NRicher™: Family Specific Enrichment For Targeted Proteomics

Haiyan Zheng[1]; Swapan Roy[2]; Amenah Soherwardy[1]; Matthew Kuruc[2]
[1]Rutgers Center for Integrative Proteomics, Piscataway, NJ; [2]Biotech Support Group LLC, Monmouth Junction, NJ
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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 adaptability to 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:

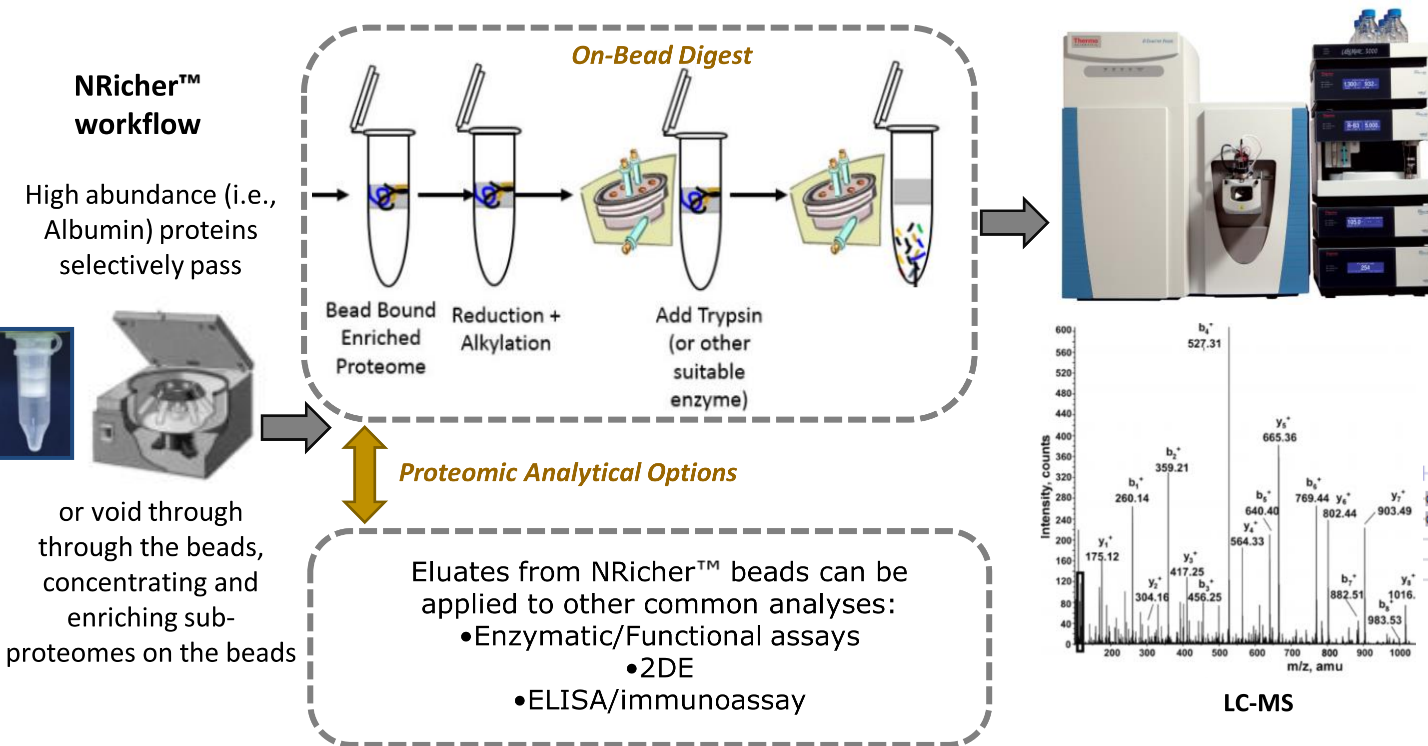
NRicher™ C: Enrichment Complement & Related Proteins

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-type 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 and 50 µl for Apolipoproteins and Complement respectively, from pooled normal human serum. The samples were analyzed at the Rutgers Center for Integrative Proteomics using nano-LC-MS/MS and DIA for recovery tests, and targeted proteomics for specific peptide enrichment tests.



