Supporting Information

Towards biomarker development in large clinical cohorts: An integrated high-throughput 96-well plate based sample preparation workflow for versatile downstream proteomic analyses

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Supplemental Figure S1, S2, S3

Supplemental Tables, S1, S2, S3

Supplemental Methods and Results

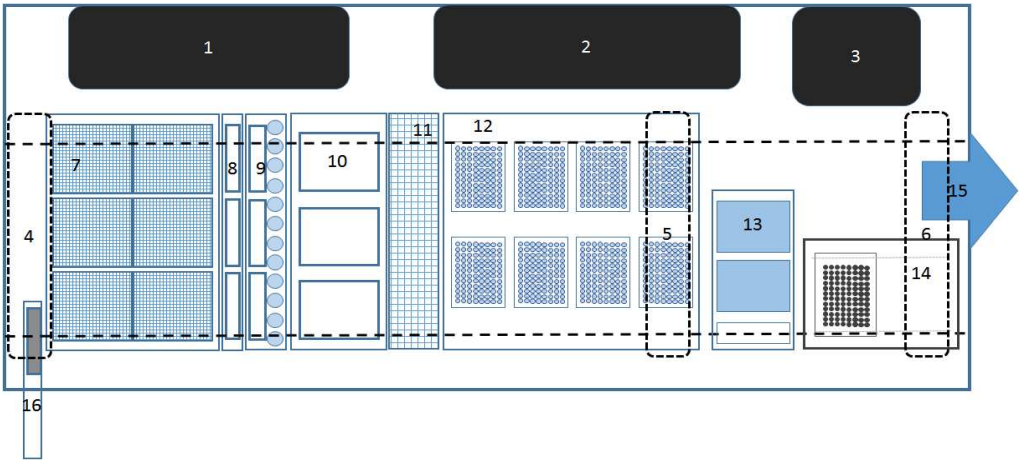

Figure S-1, Schematic diagram of the automated system with several key components: 1, pipette tips reservoir; 2, microplate hotel; 3, incubator (dark room) with temperature control for 96 well plates; 4, 8-channel liquid handling arm for disposable tips; 5, 8-channel liquid handling arm with steel needles; 6, 360°rotating microplate robotic arm; 7, pipette tips racks; 8, reagent carriers/wash station; 9, reagent carriers with temperature control; 10, Te-shaker; 11, tube rack for samples; 12, 96-well plate carrier; 13, on-deck heating and cooling blocks; 14, positive pressure solid extraction module; 15, connect to the N_2 evaporator; 16, pipette tips chute. All on-deck components are marked by solid lines, while handover robotic arms are marked by dash lines.

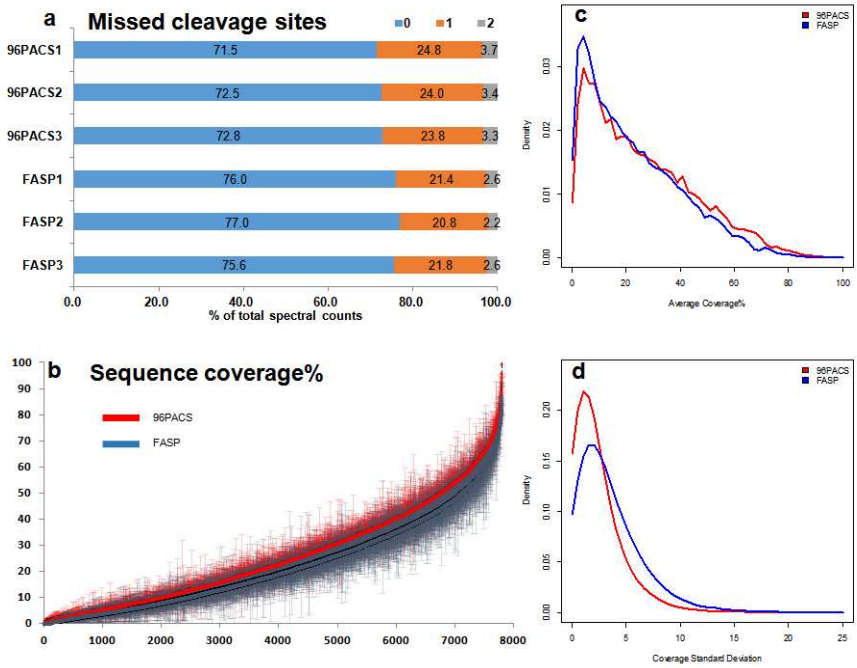

Figure S-2, Comparison of protein digestion efficiency of 96PACS and

FASP workflow. The percentages of MSMS spectra containing peptides with miss cleavage sites were compared between 3 96PACS and 3 FASP preparation using the Huh-7 lysate (a). Sequence coverage of 6596 proteins commonly identified in all 6 96PACS or FASP processed Huh-7 lysates were compared (b). The percentage of high sequence coverage proteins in 96PACS datasets was slightly higher than that in the FASP datasets (c), while the standard deviation of coverage in 96PACS datasets was slightly lower than that of FASP datasets (d).

