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Efficiencies gained in targeted serum proteomics using NRicher™ Beads – simplified and diversified workflows for sub-proteome and biomarker enrichment

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Introduction

The need for new biomarkers to support personalized healthcare, has fostered numerous proteomic innovations. Still, a number of challenges remain. One is the preponderance of high abundance proteins and, concurrently in targeted proteomic workflows, efficiency and consistency in quantifying target peptides from different sample cohorts. This is in part due to the changing landscape of proteins/peptides not associated with the selected targets. A solution for both these challenges is now available through a suite of products called **NRicher™**. This bead-based technology is derived from experience of over 10 years at the forefront of manufacturing beads (i.e., ionic, hydrophobic, hydrogen bonding, aromatic, polymeric) with differential proteome binding properties[1,2]. Several commercial products based on singular bead surface features have been derived, notably **AlbuVoid™** and **HemoVoid™**[3,4]. Like these products, **NRicher™** now offers a solution that stands out in its simplicity and versatility. **NRicher™** consists of consumable chemically derived porous beads, and an adaptability to bead cocktails, even with seemingly incompatible surface features; an important distinction of porous, over non-porous magnetic beads. **Nricher™** products do not require any specialized instruments, can be processed using a standard microfuge, with protocols suitable for automated liquid handlers.

Methods

We have previously reported that **NRicher™** significantly reduces the influence of high abundance proteins like Albumin and Hemoglobin, and can enrich certain families of proteins 2-30X compared to neat (not enriched) serum[5].
We now report on improvements in targeted proteome workflows by using a series of differentiated beads, called **NRicher™**, with derivative fit for purpose products:

NRicher™ C: Enrichment Complement & Related Proteins

NRicher™ Ig: Immunoglobulin Enrichment

NRicher™ APO: Enrichment of Apolipoproteins

NRicher™ Mx: Low Abundance Enrichment >Soluble Membrane Proteins

A brief review of the **NRicher™** sample prep protocol. Each **NRicher™** bead is processed the same, and follows a bind/wash protocol using a spin-filter format. Reduction, alkylation and overnight digestion is performed on the bead-bound proteome, without the use of detergents, according to protocols developed from previous products[6,7,8]. Results reported here, are based on processing 25 µl of pooled normal human serum. The samples were analyzed at the Rutgers Center for Integrative Proteomics using nano-LC-MS/MS, DIA and targeted proteomics.

The NRicher™ platform was designed to help investigate outside the Venn Diagram box.

Not derived from immuno-affinity:
NRicher™ beads, are not species-specific. This allows a wider applicability across various sample types.

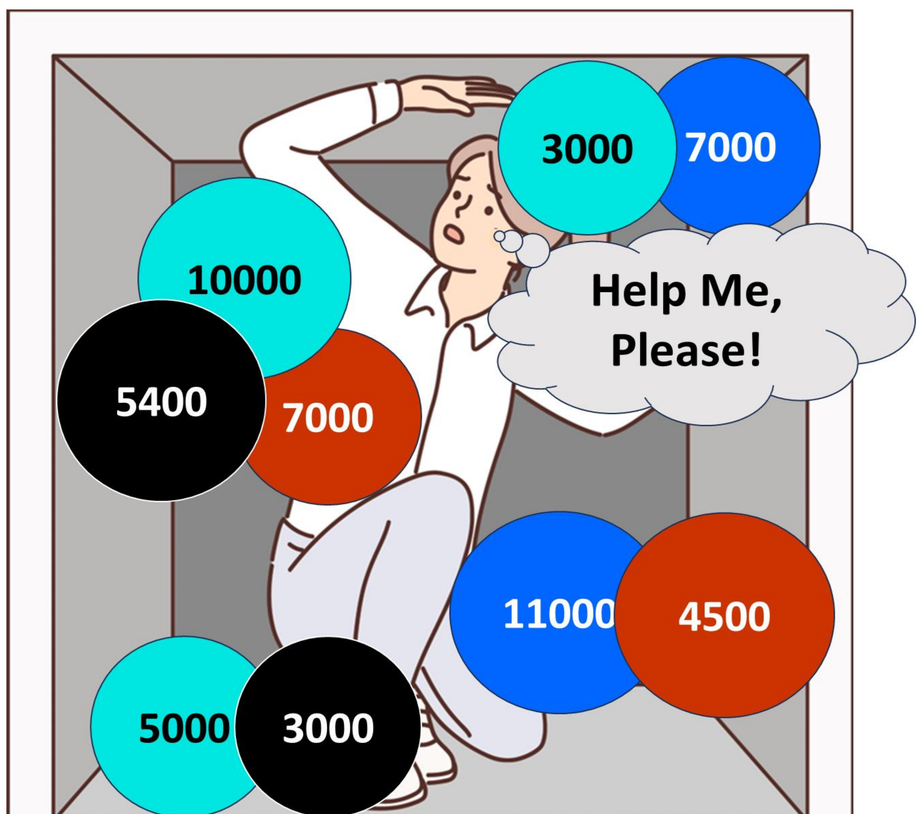
Streamlined Analysis:
Through the use of bead cocktails, NRicher™ products serve virtually all applications, starting with sample volumes as low as 25 µl.

