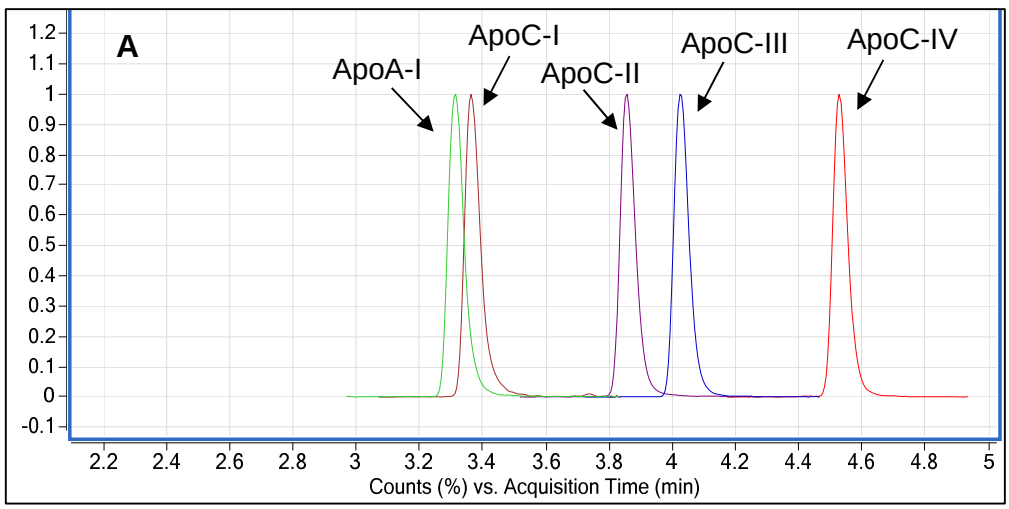
Supplement Figure 6. Peptide to Peptide Ratios

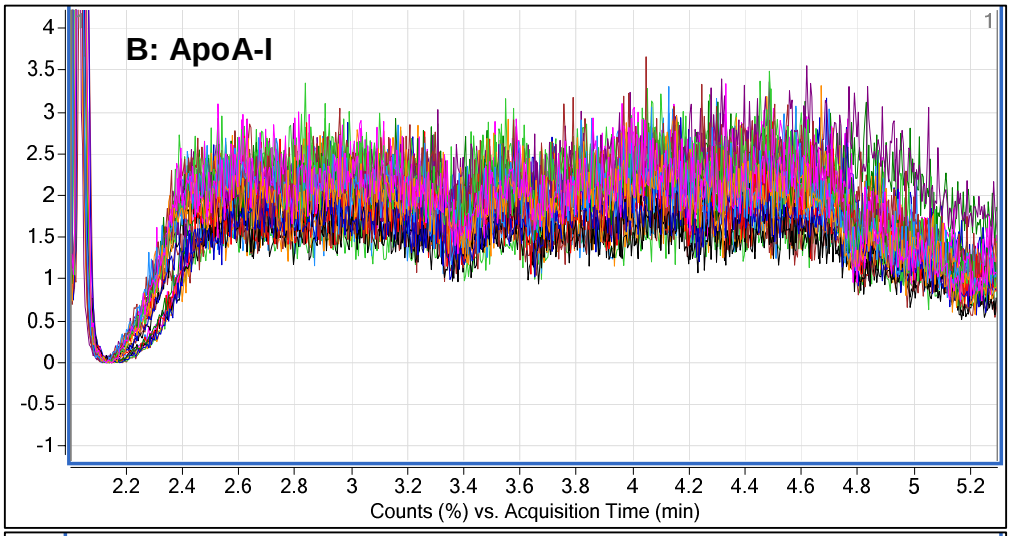


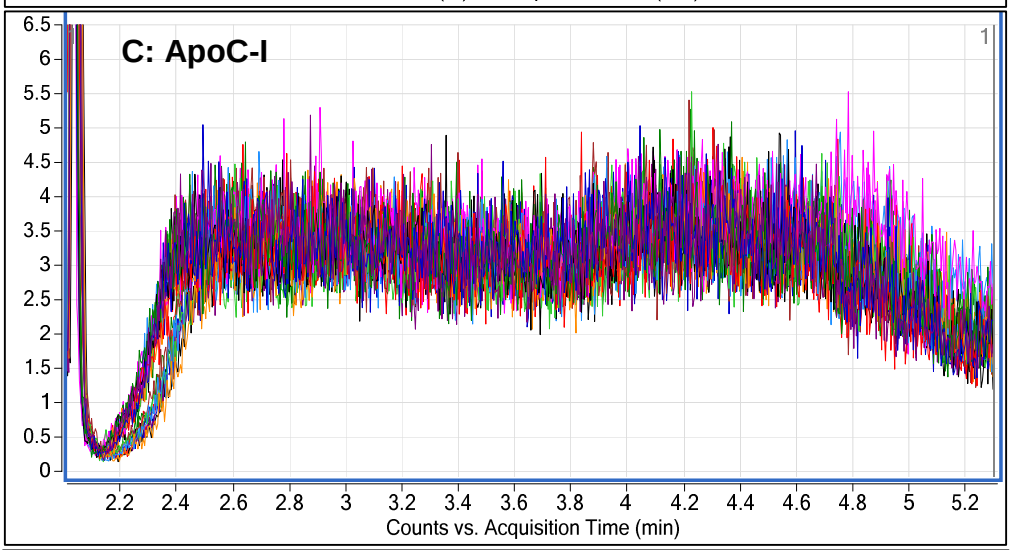
Supplement Figure 7. Fragment ion ratio of each signature peptide.