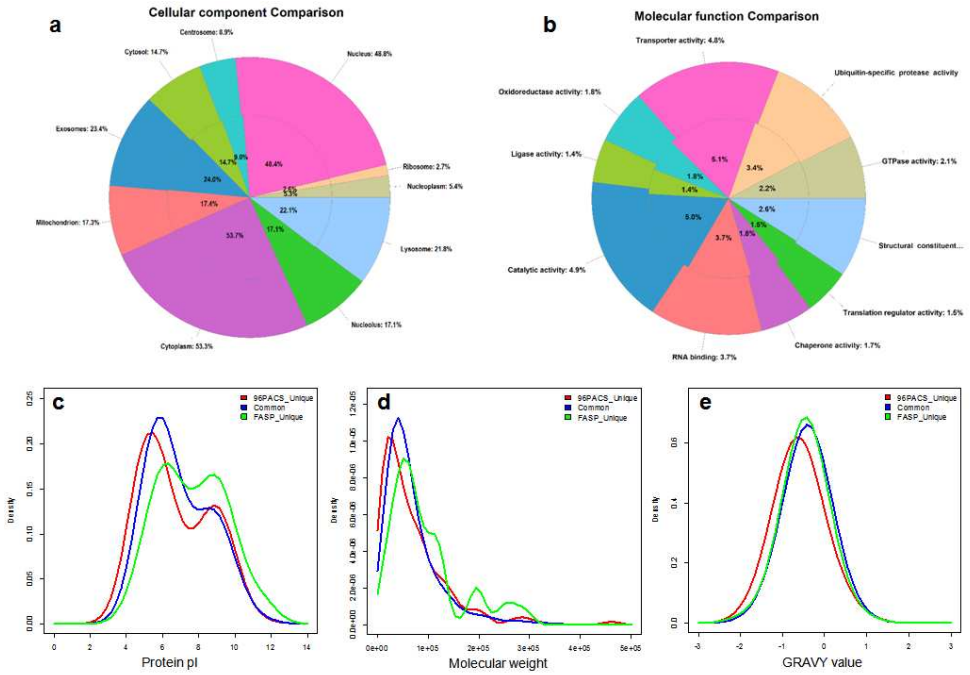

Figure S-3, GO annotations and physichemical properties of Huh-7 proteins detected in 96PACS and FASP proteomics workflow. The top 10 most enriched cellular component (a) and molecular function (b) GO terms based on proteins identified from 96PACS (outer circle) or FASP (inner circle) processed samples were shown in pie charts. Distribution of pI (c), molecular weight (d) and hydrophobicity measured in GRAVY value (e) of proteins identified only from in 96PACS (red) or FASP (green) processed samples or commonly found in both (blue) were shown in frequency density plots.

Table S-1, List of peptides targeted in 96PACS-PRM study

Proteins	peptide	Mass [m/z]	CS [z]	Start [min]	End [min]
sp|P0AG93|SECF_ECOLI	VINESTNQNAAVK	694.36243	2	34.42	40.42
sp|P77522|SUFB_ECOLI	NNSAQLEHEATTSR	519.913247	3	38.57	44.57
sp|P64429|YPFJ_ECOLI	QLQQNATQAEVNR	750.381685	2	38.64	44.64
sp|P0C8J6|GATY_ECOLI	DYLQSAK	412.711068	2	42.4	48.4
sp|P02769|ALBU_BOVIN	DDSPDLPK	443.711265	2	45.96	51.96
sp|P0A6Y8|DNAK_ECOLI	ASSGLNEDEIQK	645.812238	2	47.82	53.82