Cost-efficient:
There's no need for an investment in high-end specialized equipment; a standard laboratory microfuge will suffice.

On-bead digestion:
BSG pioneered Bead-Assisted Sample Prep (BASP™), offering workflow efficiencies (i.e., no added denaturants) for LC-MS proteomics of bead enriched sub-proteomes.

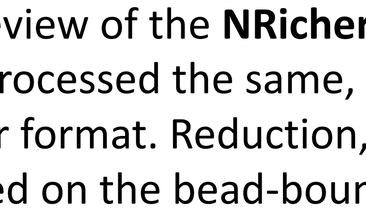
Knowledgebase of 2000+ Serum Proteins
Supports targeted and quantitative protein markers from serum/plasma

Serves All Proteomic Analytical Platforms:
Maintains the functional activity of proteins post-separation. This makes it ideal for orthogonal analyses, be it functional, enzymatic, or immunoassay-based.

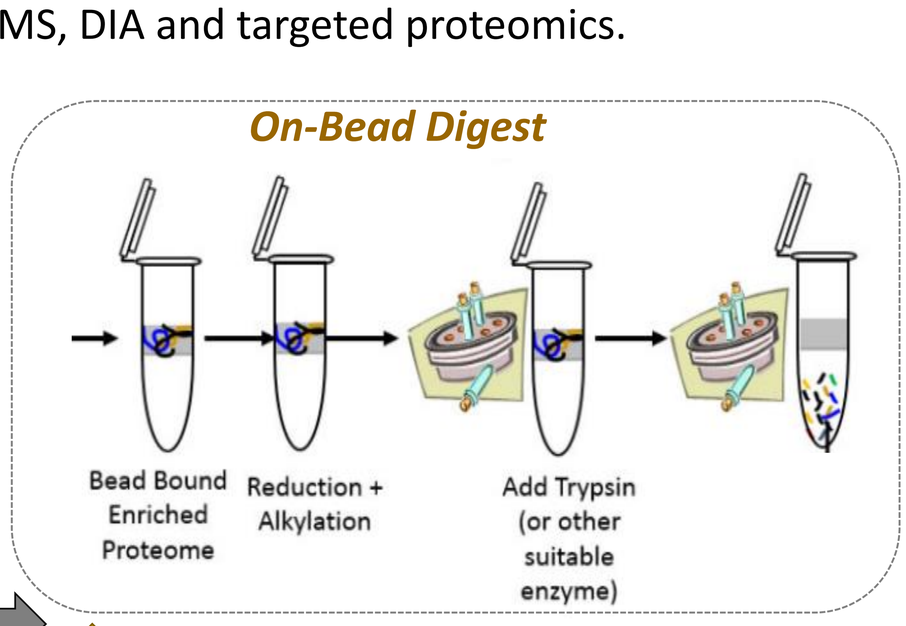


Nricher™ workflow

High abundance (i.e., Albumin) proteins selectively pass

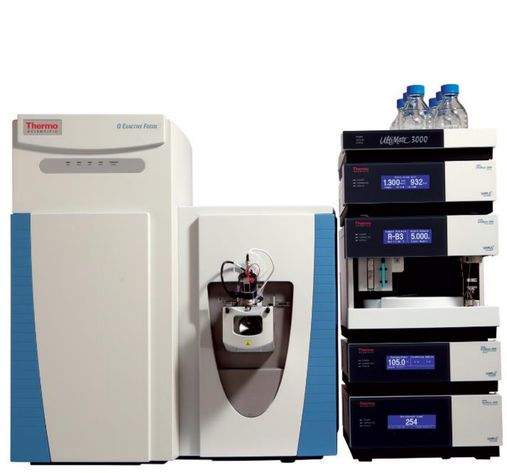


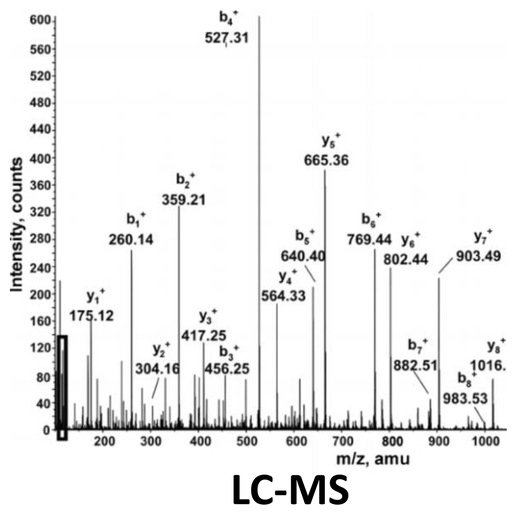
or void through the beads, concentrating and enriching sub-proteomes on the beads



Proteomic Analytical Options

Eluates from **NRicher™** beads can be applied to other common analyses:
- Enzymatic/Functional assays
- 2DE
- ELISA/immunoassay