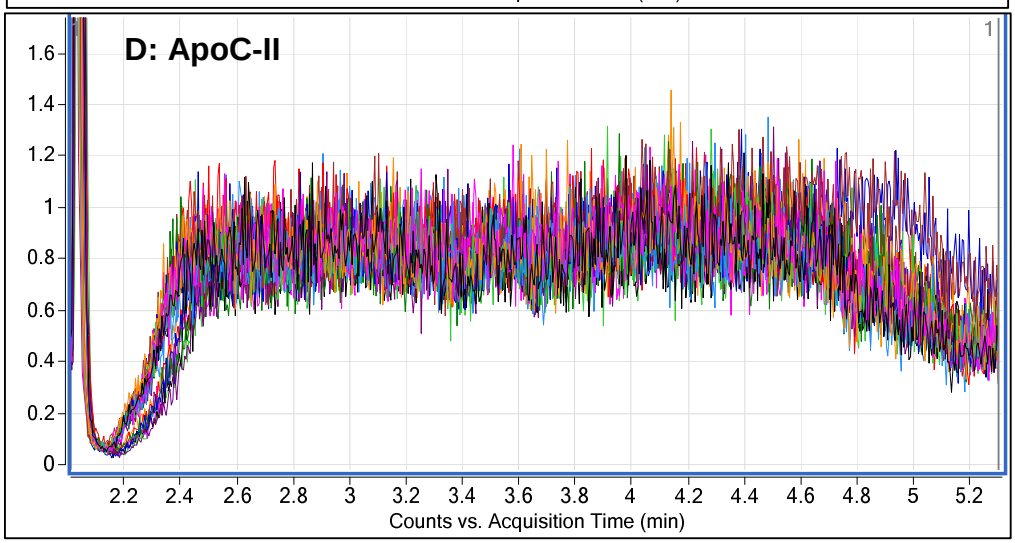
Matrix Effect

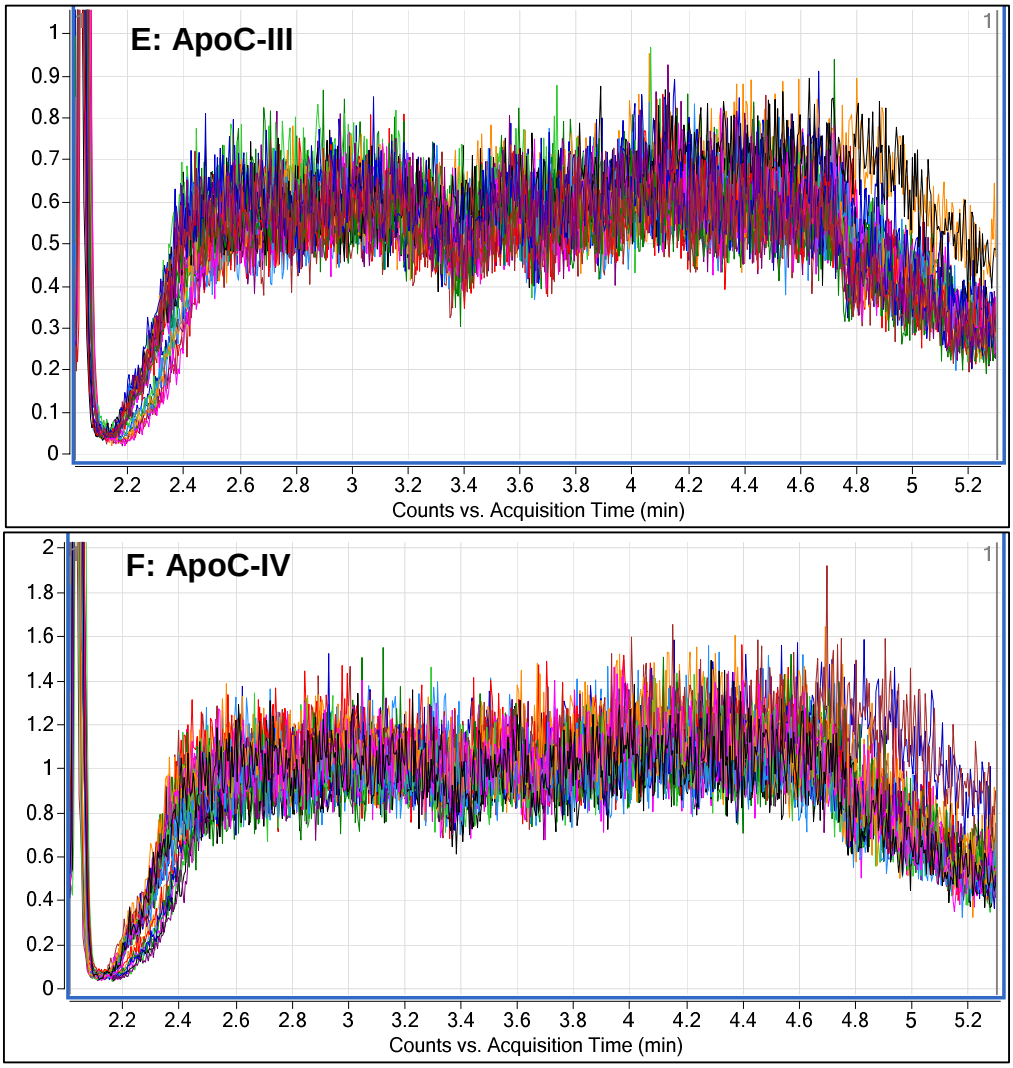
Twenty-four unique patient serum samples were prepared without the addition of internal standard. While authentic sample matrix was eluted from the analytical column, internal standard peptides were introduced to mass spectrometer via direct infusion and matrix suppression of internal standard was evaluated. The matrix suppression results are shown with the internal standard response plotted as a function of chromatographic time. By examination internal standard response to the elution of matrix from authentic samples, regions of perturbation can be identified. Slight matrix suppression was observed at 3.3 to 3.4 min when ApoA-I and ApoC-I were eluted from column (**Supplement Figure 8**). No regions of substantial suppression or enhancement were identified for any protein. Graphical representation for the extracted ion chromatograms for the quantifier for each protein are given in Supplement Figure 8A.