sp|P64429|YPFJ_ECOLI	VVPDSFTHGTSQQR	779.884061	2	48.86	54.86
sp|P68066|GRCA_ECOLI	FNSLTPEQQR	610.306926	2	51.92	57.92
sp|P0A738|MOAC_ECOLI	VEVNLQAEPEHNR	767.884061	2	52.65	58.65
sp|P0A6N4|EFP_ECOLI	GDTAGTGGKPATLSTGAVVK	596.654773	3	52.79	58.79
sp|P0C8J6|GATY_ECOLI	NYLTEHPEATDPR	771.862794	2	54.04	60.04
sp|P32132|TYPA_ECOLI	VEETEDADAFR	641.283314	2	54.83	60.83
sp|P68066|GRCA_ECOLI	EVPVEVKPEVR	640.864077	2	56	62
sp|P32132|TYPA_ECOLI	VKPNQQVTIIDSEGK	828.451776	2	56.31	62.31
sp|P32132|TYPA_ECOLI	LLQQSGTFDSR	626.320033	2	57.84	63.84
sp|P02769|ALBU_BOVIN	AEFVEVTK	461.74765	2	58.26	64.26
sp|P0CE48|EFTU2_ECOLI	AFDQIDNAPEEK	688.820063	2	58.82	64.82
sp|P0ABT2|DPS_ECOLI	YAIVANDVR	510.777273	2	59.96	65.96
sp|P0A6F5|CH60_ECOLI	ATLEDLGQAK	523.279846	2	60.76	66.76
sp|P37773|MPL_ECOLI	DDLAPSLGR	472.245806	2	62.44	68.44
sp|P0CE48|EFTU2_ECOLI	ALEGDAEWEAK	609.785492	2	63.23	69.23
sp|P0C8J6|GATY_ECOLI	NAFSQALK	439.740159	2	63.31	69.31
sp|P09373|PFLB_ECOLI	GAVASLTSVAK	502.292756	2	64.1	70.1
sp|P68066|GRCA_ECOLI	AGYAEDEVVAVSK	669.332807	2	65.37	71.37
sp|P09373|PFLB_ECOLI	GDWQNEVNVR	608.788901	2	66.76	72.76
sp|P0A6A8|ACP_ECOLI	IIGEQLGVK	478.792392	2	67.07	73.07
sp|P0A6F5|CH60_ECOLI	AIAQVGTISANSDETVGK	880.95488	2	67.25	73.25
sp|P68066|GRCA_ECOLI	VEGGQHLNVNVLR	717.894231	2	67.88	73.88
sp|P0A978|CSPG_ECOLI	VEFSIEQGQR	596.801477	2	69.05	75.05
sp|P02769|ALBU_BOVIN	HLVDEPQNLIK	653.361701	2	70.04	76.04
sp|P02769|ALBU_BOVIN	YLYEIAR	464.25036	2	72.02	78.02
sp|P0A8G3|UXAC_ECOLI	LAGETLSEHEVAQFK	829.922851	2	72.45	78.45
sp|P22255|CYSQ_ECOLI	DARPPLVVISR	611.866954	2	73.06	79.06
sp|P76049|YCJY_ECOLI	AQYPTAPGYATLR	704.864608	2	73.48	79.48
sp|P0ACT6|UIDR_ECOLI	GEISPDINTAMTSR	746.35903	2	73.71	79.71
sp|P0A853|TNAA_ECOLI	SYYALAESVK	565.790046	2	75.33	81.33
sp|P0AGG8|TLDD_ECOLI	EGQSLPVGVGQPTLK	755.417205	2	75.48	81.48
sp|P0A6F5|CH60_ECOLI	GVNVLADAVK	493.287474	2	76.72	82.72
sp|P0CE48|EFTU2_ECOLI	AGENVGVLLR	514.298373	2	77	83
sp|P0A853|TNAA_ECOLI	YADMLAMSAK	550.759377	2	77.21	83.21
sp|P0A9D2|GSTA_ECOLI	QLLAPVNSISR	599.351137	2	77.79	83.79
sp|P0A9D2|GSTA_ECOLI	MAERPEVQDALSAEGLK	615.31213	3	79.17	85.17
sp|P0ABT2|DPS_ECOLI	SYPLDIHNVQDHLK	839.931011	2	79.58	85.58
sp|P32132|TYPA_ECOLI	INIVDTPGHADFGGEVER	963.471228	2	80.18	86.18
sp|P04036|DAPB_ECOLI	EGSSLLGSDAGELAGAGK	809.899573	2	81.09	87.09
sp|P0ABT2|DPS_ECOLI	DDDTADILTAASR	455.216044	3	82.37	88.37
sp|P0A8G3|UXAC_ECOLI	FADLQTALTK	554.305863	2	83.94	89.94
sp|P02769|ALBU_BOVIN	VPQVSTPTLVEVSR	756.42503	2	84.74	90.74
sp|P0ABT2|DPS_ECOLI	TALIDHLDTMAER	743.37194	2	85.47	91.47