Complement and Related Sub-Proteome Enriched and Recovered Using NRicher™ C

Gene Name	Bead-Bound/ Total	Flow-through/ Total	Recovery %	Bead Enr Factor
C3	1.1E+09	1.1E+08	91%	5.4
C4A	1.1E+08	1.1E+07	91%	1.9
CFH	1.3E+08	7.7E+06	95%	4.9
CFB	2.7E+08	1.4E+07	95%	4.2
C4BPA	4.8E+07	1.5E+07	76%	0.8
C6	2.1E+07	1.4E+06	94%	2.7
C5	1.8E+07	7.9E+06	69%	0.7
C9	2.4E+07	6.0E+06	80%	1.0
C1S	1.5E+07	2.1E+07	41%	1.1
C1R	8.7E+06	1.2E+07	41%	1.2
C1QC	1.7E+07	6.1E+06	74%	2.5
C8A	1.4E+07	1.9E+06	88%	1.5
C8B	1.3E+07	2.4E+06	85%	1.7
C7	2.3E+07	3.7E+06	86%	1.5
C1QB	1.2E+07	4.2E+06	75%	0.9
CFI	3.2E+07	5.6E+06	85%	2.9
C2	4.4E+06	1.4E+07	24%	1.2
CFP (Properdin)	1.6E+07	3.0E+05	98%	5.4
C4BPB	8.4E+06	2.4E+05	97%	5.9
C1RL	6.9E+05	1.1E+06	39%	0.5
C1QA	8.8E+06	4.3E+06	67%	1.3
CFHR5	1.6E+06	nd	100%	4.1
CFHR2	1.0E+07	nd	100%	8.4
CFD	2.1E+06	nd	100%	6.5
CFHR1	3.4E+05	nd	100%	∞
CFHR4	9.4E+06	nd	100%	∞
MASP1	2.4E+06	nd	100%	1.0
MASP2	7.9E+05	1.6E+06	33%	0.8
CLU	4.1E+05	6.5E+05	38%	5.0
VTN	5.0E+07	8.5E+06	85%	0.7
Total Complement	3.8E+07	9.0E+06	81%	3.3

References
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Apolipoprotein Sub-Proteome Enriched and Recovered Using NRicher™ Apo

Gene Name	Bead-Bound/ Total	Flow-through/ Total	Recovery %	Bead Enr Factor
APOA1	2.3E+09	2.6E+07	99%	82
APOB	1.5E+08	3.6E+07	87%	8
APOA2	6.1E+08	1.8E+06	100%	358
APOA4	1.5E+08	3.1E+06	99%	42
APOE	8.9E+07	1.0E+06	99%	88
APOC1	5.8E+07	8.4E+05	99%	27
APOC3	7.3E+07	3.5E+06	97%	83
APOC2	7.9E+07	2.8E+05	100%	349
APOD	6.4E+07	3.4E+06	97%	39
APOL1	7.8E+07	1.5E+06	99%	94
APOF	1.6E+07	8.0E+04	100%	318
APOM	9.4E+07	6.5E+05	100%	106
APOC4	5.1E+06		100%	∞
APOA5	2.9E+05	3.5E+04	93%	∞
LPA (apo(a))	7.9E+06	1.5E+06	90%	10
Total Apo	3.8E+09	8.0E+07	99%	73