B: ApoA-I
1
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
2.2 2.4 2.6 2.8 3 3.2 3.4 3.6 3.8 4 4.2 4.4 4.6 4.8 5 5.2
Counts (%) vs. Acquisition Time (min)


C: ApoC-I
1
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
2.2 2.4 2.6 2.8 3 3.2 3.4 3.6 3.8 4 4.2 4.4 4.6 4.8 5 5.2
Counts vs. Acquisition Time (min)


D: ApoC-II
1
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2.2 2.4 2.6 2.8 3 3.2 3.4 3.6 3.8 4 4.2 4.4 4.6 4.8 5 5.2
Counts vs. Acquisition Time (min)



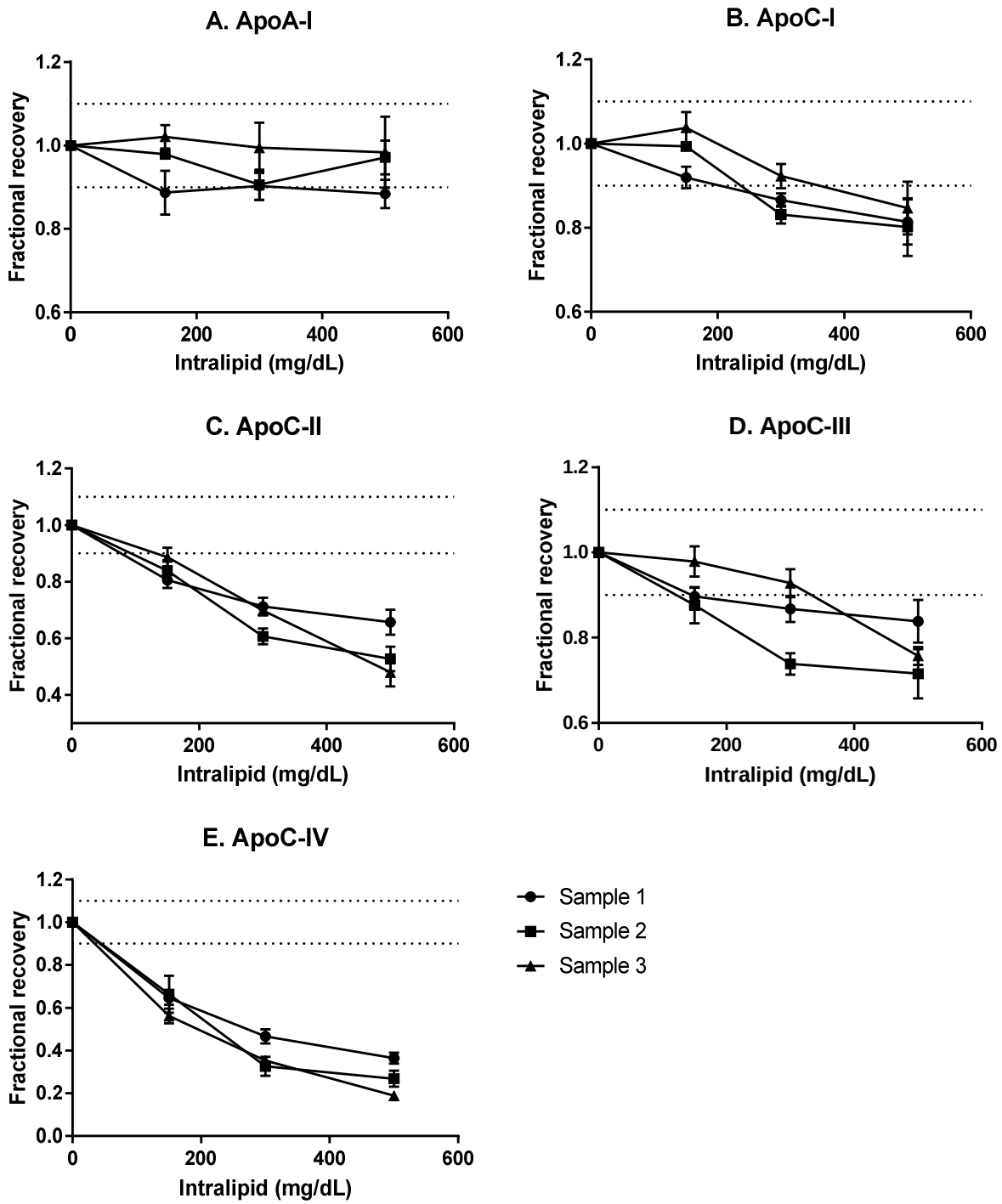
Supplement Figure 8 A-F. Internal standard signal suppression. A) Extracted ion chromatogram for the signature peptide of each protein. B) to F) Suppression of each internal standard peptide.