sp|P0ABP8|DEOD_ECOLI	GMLGFTGTYK	537.76806	2	86.49	92.49
sp|P0AGG2|TESB_ECOLI	KPIIYDVETLR	673.887552	2	86.89	92.89
sp|P22255|CYSQ_ECOLI	ESFLNPGFR	533.769448	2	88.27	94.27
sp|P0A853|TNAA_ECOLI	GNFDLEGLER	575.280377	2	88.33	94.33
sp|P02769|ALBU_BOVIN	LVNELTEFAK	582.318971	2	89.39	95.39
sp|P0A9D2|GSTA_ECOLI	GFTPLFRPDTPEEYKPTVR	750.723299	3	89.75	95.75
sp|P0A9X9|CSPA_ECOLI	GFGFITPDDGSK	620.79586	2	89.97	95.97
sp|P0A6F5|CH60_ECOLI	AAVEEGVVAGGGVALIR	784.443754	2	91.7	97.7
sp|P0A6Y8|DNAK_ECOLI	QAVTNPQNTLFAIK	772.925197	2	93.94	99.94
sp|P0A6F5|CH60_ECOLI	DTTTIIDGVGEEAAIQGR	923.463069	2	94.45	100.45
sp|P09373|PFLB_ECOLI	VDDLAVDLVER	622.330067	2	95.34	101.34
sp|P45748|TSAC_ECOLI	AQFGAAFPVVPGETGGR	830.925728	2	95.51	101.51
sp|P32132|TYPA_ECOLI	ASGTDEAVVLVPPIR	762.42503	2	96.33	102.33
sp|P0AGG2|TESB_ECOLI	QVFGGQVVGQALYAAK	818.446297	2	96.78	102.78
sp|P0ABP8|DEOD_ECOLI	YIAETFLEDAR	664.330067	2	97.63	103.63
sp|P0A6Y8|DNAK_ECOLI	IINEPTAAALAYGLDK	830.451245	2	98.06	104.06
sp|P0ABT2|DPS_ECOLI	AVQLGGVALGTTQVINSK	878.5018	2	98.36	104.36
sp|P0A8G3|UXAC_ECOLI	LLGPDVGFDSINDRPMAEELSK	801.733535	3	98.5	104.5
sp|P0A6Y8|DNAK_ECOLI	TTPSIIAYTQDGETLVGQPAK	1095.568065	2	99.38	105.38
sp|P0AE37|ASTA_ECOLI	LILTAAQLDALK	635.39247	2	99.48	105.48
sp|P02769|ALBU_BOVIN	LGEYGFQNALIVR	740.401358	2	99.94	105.94
sp|P09373|PFLB_ECOLI	THNQGVFDVYTPDILR	937.973407	2	100.32	106.32
sp|P0A8G3|UXAC_ECOLI	AFNIEQATFNDYMAK	881.908887	2	100.47	106.47
sp|P77522|SUFB_ECOLI	EVEAAFEQLGVPVR	772.40938	2	100.92	106.92
sp|P02769|ALBU_BOVIN	DAIPENLPPLTADFAEDK	978.48347	2	101.12	107.12
sp|P0AGG8|TLDD_ECOLI	VQTLGAVEHSPLYTSVDPLQSMSR	872.442643	3	101.56	107.56
sp|P0C8J6|GATY_ECOLI	SVMIDASHLPFAQNISR	943.483085	2	102.4	108.4
sp|P22255|CYSQ_ECOLI	TLTPDVPVLSEEDPPGWEVR	1118.56024	2	102.82	108.82
sp|P0A6A8|ACP_ECOLI	ITTVQAAIDYINGHQA	857.941575	2	103.61	109.61
sp|P0C8J6|GATY_ECOLI	QWVNLPLVLHGASGLSTK	960.538717	2	103.83	109.83
sp|P22255|CYSQ_ECOLI	ADNSPVTAADIAAHTVIMDGLR	746.710953	3	103.92	109.92
sp|P09373|PFLB_ECOLI	DAIPTQSVLTITSNVVYGK	1003.543862	2	104.82	110.82
sp|P0A6N4|EFP_ECOLI	VPLFVQIGEVIK	671.410663	2	105.08	111.08
sp|P0A6Y8|DNAK_ECOLI	VALQDAGLSVSDIDDVILVGGQTR	814.434254	3	105.96	111.96
sp|P0A853|TNAA_ECOLI	YDIPVVMDSAR	633.313363	2	106.76	112.76
sp|P0A8G3|UXAC_ECOLI	WVEAGEAPADINLLGEMVK	1021.51679	2	106.81	112.81
sp|P02769|ALBU_BOVIN	HPYFYAPELLYYANK	944.967062	2	107.19	113.19
sp|P02769|ALBU_BOVIN	TVMENFVAFVDK	700.349945	2	107.31	113.31
sp|P45748|TSAC_ECOLI	DAIAAAIDVLNEER	750.388644	2	107.45	113.45
sp|P37773|MPL_ECOLI	TLILNNLEFDHADIFDDLK	749.382831	3	107.76	113.76
sp|P0AGG2|TESB_ECOLI	NLLTLLNLEK	585.858263	2	107.8	113.8