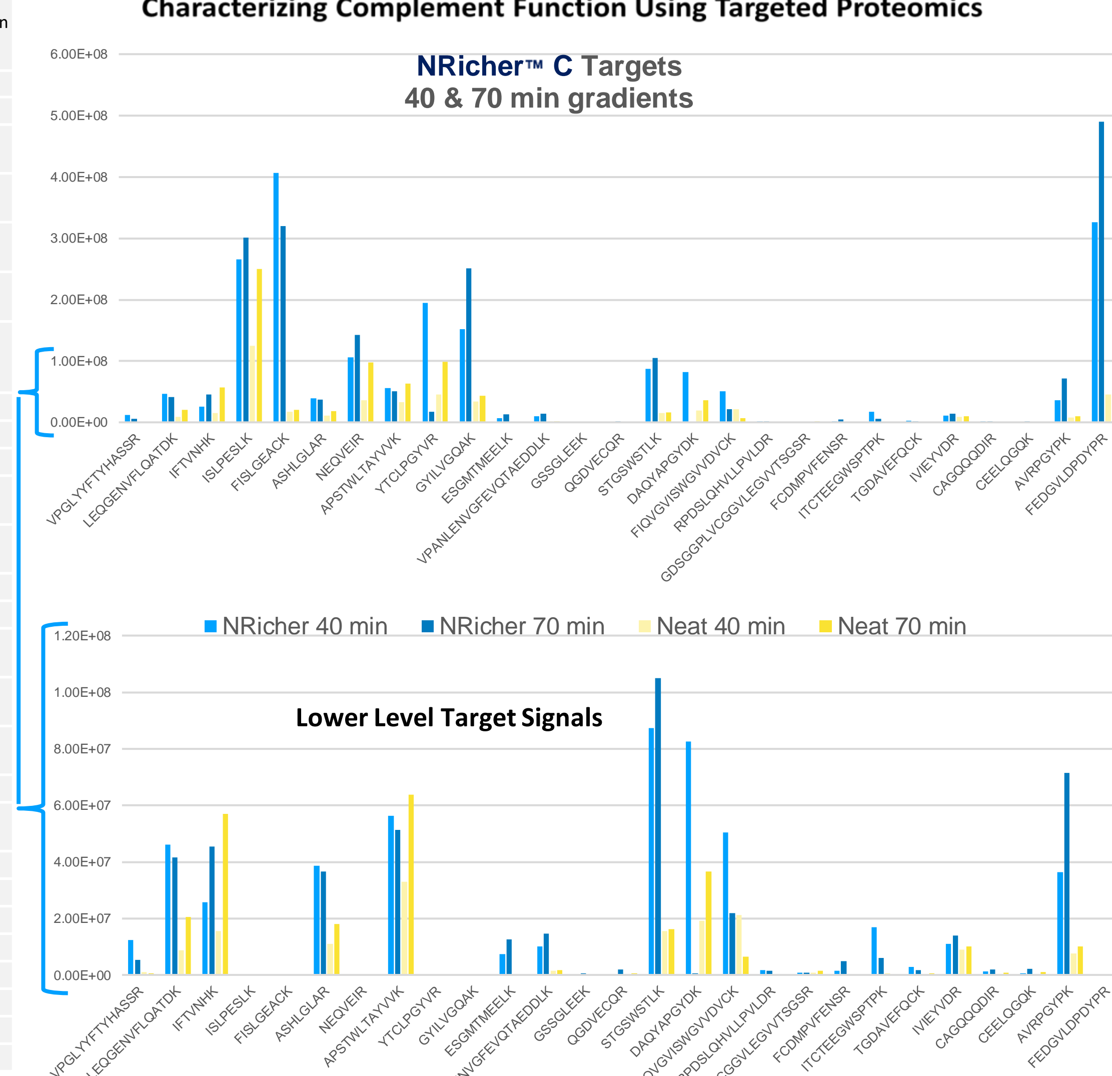
Recovery % is a metric to report how quantitative the beads are in capturing the gene-specific protein. With few exceptions (C1,C2, MASP), most are 80 to 90+% indicating excellent quantitative recovery. For targeted quantitation, high and consistent recovery is most desirable. The Bead Enrichment Factor relates the signal for the observed protein relative to the total signal from the Top 10 proteins in the prep, relative to the same in neat (untreated) serum; the top 10 being adjusted accordingly.