Interferences

The effect of common endogenous interferences, i.e. intralipid, unconjugated bilirubin, and hemoglobin, on the peptide quantification results were evaluated. Three serum samples were spiked with intralipid to the final concentrations of 150, 300, and 500 mg/dL, or spiked with unconjugated bilirubin to the final concentrations of 0.2, 2, and 5 mg/dL, or spiked with hemoglobin to the final concentrations of 100, 150, and 200 mg/dL, respectively. These samples were analyzed in 3 replicates and the recovery

(relative to non-spiked samples) was examined. For evaluation of intralipid interference, graphical representation is shown in **Supplement Figure 9**. All proteins, other than ApoA-I, showed an Intralipid concentration dependent decrease with ApoC-IV most significantly influenced, declining by 40% at 150 mg/dL of intralipid. The changes in protein abundance lead to a steady decrease in pCE and pCAD values as a function of intralipid spike levels. These results indicate that only minor amounts of lipemia are acceptable.

For unconjugated bilirubin, all proteins demonstrated good stability as a function of increasing bilirubin concentration up to 5 mg/dL. For hemoglobin interference, all proteins measurement showed good stability with hemoglobin up to 200 mg/dL. The stability in protein abundance leads to constant levels of pCE and pCAD values as a function of bilirubin or hemoglobin spike levels. These results indicate that specimens with moderate levels of icterus or hemolysis are acceptable.



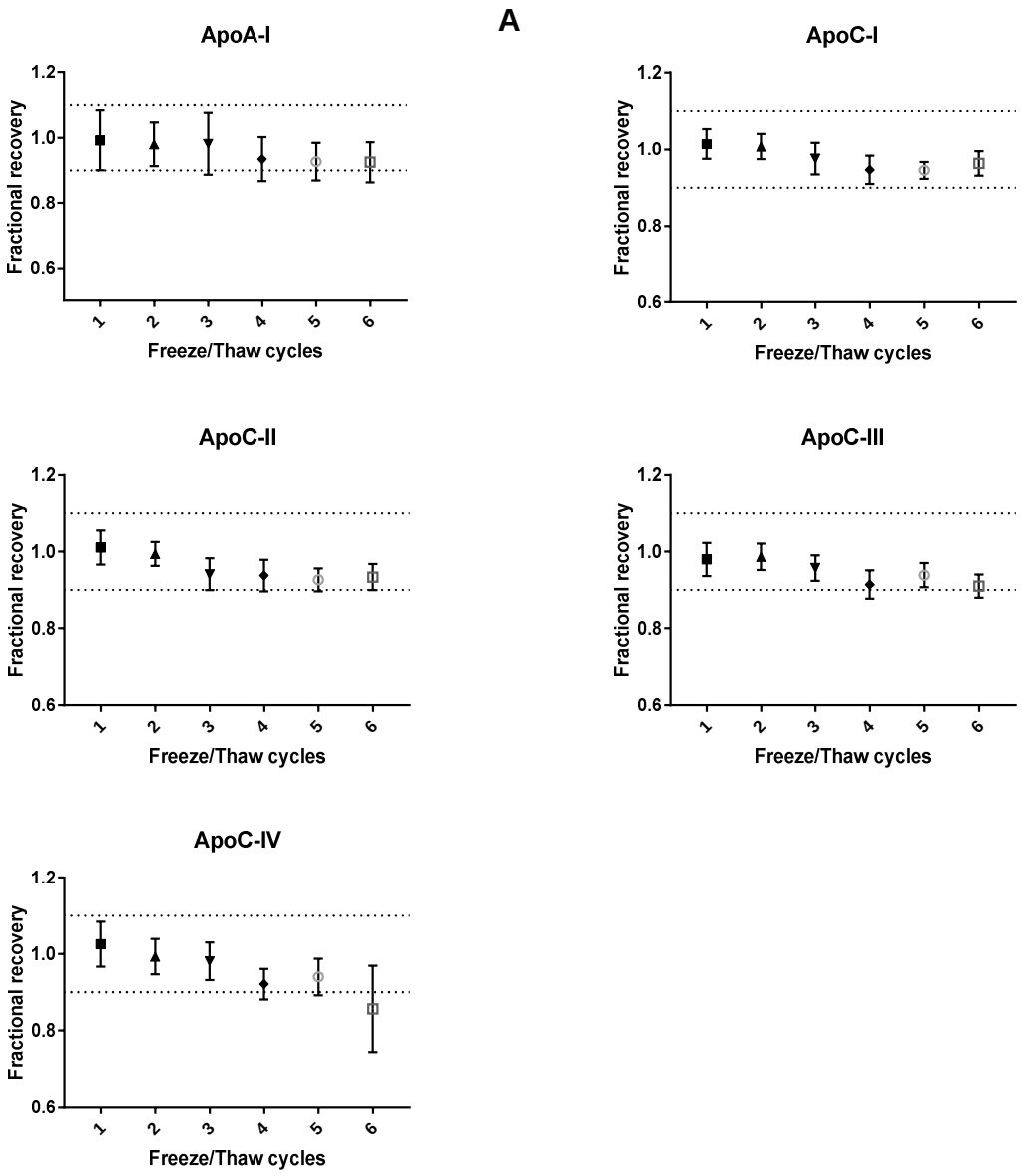
Supplement Figure 9 A-E Intralipid interference (n=9 at each data point)