sp|P02769|ALBU_BOVIN	DAFLGSFLYEYSR	784.375006	2	107.9	113.9
sp|P0AC23|FOCA_ECOLI	ADNPFDLLLPAAMAK	793.915983	2	108.16	114.16
sp|P68066|GRCA_ECOLI	AANDDLLNSFWLLDSEK	975.975812	2	108.41	114.41
sp|P0A853|TNAA_ECOLI	GIEEVGPNNVPYIVATITSNSAGGQPVSLANLK	1103.915688	3	108.88	114.88
sp|P0CE48|EFTU2_ECOLI	TTDVTGTIELPEGVEMVMPGDNIK	849.416995	3	109.34	115.34
sp|P0CE48|EFTU2_ECOLI	ILELAGFLDSYIPEPER	981.514573	2	109.35	115.35

Table S-2, 96PACS automation steps

1	manual placement of samples (~25μg Proteins) in 96T format (hereafter plate A) and freshly prepare reagents : 100 mM dithiothreitol (DTT) stock 300 mM iodoacetamide (IAA) stock 100 mM triethylammonium bicarbonate (TEAB) stock 20 μg MS-grade trypsin (need to store in 4 °C) pure acetone (need to store in 4 °C) 2% ACN, 0.1% FA 60% ACN 100% ACN
2	add 2 μL 100 mM DTT stock to each well in plate A.
3	place plate A on Te-shaker, vortex for 10 s.
4	transfer plate A to incubator, incubate @RT for 30 min.
5	add 2 μL IAA stock to each well in plate A.
6	place plate A on Te-shaker vortex for 10 s.
7	transfer plate A to incubator, incubate in dark @RT for 30 min.
8	add 200 μL cold acetone to each well in plate A.
9	place plate A on Te-shaker, vortex for 10 s.
10	transfer plate A to cooler, incubate @ 4°C for 30 min.
11	*transfer the plate A to a microplate centrifuge, spin at 1,000xg for 30 min @4°C.
12	*transfer the plate A back to the workstation.
13	aspirate supernatant in plate A, using tips positioned at a pre-defined 1 mm above Z-max position, discard the supernatant.
14	add 200 μL cold acetone at speed of 50 μL/min to wash the pellet in plate A.
15	repeat wash step 13-14-13 one more time.
16	add 200 μL 100 mM TEAB to each well in plate A.
17	add 200 μL 100 mM TEAB to the 20 μg MS-grade trypsin, vortex for 30 s.
18	transfer 4 μL 0.1 μg/μL trypsin to each 96 well in plate A.
19	place plate A on Te-shaker, vortex for 1 min.
20	transfer plate A to incubator, for 14 hr trypsin digestion @37°C.
21	about 10 min before ending the digestion, manually place the OASIS HLB 96-μElution plate (Waters, plate B) to the positive pressure SPE module.
22	add 50 μL 100% ACN to each well in plate B, push it through.
23	add 50 μL 60% ACN to each well in plate B, push it through.