The calculation is:

((Gene-specific Bead-Bound Signal)/(Prep Top 10 Total Signal))/((Gene-specific Neat Signal)/(Neat 10 Top Total Signal)). Bead-Bound Signals in the the Top 10 (i.e, C3, APOA1) are removed. This factor serves as a metric to how well enrichment reduces the signal influence of the remaining top 10 most abundant proteins. Most of the relatively lower abundance Complement regulating proteins (CFH,CFB,CFP,CFHRs) have enrichment factors of 4-8X. nd - not detected

∞ - division by 0, not detected in neat

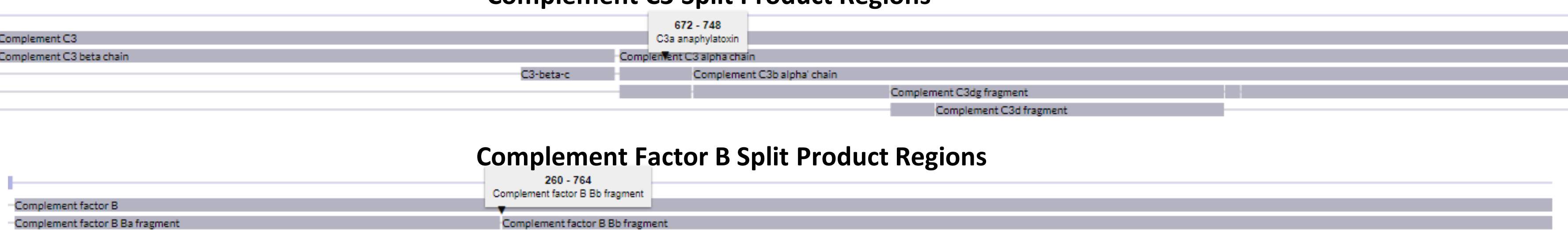
Gene name	First Pos	Peptide	Last Pos	Prec'r Chr	Prec'r m/z	70 min RT	40 min RT
C1QB	164	VPGLYYFTYHASSR	177	2	831	38	27
C1QB	216	LEQGENVFLQATDK	229	2	796	33	25
C3 beta chain region	149	IFTVNHK	155	2	430	18	17
C3 beta chain region	282	ISLPESLK	289	2	444	31	24
C3a anaphylatoxin region	713	FISLGEACK	721	2	513	29	23
C3a anaphylatoxin region	741	ASHLGLAR	748	2	413	18	17
Complement C3c alpha' chain fragment 1	842	NEQVEIR	848	2	444	18	17
C3dg region	1073	APSTWLTAYVVK	1084	2	668	44	30
C4BPA	79	YTCLPGYVR	87	2	565	28	22
C4BPA	457	GYILVGQAK	465	2	475	27	22
C4b-binding protein beta	228	ESGMTMEELK	237	2	578	26	21
C6	232	VPANLENVGFEVQTAEDDLK	251	2	1095	45	30
C6	460	GSSGLEEK	467	2	404	15	22
C6	694	QGDVECQR	701	2	496	14	17
Factor B Ba fragment	78	STGSWSTLK	86	2	484	23	20
Factor B Bb fragment	659	DAQYAPGYDK	668	2	564	18	17
Factor B Bb fragment	711	FIQVGVISWGVVDVCK	726	2	903	53	34
CFD	162	RPDSLQHVLLPVLDR	176	3	587	41	28
CFD	206	GDSGGPLVCGGVLEGVVTSGSR	227	2	1031	37	27
CFHR4	148	FCDMPVFENSR	158	2	701	35	25
CFHR5	70	ITCTEEGWSPTPK	82	2	753	24	20
CFHR5	533	TGDAVEFQCK	542	2	578	20	19
CFI	407	IVIEYVDR	414	2	504	29	23
CFP	360	CAGQQQDIR	368	2	538	16	15
CFP	439	CEELQGQK	446	2	496	16	15
VTN	187	AVRPGYPK	194	2	444	17	16
VTN	230	FEDGVLDPDYPR	241	2	712	33	25