LC-MS

A Partial List of the >100 Biomarkers Annotated in NRicher™ Knowledgebase

Gene Name	Uniprot Identifier	Gene Description	Optimal Product	Potential biofluid biomarkers for:	References
PARK7	Q99497	Parkinson disease protein 7, (also called DJ-1)	NRicher D	Neurodegenerative, Parkinsons	Potential Biomarker of the Earliest Clinical Stages of Parkinson's Disease
SNCA	P37840	Alpha-synuclein	NRicher E	Neurodegenerative, Parkinsons	Chang, Chun-Wei, et al. "Plasma and serum alpha-synuclein as a biomarker of diagnosis in patients with Parkinson's disease." Frontiers in neurology 10 (2020): 1388.
PRTN3	P24158	Myeloblastin (aka Leukocyte proteinase 3)	AlbuVoid	Oncology, Covid-19, Sepsis, COPD	Neutrophil elastase, proteinase 3, and cathepsin G are three hematopoietic serine proteases stored in large quantities in neutrophil cytoplasmic azurophilic granules. These proteases are also released during neutrophil activation at inflammatory sites. As multifunctional proteases, they also play a regulatory role in non-infectious inflammatory diseases.
CTSG	P08311	Cathepsin G	NRicher E	Autoimmune, Covid-19, Sepsis, COPD	
ELANE	P08246	Neutrophil elastase	NRicher MX5, NRicher C	Covid-19, Sepsis, Oncology, COPD	
CDH1	P12830	Cadherin-1 (E-cadherin)	NRicher Mx	Oncology, COPD, Asthma	In tumors, epithelial–mesenchymal transition (EMT) describes the transformation of tumor cells from a non-motile epithelial phenotype to a migratory mesenchymal phenotype. A common characteristic of EMT is the loss of epithelial calmodulin (E-cadherin) expression, which is accompanied by the upregulation of N-cadherin expression; a so-called "cadherin switch". This change in cadherin expression increases the ability of tumor cells to invade and metastasize to distant sites, predicating poor prognosis. In comparison with normal epithelia, cancer cells almost invariably show diminished cadherin-mediated intercellular adhesion. Proteolytic shedding of the extracellular domain results in soluble cadherins, circulating in blood. Elevated levels of sCDH1, sCDH2 and other cadherins have been detected in cancer patient serum when compared with healthy persons. Therefore, soluble cadherins might serve as important biomarkers for cancer metastasis and prognosis.
CDH2	P19022	Cadherin-2 (aka neural cadherin or N-cadherin)	NRicher Mx	Oncology	
FAP	Q12884	Prolyl endopeptidase FAP	NRicher Mx	Oncology	FAP, LRG1, LBP and COMP can effectively predict response to anti-PD-1/PD-L1 in TNBC and NSCLC patients
LBP	P18428	Lipopolysaccharide-binding protein	NRicher Mx, NRicher D	Oncology	
LRG1	P02750	Leucine-rich alpha-2-glycoprotein	NRicher E, NRicher H	Oncology	
COMP	P49747	Cartilage oligomeric matrix protein	NRicher D	Oncology, Long Covid	
CD163	Q86VB7	Scavenger receptor cysteine-rich type 1 protein M130	NRicher H, NRicher Mx	Oncology, COPD, Covid-19, Sepsis, Liver Disease	CD163, a hemoglobin-haptoglobin scavenger receptor, is exclusively expressed by monocytes and macrophages whose main biological function is the elimination of hemoglobin-haptoglobin complexes during hemolysis. The mannose receptor (CD206) is primarily expressed by M2-like macrophages, dendritic cells and endothelial cells. Both CD163 and Mannose Receptor exist as soluble serum proteins (sCD163 and sMR/sCD206) that may reflect the M2-like state of tissue macrophages, including Tumor-associated macrophages (TAMs).
MRC1 (aka CD206)	P22897	Macrophage mannose receptor 1 (aka mannose receptor)	NRicher E	Sepsis, Liver Disease, Oncology	
MCAM	P43121	Cell surface glycoprotein MUC18 (aka CD146)	AlbuVoid	Oncology	The plasma concentration of soluble CD146 (sCD146) is modulated in inflammatory diseases associated with endothelial alterations, including inflammatory bowel disease, chronic renal failure, chronic liver disease and cancer. The transmembrane CD146 glycoprotein is expressed by a variety of cancer cell types and is correlated with poor cancer prognosis and tumor aggressiveness. Soluble CD146, was found to induce epithelial–mesenchymal transition (EMT), cancer stem cell generation, and tissue factor expression in cancer cells.

Comparison of NRicher™ Apo vs. Neat (untreated) pooled human serum
Multiplex Targeted Peptides Demonstrate Substantially Improved Spectral Signals After Enrichment
A Selection of Peptide Levels with Example Variations That Represent Common Functional Level Reporting Features