24 add 200 μL 2% ACN 0.1%FA to each well in plate B, push it through.
25 transfer all liquid from plate A to the plate B, push it through, collect eluent by the plate A.
26 repeat loading step 25 one more time.
27 add 200 μL 2% ACN 0.1%FA to each well in plate B, push it through.
28 add 50 μL 60% ACN to each well in plate B, push it through, collect eluent by plate C.
29 transfer the plate C to Nitrogen Evaporator to dry the sample.
30 add 20 μL 2% ACN 0.1%FA to each well in plate C.
31 transfer all liquid from plate C to sample loading vial (waters). Ready for LC-MSMS analysis.

* These two steps can be automated if the workstation is augmented with microplate centrifuge equipped with robot-accessible automated centrifuge loader.

Table S-3, 235 serum proteins quantified by 96PACS-LC-MSMS to develop potential CHB markers

Gene	Fold-Change	p-value	adjusted p-value*
TRFE	0.28	1.61E-09	4.98E-07
APOC4	-0.77	8.54E-09	2.65E-06
APOA1	0.27	1.29E-08	4E-06
THBG	0.42	1.76E-08	5.45E-06
A2MG	0.43	1.94E-08	6.01E-06
PLMN	-0.19	3.08E-07	9.54E-05
VTNC	-0.24	6.51E-07	0.000202
APOB	-0.42	8.68E-07	0.000269
APOM	-0.30	9.38E-07	0.000291
CD44	0.39	1.94E-06	0.000602
CO7	0.26	4.25E-05	0.013176
ANT3	0.26	7.03E-05	0.021782
CD14	0.26	7.71E-05	0.023895
CO8B	-0.21	0.000108	0.03336
CO4B	-0.27	0.000124	0.038476
ANGT	-0.28	0.000143	0.044269
APOA4	0.30	0.000147	0.045562
LUM	0.26	0.000173	0.053562
FINC	-0.21	0.000178	0.055225
CNDP1	-0.36	0.00091	0.28222
PROS	-0.16	0.000955	0.295987
PON1	-0.19	0.002179	0.675583
A1AT	0.14	0.002185	0.677407
FETUB	0.26	0.002465	0.764196
SAA4	-0.21	0.002622	0.812731
LYAM1	0.24	0.002673	0.828495
CBPB2	0.13	0.002935	0.909967
CO8A	-0.16	0.003118	0.966498

IGLL5	0.70	0.0404	1
QSOX1	0.09	0.064453	1
APOL1	-0.17	0.005993	1
CD5L	0.36	0.037261	1
FCN3	-0.07	0.513298	1
ATRN	0.00	0.957266	1
CERU	-0.01	0.856452	1
THRB	-0.11	0.004207	1
C1R	-0.02	0.642323	1
HPT	0.24	0.04729	1
HPTR	-0.21	0.09139	1
FA9	-0.05	0.307326	1
FA10	-0.16	0.007538	1
CFAD	-0.01	0.85878	1
FA12	-0.14	0.314059	1
CFAB	-0.16	0.019243	1
CAH1	0.13	0.437156	1
AACT	-0.01	0.844027	1
CO3	0.01	0.904075	1
CO5	-0.02	0.679249	1
KNG1	0.01	0.717574	1
IGF2	-0.10	0.121651	1
IGJ	0.37	0.080954	1
KV204	0.61	0.088923	1
KV305	0.85	0.040628	1
KV402	0.89	0.026533	1
HV303	1.08	0.017023	1
HV305	0.94	0.028221	1
HV315	0.69	0.03776	1
IGKC	0.92	0.034144	1
IGHG1	1.56	0.06781	1
IGHG3	1.87	0.097826	1
IGHG4	1.42	0.08385	1
IGHM	0.43	0.017638	1
IGHA1	0.68	0.110993	1
HBD	0.24	0.173503	1
APOE	0.02	0.855241	1
APOA2	0.08	0.288087	1
APOC1	-0.27	0.006605	1
APOC2	-0.29	0.010783	1
APOC3	-0.28	0.007878	1
FIBA	-0.21	0.026201	1
SAMP	0.08	0.512137	1