Pre-analytical stability

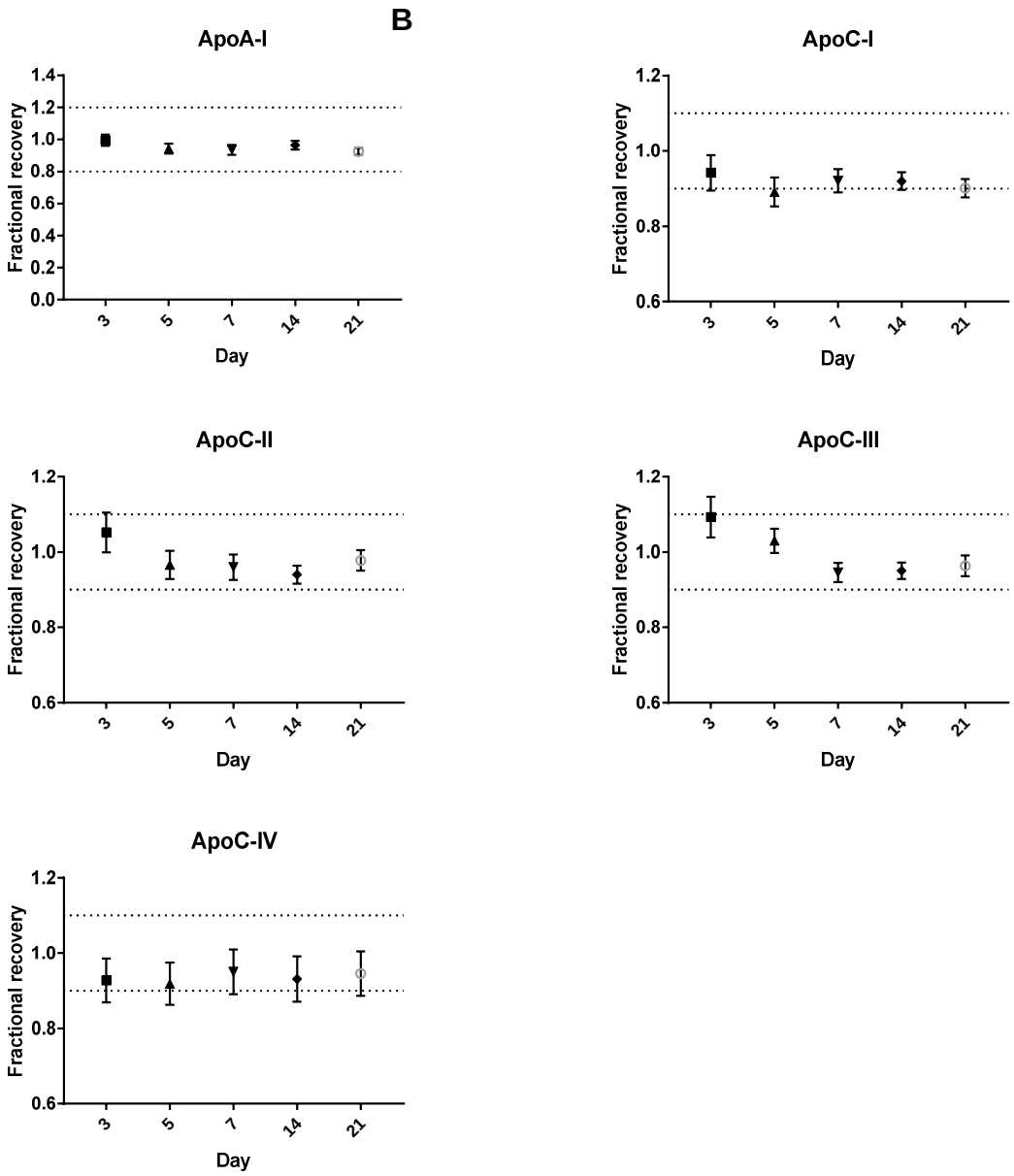
Two serum samples and three QC specimens were used to evaluate sample stability at various storage conditions. The following conditions were tested: freeze thaw cycle (up to 6 cycles), storage at ultralow (≤ -60 °C), frozen (-18 to -25 °C), refrigerator (2 to 8 °C), and room temperature (20 to 26 °C). On the day zero, stability samples were run and assigned as reference. Other time point data were compared with the Day 0 data to determine the stability. Stabilities of peptide concentrations, pCE, and pCAD values are evaluated.