Protein	First Position	Peptide	Last Position	NRicher/Neat Total Signal Peak Area
APOA1	86	EQLGPVTQEFWDNLEK	101	0.2
	189	ELFHPYAESLVSGIGR	204	3.6
	231	AKPALEDLR	239	2.9
APOA2	54	SPELQAEAK	62	13.3
	70	EQLTPLIK	77	71.0
APOA4	267	LAPLAEDVR	275	2.1
APOA4 canon	144	TQV**S**TQAEQLR	144	5.5
APOA4 variant	144	TQV**N**TQAEQLR	154	11.7
APOA5	189	ELFHPYAESLVSGIGR	204	1.9
	290	RQDTYLQIAAFTR	301	3.6
APOB	2898	LDFSSQADLR	2907	4.2
APOB canon	94	TSQC**T**LK	100	7.1
	617	E**A**LK ESQLPTVMDFR	631	0.4
	2310	IND**I**LEHVK	2318	0.1
APOB variant	94	TSQC**I**LK	100	2.9
	617	E**V**LK ESQLPTVMDFR	631	0.7
	2310	IND**V**LEHVK	2318	0.2
APOB-100	779	ILGEELGFASLHDLQLLGK	797	3.0
APOB-48 cleaved	2171	LSQLQTYMI	2179	17.6
APOB-48 spanning region	2171	LSQLQTYMI QFDQYIK	2186	0.8
APOC1	57	QSELSAK	63	13.7
APOC1 non-truncated	27	**TP** DVSSALDKEFGNTLEDK	47	
APOC1 truncated	29	DVSSALDKLKEFGNTLEDK	47	16.7
APOC2	78	STAAMSTYTGIFTDQVLSVLK	98	214.0
APOC2 non-truncated	23	**TQQPQQ** DEMPSPTFLTQVK	41	58.6
APOC2 truncated	29	DEMPSPTFLTQVK	41	14.2
APOC3	20	ASEAEDASLLSFMQGYMK	37	0.3
APOC4	67	DGWQWFWSPSTFR	79	25.5
APOC4 canon	92	DLGP**L**TK	98	59.6
APOC4 variant	92	DLGP**R**TK	98	1.7
APOD	162	NPNLPPETVDSLK	164	8.4
APOE	80	ALMDETMK	87	0.4
	281	SWFEPLVEDMQR	292	2.6
APOE2 canon	176	**R**LAVYQAGAR	185	4.3
APOE2 variant	176	**C**LAVYQAGAR	185	23.5
APOE4 canon	122	LGADMEDV**C**GR	132	18.2
APOE4 variant	122	LGADMEDV**R**GR	132	3.2
APOF	114	QGGVNATQVLIQHLR	128	9.7
	251	SGVQQLIQYYQDQK	264	40.1
APOL1	306	VTEPISAESGEQVER	320	51.6
APOL1 canon	147	LKS**E**LEDNIR	156	10.2
	213	ELGITAALTGITSST**M**DYGK	233	0.1
APOL1 variant	149	S**K**LEDNIR	156	3.4
	213	ELGITAALTGITSST**I**DYGK	233	0.0
APOM	58	EELATFDPVDNIVFNMAAGSAPMQLHLR	85	7.8
LPA (apo(a)) canon	1398	G**T**LSTTITGR	1407	3.7
LPA (apo(a)) variant	1398	G**P**LSTTITGR	1407	0.4
LPA (apo(a)) canon	1667	NPDADTGPWCFT**M**DPSIR	1684	
LPA (apo(a)) variant	1667	NPDADTGPWCFT**T**DPSIR	1684	6.8