C1QA	0.16	0.008228	1
C1QB	-0.01	0.804683	1
C1QC	0.06	0.186654	1
CO9	0.12	0.147313	1
APOH	0.12	0.019438	1
A2GL	-0.04	0.625417	1
RET4	-0.13	0.246924	1
AMBP	0.00	0.920522	1
A1AG1	-0.05	0.524112	1
FETUA	0.13	0.038508	1
TTHY	0.05	0.597529	1
ALBU	0.36	0.414745	1
VTDB	0.05	0.174263	1
CXCL7	0.00	0.977908	1
PLF4	-0.25	0.031187	1
HEMO	0.01	0.834008	1
KLKB1	0.03	0.553379	1
C4BPA	-0.11	0.038037	1
PROC	-0.15	0.008919	1
LCAT	-0.01	0.856263	1
HRG	-0.21	0.00993	1
LV106	0.32	0.294764	1
A1BG	0.01	0.74022	1
MUCB	0.41	0.021481	1
VWF	0.07	0.521379	1
SHBG	0.21	0.155255	1
KV309	1.16	0.046898	1
APOD	0.02	0.854415	1
IC1	0.06	0.651475	1
CFAI	-0.07	0.167105	1
F13B	0.03	0.487447	1
TETN	0.05	0.473937	1
HEP2	-0.04	0.616093	1
CHLE	0.28	0.003588	1
GELS	0.15	0.011914	1
CO2	-0.05	0.217942	1
GP1BA	0.07	0.594516	1
CO8G	-0.11	0.078883	1
TSP1	-0.23	0.018591	1
CBG	0.04	0.275045	1
CFAH	-0.02	0.724963	1
A2AP	0.03	0.390547	1
SPRC	-0.13	0.104797	1

C1S	-0.06	0.281743	1
CO4A	-0.19	0.125863	1
LAC2	0.73	0.022964	1
SAA1	-0.36	0.218279	1
CLUS	-0.03	0.373266	1
CETP	0.30	0.009898	1
FA5	-0.03	0.509055	1
CO6	0.12	0.009839	1
CBPN	0.02	0.698997	1
IBP3	-0.12	0.1148	1
LBP	-0.23	0.004917	1
A1AG2	-0.18	0.022008	1
ITIH2	-0.03	0.407782	1
ITIH1	-0.09	0.018294	1
C4BPB	0.06	0.466204	1
GPX3	0.18	0.012027	1
CPN2	-0.08	0.135306	1
FBLN1	0.14	0.105661	1
ZA2G	0.11	0.081505	1
HGFL	-0.08	0.067938	1
PROP	-0.02	0.812745	1
KAIN	-0.05	0.294781	1
CADH5	0.23	0.076972	1
ALS	0.03	0.686579	1
PEDF	0.01	0.839213	1
FHR2	-0.16	0.454486	1
BTD	-0.03	0.629806	1
AFAM	-0.04	0.701145	1
MASP1	-0.07	0.287584	1
COMP	0.04	0.761679	1
SEPP1	-0.10	0.165811	1
PLTP	-0.02	0.799264	1
LYSC	0.08	0.333484	1
B2MG	0.06	0.570531	1
ACTG	0.19	0.07693	1
HBB	0.29	0.066908	1
HBA	0.20	0.191329	1
PHLD	-0.04	0.503859	1
SPTB2	-0.14	0.273852	1
FHR1	-0.06	0.480393	1
HGFA	-0.18	0.040432	1
ITIH3	-0.01	0.950118	1
LG3BP	0.16	0.097569	1

FBLN3	0.01	0.890536	1
HABP2	-0.05	0.2423	1
ITIH4	-0.03	0.5392	1
FCN2	0.26	0.063623	1
BGH3	0.05	0.456683	1
ECM1	0.13	0.061507	1
VASN	0.19	0.079975	1
PI16	0.19	0.185342	1
TITIN	0.08	0.511289	1
GGH	0.07	0.55206	1
PRG4	-0.30	0.015076	1
PGRP2	0.00	0.989013	1
COL11	0.01	0.906697	1
C1RL	0.05	0.304718	1
PCYOX	-0.03	0.723463	1
ZPI	0.05	0.452945	1
FA11	-0.37	0.000396	0.122835
APOF	-0.42	0.001253	0.388381
IGHA2	-0.75	0.191707	1
TFR1	0.14	0.324542	1
CTR1	0.42	0.049086	1
CRIS3	0.06	0.603278	1
ACTS	-0.06	0.691385	1
SPP24	0.19	0.241959	1
ADIPO	0.01	0.95135	1
LYVE1	0.19	0.173696	1
KV101	1.19	0.016317	1
KV303	0.37	0.297108	1
IGHG2	0.90	0.246355	1
LAMP2	-0.12	0.26229	1
AMPN	-0.13	0.287727	1
GPV	-0.21	0.170323	1
PTGDS	0.13	0.269689	1
CP046	0.29	0.111846	1
PON3	0.65	0.000366	0.113517
MASP2	-0.28	0.025189	1
CYTC	0.28	0.011984	1
KV401	0.53	0.007606	1
APOA	-1.44	0.028464	1
FCG3B	0.94	0.000373	0.115637
LV301	0.62	0.248645	1
PVR	-0.27	0.071086	1
PZP	-0.09	0.821887	1