Each protein showed a freeze/thaw cycle dependent decrease in percentage recovery compared to the starting value (**Supplement Figure 10A**). Except for ApoC-IV at freeze thaw cycle 6, fraction recovery was better than 90% of Day 0 data. Based on inspection of protein stability, pCE and pCAD data, three freeze thaw cycles are considered acceptable.

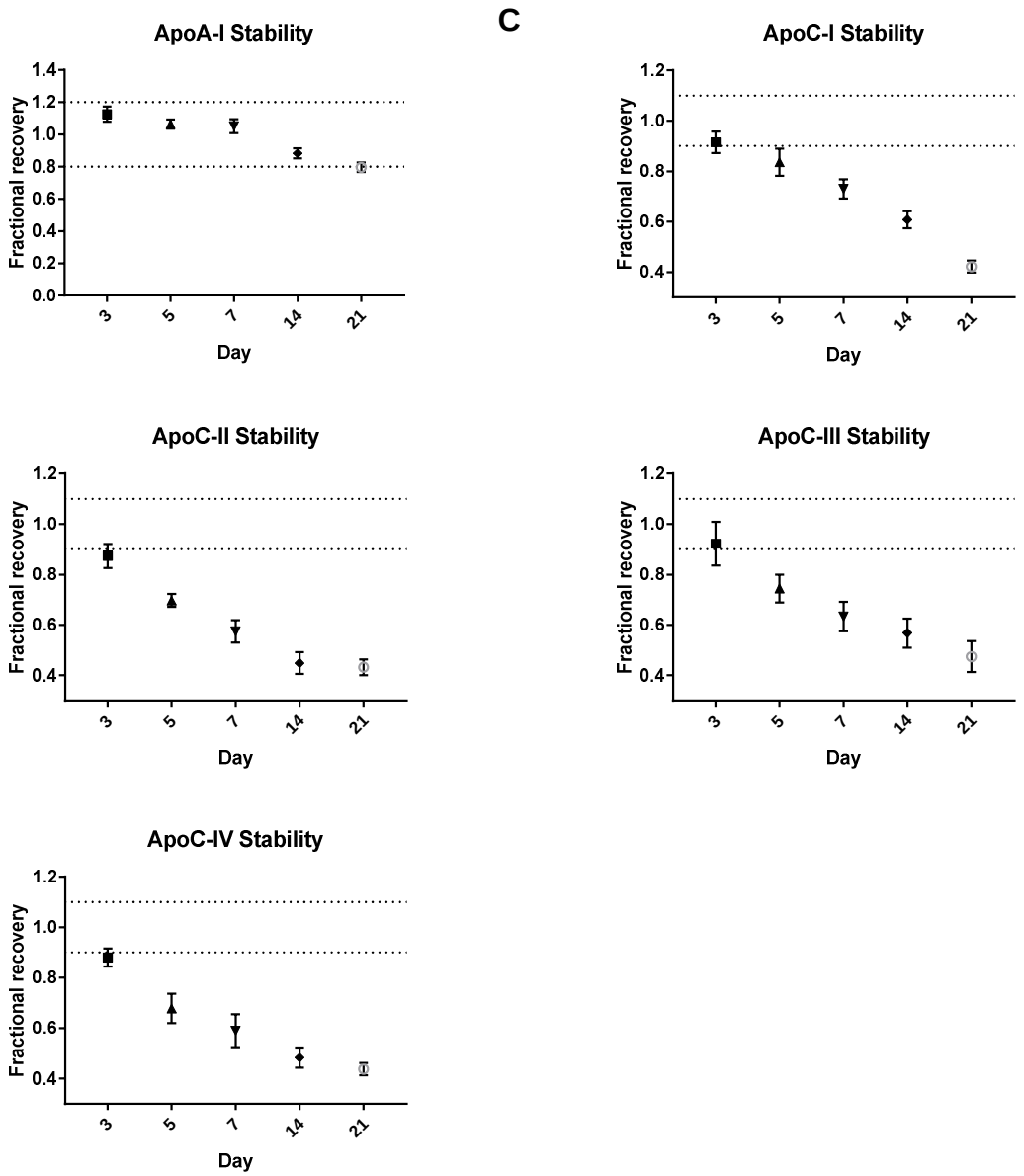
Each protein demonstrated good stability at ultralow temperature, frozen, and refrigerator storage over 21 days. As an example, ultralow temperature storage data were presented in **Supplement Figure 10B**. The associated pCE and pCAD values were also stable as. When stored at room temperature, each protein showed poor stability with ApoC-II and ApoC-IV declining below the 10% threshold by Day 3 (**Supplement Figure 10C**). Both pCE and pCAD values showed substantial perturbations due to protein abundance changes. These results indicated that samples are stable for 21 days at ultralow (≤ -60 °C), frozen (-18 to -25 °C), refrigerator (2 to 8 °C) storage. But samples are only stable for 1 day at room temperature between 20 to 26 °C.