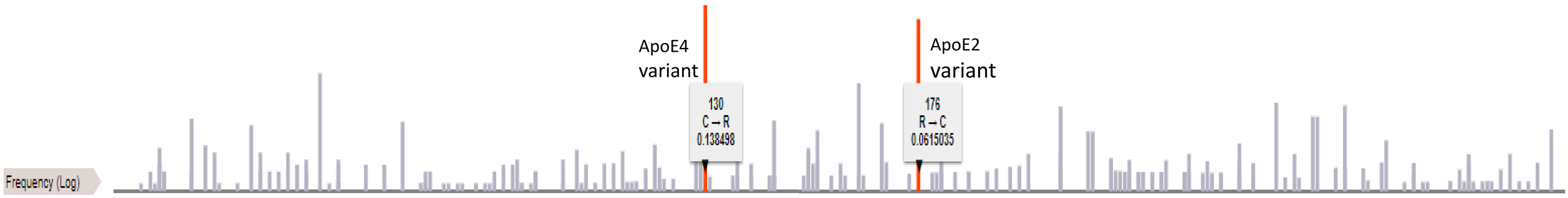
Highly signal enriched >4X

Signal Enriched 1-4X

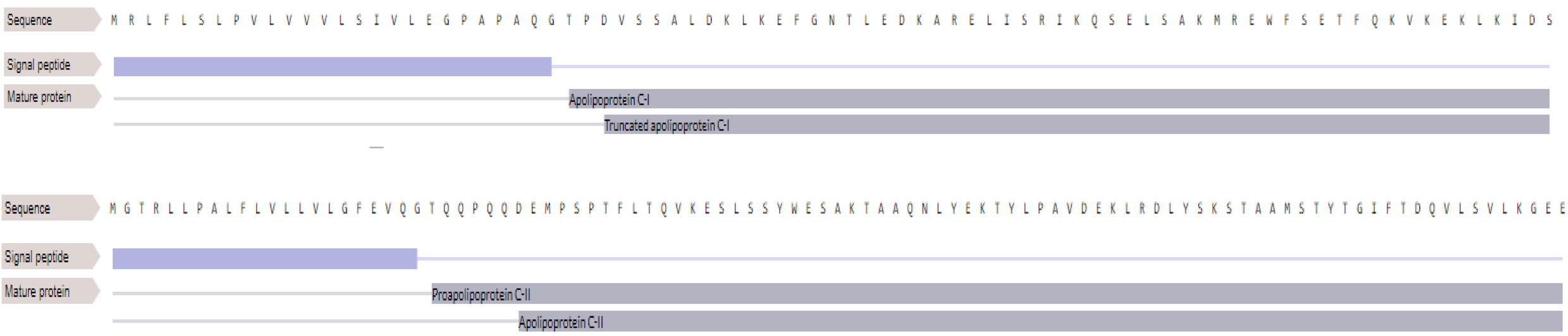
Highly signal depleted >4X

Signal depleted 1-4X

Not observed



ApoE is associated noncovalently with lipids as part of lipoprotein complexes. Single residue gene level variant substitutions alter interactions with specific lipoprotein sub-populations. ApoE4 is widely recognized as the major genetic risk factor for AD, while ApoE2 may be protective.



Both apoC-I and C-II appear in the circulation predominantly as full length native proteins along with minor truncated proteoforms. Ratios of these proteoforms are under investigation in atherosclerosis and coronary heart disease (CHD).

Results and Discussion

We demonstrate that it is desirable to raise lower level peptide peak area signals to those typically observed for mid-abundance proteins, a purpose fulfilled by **NRicher™** in a simple and efficient way. Notwithstanding those efficiencies, as demonstrated here using Apolipoprotein enrichment as a representative example, investigators are not limited to just measurements of tryptic peptides that are highly observable. Now through proper selection of **NRicher™ beads,** multiplex peptide targets from specific functional regions, such as spanning regions of proteolysis, truncations, amino acid variants or other PTMs can be explored in depth. This extends research beyond strict abundance level quantitation, to more discretionary functional levels. For this, **NRicher™- derived sub-proteomes** ultimately generate better peptide signal to noise levels, with reduced acquisition time and enhanced productivity, compared to neat (not enriched) serum or plasma.