MDN1	0.28	0.147721	1
PRDX2	0.78	0.001757	0.5448
HV102	1.75	0.014742	1
APMAP	-0.01	0.96469	1
PLSL	0.25	0.112136	1
PROZ	-0.20	0.416518	1
IGF1	0.35	0.054572	1
MBL2	0.17	0.516462	1
MA1A1	-0.32	0.035264	1
CAMP	-0.08	0.675928	1
DEF3	0.12	0.558983	1
K1C10	2.94	0.190449	1
CRAC1	0.09	0.641383	1
EPCR	0.18	0.322334	1
HV311	0.69	0.094721	1
ANGI	0.48	0.015976	1
CAD13	0.56	0.006831	1
KV106	0.99	0.069145	1
TYB4	0.65	0.125354	1
LV302	0.65	0.156887	1
ICAM2	0.83	0.00054	0.167509
FHR3	0.34	0.128173	1
PCSK9	0.11	0.633078	1
DOPO	0.18	0.605821	1
K1C9	2.86	0.094047	1
SRGN	-0.42	0.071428	1
CRP	-1.52	0.010987	1
K2C1	2.70	0.113046	1
LDHB	0.14	0.542136	1
TRI22	-0.53	0.072641	1
HV103	0.39	0.290255	1
LV403	0.94	0.101535	1
K22E	3.48	0.195044	1
MENT	-0.14	0.606064	1
MMRN2	0.22	0.384747	1

* Bonferroni adjusted p-value for multiple comparison correction.

Supplemental Methods and Results

Automated sample preparation. The automation setup was built on a Freedom Evo 200 platform (Tecan, Switzerland) equipped with a microplate robotic arm and two 8-Channel liquid handling arms, one for sample transfer with disposable tips, one with steel needles for large volume solvent transfer and SPE operations. The workbench was also equipped with several on-deck peripheral devices including a plate shaker, a cooling block, an incubator with temperature control, a 96-well based SPE system and a N_2 evaporator as detailed in Figure S-1. Liquid handling parameters (aspiration and dispensing rates, air gap volumes, etc.) were calibrated for water, serum, pure acetonitrile, pure cold acetone, and 60% acetonitrile in water. EVOware® was used for the main operating interface. The whole shotgun proteomics workflow design was detailed in Table S-2**.**

LC-MSMS experiment for CHB biomarker discovery. Peptides processed in parallel from individual serum samples by automated 96PACS protocol were dissolved in 0.1% FA and directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific). Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific). The gradient was comprised of an increase from 4% to 20% solvent B (0.1% FA in 98% ACN) over 45 min, 20% to 35% in 8 min and climbing to 80% in 4 min then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system, The resulting peptides were analyzed by Q-Exactive™ hybrid Quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific). Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using NCE setting as 28; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 1E4 in the MS survey scan with 30.0 s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control was used to prevent overfilling of the Orbitrap; 5E4 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1,800 Th.

Analysis of proteins lost during the 96PACS sample processing. To investigate if any protein groups were difficult to be precipitated using the 96PACS protocol with short acetone incubation time and low speed centrifugation, we collected the supernatant of 96PACS processed *E.coli* lysate containing 200 µg proteins, and further incubated in acetone on ice overnight and centrifuged at 14,000 xg for 30 min. The resulting residual was resuspended in 50 µL 50 mM TEAB and digested by 1µg trypsin for 14 h at °C. Peptides were then desalted by house-made C18 stagetips. LC-MSMS and

data analysis were proceeded as described in the experimental section. We compared the identified protein list from this experiment to the list from 6 96PACS *E.coli* datasets described in the main text. To avoid spurious IDs, proteins with spectral counts ≥3 were compared.

A total of 737 proteins were detected in the supernatant fraction, of which 550 have spectral counts ≥3. Compared to the pellet fractions, only three proteins (P0AFU4, Transcriptional regulatory protein GlrR, 8 spectral counts; P76004, Uncharacterized protein YcgM, and P40191, pyridoxamine kinase, both 3 spectral counts) were found in the acetone supernatant but not in the pellet. Therefore, we concluded the bias selection of 96PACS method is very limited.