Supplement Figure 10 (A) Freeze/Thaw Cycle Sample Stability (n=9 at each timepoint)



Supplement Figure 10 (B) Ultralow Temperature (≤ 60 °C) Sample Storage Stability (n=9 at each timepoint)

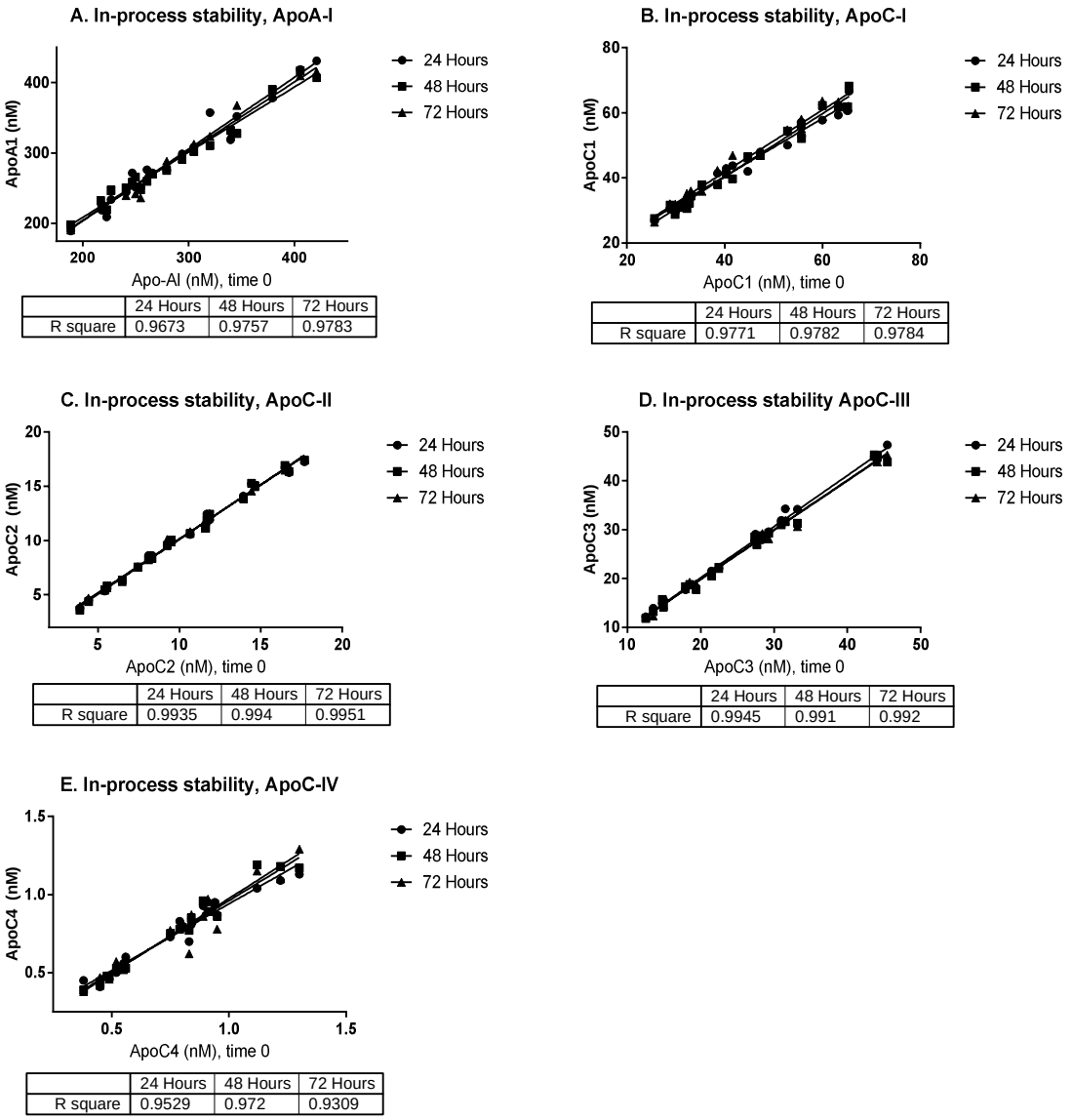


Supplement Figure 10 (C) Room Temperature (20 to 26 °C) Sample Stability (n=9 at each timepoint)

In-process stability

A set of 20 patient samples and QC pools is prepared and placed in the autosampler at 2-8 ˚C. The plate is run and subsequently reinjected at 24, 48, and 72 hours. Graphic representation for each protein are shown in **Supplement Figure 11**. Correlations at the peptide level indicate exemplary

stability over the course of 72 hours. No statistically significant difference in slope is indicated and $r^2 > 0.93$ at every time point. These results indicate that fully prepared samples are stable for up to 72 hours when stored at 2-8 ˚C.