NRicher™ C Functional Profiling of the Complement Cascade

The complement cascade is a major component of the innate immune response requiring a delicate balance between activation and inhibition, necessary to counteract infectious agents or inflamed tissues, while also protecting healthy self/host tissue. As activation is achieved through proteolysis, except for a few rare genetic deficiencies, concentrations do not serve well as metrics for functionality. Rather, in order to characterize Complement functionality in disease cohorts, specific regions of Complement proteolytic split-products need to concurrently be monitored, so that functionality can be inferred from the relative amounts of the split-products from different functional regions, for example the signal from C3a region relative to C3 beta chain (and/or C3b alpha chain), see below. Similarly, Factor B can be monitored functionally. As a proof of principal, such a target cross selection from predominantly low signal level regulating factors is demonstrated here.

Complement C3 Split Product Regions



Limit of quantification improves using NRicher™

The dynamic range for most LC-MS proteomic identifications is about 5-6 orders of magnitude, with limits of detectability in the ng/ml range. As biological variance can be an order of magnitude or greater, it necessitates that the limit of quantification be about an order of magnitude higher than the limit of detectability, so that any decrease in signal is accountable in much the same way as any increase. Consequently, the LC-MS dynamic range for targeted quantification demands a more compressed range, about 3-4 orders of magnitude. It therefore is desirable to raise the lower level signals to a greater extent than the higher level signals, a purpose fulfilled by **NRicher™** in a simple and efficient way. Like Complement presented here, investigators can now use **NRicher™** for multiplex targeting, extending research beyond just abundance levels, which often do not directly correlate to functional levels. As demonstrated, functional level features such as PTMs, can be acquired at high signal to noise levels, with reduced acquisition time and greater productivity.

Conclusion For **NRicher™ C**, we establish a proof of principle for quantitative recoveries of most Complement and Complement regulating proteins, and especially those of relatively low abundance (i.e., C4BPB, CFP (Properdin), CFHRs). Furthermore, specific peptides that report functional regions are especially noteworthy after **NRicher™ C** enrichment; for example - C3a, C4BPB, CFB (Ba fragment), reporting peptide functional regions have strong signal, even as gradient time is reduced. With protein level enrichment, peptide injection loads can be much higher than with neat achieving better signal to noise especially at lower end signals. Similarly, for **NRicher™ Apo**, enrichment of Apolipoproteins is an exceptional 10 >300x compared to neat, and for **NRicher™ Ig**, enrichment of most Immunoglobulins subclasses achieves about 4-10x (data not shown). 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 specific protein level enrichment can be achieved through optimized buffer systems, and at the peptide level through gradient optimization.

Furthermore, with 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. Finally, through a searchable knowledgebase of >2000 serum proteins, 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. For these reasons, sample prep workflows combining **NRicher™** sub-proteome enrichment with on-bead digestion will become especially desirable.

Future Work

Proteolysis is the primary triggering post-translational modification for activating pathway cascades in blood such as Complement and Coagulation. Proteases are also released from granulocytic cargo from Neutrophils and Lymphocytes. As proteolysis is irreversible, protease inhibitors play a key role to regulate and maintain normal healthy homeostasis. Nevertheless, protease inhibitors are also tightly regulated and display functionality very often not proportional to their abundances (i.e. Serpins). This finely tuned regulatory system, the central control of innate immunity, when dysregulated, orchestrates chronic inflammation and a range of pathologies, from cardiovascular disease to cancer. Therefore, like Complement, future studies using **NRicher™** products will focus on a targeted collection of specific functional proteoform features, especially common amino acid variants and proteolytic cleavage regions, related to protease/inhibitor functionality, ectodomain shedding of membrane proteins, and apolipoprotein isoforms. Such functional profiling cannot be ascertained through evidence collected from conventional counting of protein level abundances, as this can be either uninformative or terribly misleading when investigating the innate immune proteome landscape in blood and biofluids.

Highlights

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

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

Investigate out of the Venn Diagram box. Specific target peptides that report functional and variant regions **promise actionable insights and potential multiplex biomarkers for disease.**