Similarly, enrichment of specific disease associated biomarkers can be selected from the publicly accessible **NRicher™ Knowledgebase of >2000 serum proteins**, most of which are not observable without enrichment. A selection of one or more **NRicher™** bead(s) and/or our depletion products can be investigated for enrichment of specific protein targets of interest. Another advantaged feature is that Trypsin digestion can be done while the enriched proteome remains on the bead ('on-bead digestion'), without detergents (i.e., SDS), achieving a seamless workflow to LC-MS analysis. Consequently. the simplified **NRicher™** workflows help to minimize technical variance or bias in the data, which can arise from LC co-eluting peptides that both suppress ion signals, and interfere with spectral identifier assignments. If necessary, further biomarker level enrichment can be achieved through optimized separations (buffers, load, etc.) at the protein level, and at the peptide level through gradient optimization. Finally, with an optional elution from the beads, the versatility of **NRicher™** can also be exploited in orthogonal analyses, such as enzymatic/functional assays, 2DE, or ELISA/immunoassay for further validation.

Highlights

After **NRicher™**, target peptides have **highly enhanced spectral signal,** even as gradient times are reduced.

NRicher™ sub-proteome enrichment can minimize acquisition time, collectively **improving overall throughput, cost, and productivity.**

Specific target peptides that report functional PTMs and amino acid variant regions **promise insights and potential multiplex biomarkers for disease**

References

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