	24 Hours	48 Hours	72 Hours
R square	0.9673	0.9757	0.9783

	24 Hours	48 Hours	72 Hours
R square	0.9771	0.9782	0.9784

	24 Hours	48 Hours	72 Hours
R square	0.9935	0.994	0.9951

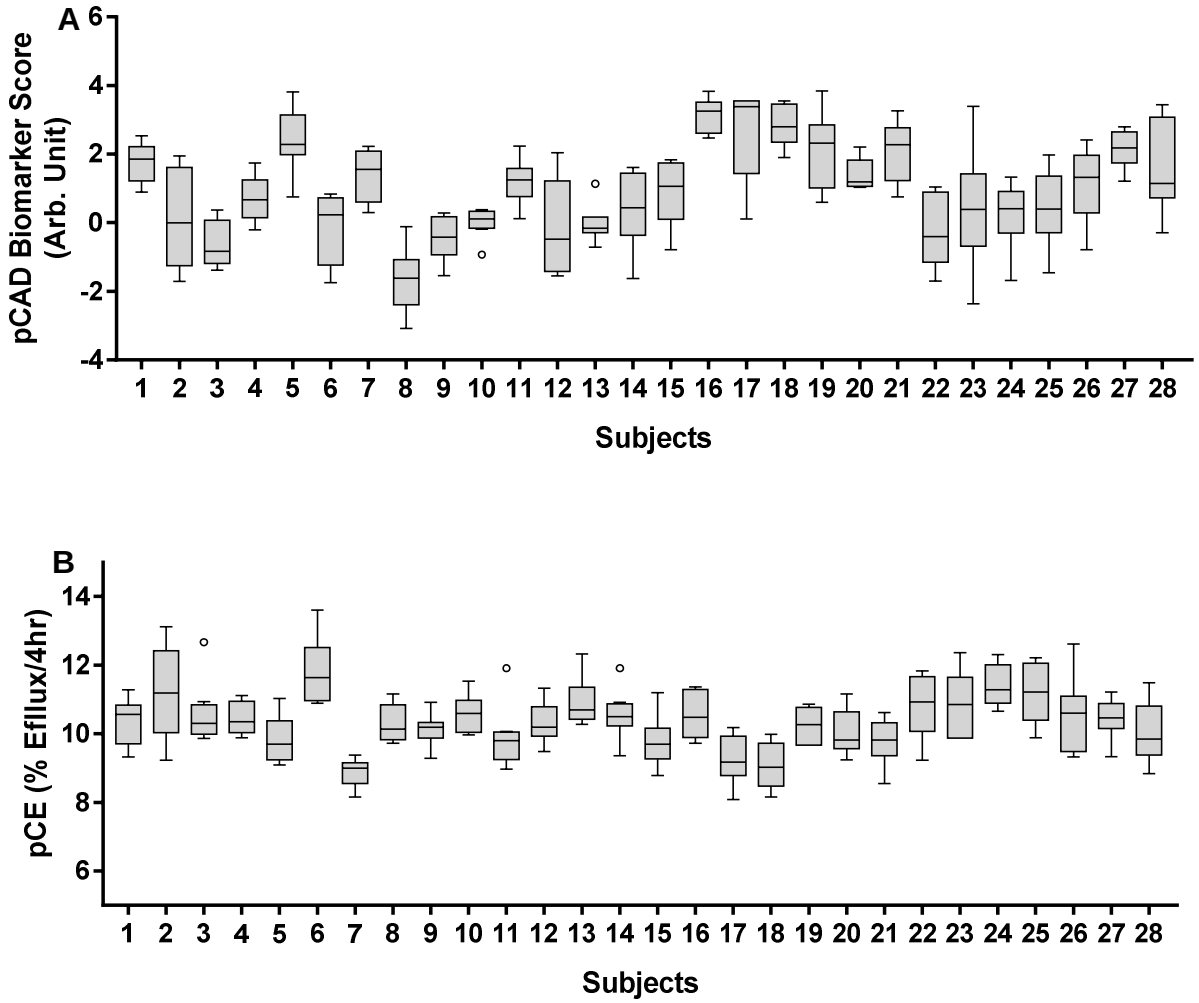
	24 Hours	48 Hours	72 Hours
R square	0.9945	0.991	0.992

	24 Hours	48 Hours	72 Hours
R square	0.9529	0.972	0.9309

Supplement Figure 11. In-process autosampler stability

Short term longitudinal study

We investigated the biological variation of the pCAD and pCE measurement in specimens collected once per week from 29 subjects over an eight-week period. Four subjects missed one of the 8 collection days. Clinical measurements (HDL-c, LDL-c, triglycerides) were evaluated to assess quality of the specimens. One subject was rejected to abnormally high triglycerides (an identified interference) for all collected specimens (>900 mg/dL). Four other individual specimens were rejected due to greater variation (>2SD) in two or more of three measurements (HDL-c, LDL-c, triglycerides) from the mean of 8 observations. The pCAD and pCE scores were calculated with box plots shown in **Supplement Figure 12**.



Supplement Figure 12. Box plots of pCAD (A) and pCE (B) biomarker scores for 28 subjects of eight-week longitudinal Study