BIOTECH SUPPORT GROUP
Sample Prep that Matters

The Functional & Chemical Proteomics Handbook

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Purposes and Definitions

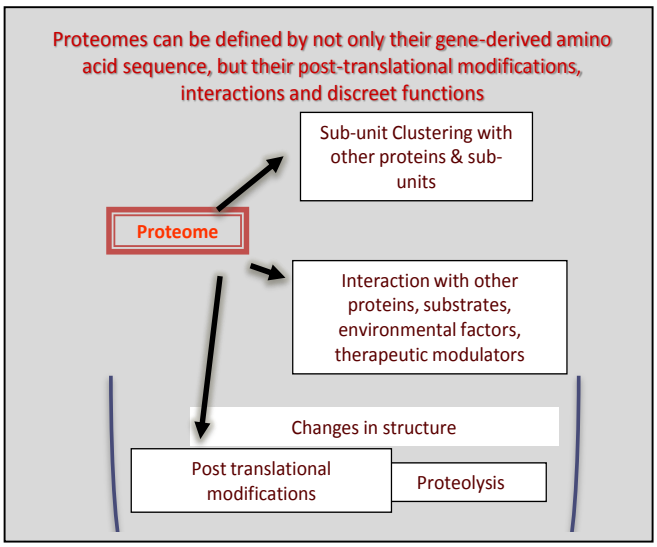
The purpose of proteomics is to find systemic differences in protein populations, and thus establish measurable markers that can define a disease, therapeutic targets and precision or personalized treatments. So its value rests in the therapeutic and diagnostic potential, as the vast majority of drugs work by modulating protein function. Therefore, proteomic analysis strives to identify differences within samples at the protein level; with such differences established at one or more levels of abundance, structure or function.

Proteomics, like other 'omic' analyses, is data driven and can generate unbiased protein profiles for a variety of end points that can contrast for example, treated vs. untreated cell models, or healthy vs. diseased tissue; all directed towards the development of measurable biomarkers. Proteomic data is generally acquired after proteolytic processing of the parent proteomes. The derived peptides are then analyzed on instruments coupling nano- Liquid Chromatography to Mass Spectrometry (LC-MS). Such instruments generate mass spectra of peptides and further fragmentation to MS2 spectra. The fragmented peptides produce a unique MS2 spectra which can be compared to a theoretical amino acid spectra definable through public gene repositories. Peptide sequence matches are thus computationally derived, and from that data, protein identifications are inferred.

From such analyses, peptide markers can be used as surrogates for the gene products from which they are derived. Through differential expression analysis of these peptide markers, Proteomics can thus help identify those gene products that define a phenotype. Many label and label-free quantitative analyses are available to compare and contrast the abundance or quantities of proteins within the starting samples. In such a manner, annotation of proteome regulation can be established between samples of different phenotypes.

While such efforts have been very productive, a critical assumption is that function is linearly proportional to protein abundance. However, protein conformational variability affects functional activity in a non-linear fashion. As a consequence, function based biomarkers may provide a higher dynamic range than abundance based biomarkers. Functional Proteomics therefore supports strategies that start with functional annotation of the structurally intact protein, and ends with sequence and structural annotation.

The term Functional Proteomics has generally been used to describe proteomics data seeking to characterize or define protein data sets within a functional context. As such, its definition is extremely broad. It has been used in pathway analysis defining the potential function and relations among identified proteins[1]. It has been used to describe quantitative protein expression data using reverse-phase protein arrays and antibody-based reporting, that can access multiple protein markers[2]. With this usage of the term, functional pathways are inferred when differential data sets of sequence specific proteins are evaluated.



In another usage of the term and for the purposes of this Handbook, we define it within the context of methods of analysis. Within this context, functional proteomics supports the characterization of proteomes derived in part from functional or structural features of intact, non-denatured proteins, rather than by differential expression alone. Examples would be the survey of substrate specificities to enzymes, or the sub-proteome binding complement to small molecule drug candidates[3-12].

While the terminology can often overlap, chemical proteomics can be considered a subset of functional proteomics and encompasses a range of methods to derive profiles that can identify and classify structurally intact proteins that interact with small molecules of synthetic or natural origin[7-12]. In this way, functional and chemical proteomics can investigate the effects of a compound on its target and any collateral off-target functions. Such information is critical to safety and efficacy.

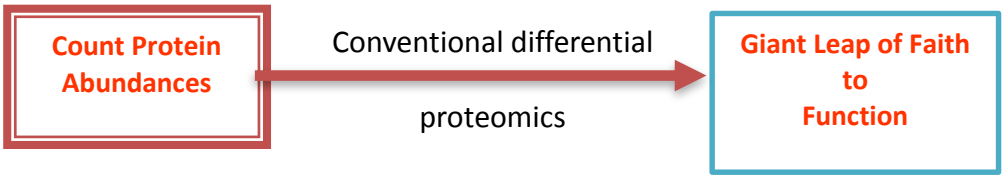
The Value of Functional Proteomics in Healthcare

The healthcare industry relies on innovations and refinements in Systems Biology technologies, such as Gene Expression and Next Gen Sequencing, to accumulate knowledge surrounding the cellular constituents of different tissue phenotypes. As the vast majority of drugs work by modulating protein function, systems biology interests are vested in characterizing proteins as discretely as possible.

Consequently, the proteomics field continues to advance more efficient methodologies to

sequence annotate subsets of proteomes. Known or suspect biomarker proteins can now be surveyed in targeted quantitative multiplexed analysis. Unique to proteomics is the additional capacity to directly assess structure, splice variants, post- translational modifications, and interactions with compounds, substrates and other regulating factors. This much deeper view of functional relevancy and phenotypic characterization cannot come from genomic information alone.

…Assumption of linearity between protein abundances and protein functions is grossly flawed



Protein conformation dynamics affects function non-linearly!

> Put simply, protein biochemistry is not so simple
> Intrinsically disordered sequence drives functional variability
> PTMs and proteolysis play a role in tuning function

Yet the foundation of modern drug development - validated protein targets defined by their amino acid sequence, rests on an oversimplifying assumption; that is, one gene presupposes one protein sequence, which further presupposes one protein function. While this assumption may be valid for some singular gene disorders or somatic mutations, it is particularly flawed for complex multi-factorial diseases like cancer and neurodegenerative disorders. Biology is simply not so simple, as proteins with common sequence can serve many functions, and conversely proteins with dissimilar sequence can perform similar functions.

Many structural features participate in defining protein function including: post-translational modification (i.e., phosphate on/off), sub-unit equilibrium, allosteric regulating factors and the unstructured (or "spatially fluid") nature of protein domains. Unstructured domains interact with environmental stimuli, and drive conformational changes in structure – promoting a multitude of alternative functions[13,14].

5.1 The Concept of Protein Disorder

The basic insight prov classical paradigm, which equated protein function with a stable 3D structure dous success in interpreting the function of teins. Decades of structure determination efforts and recent structural genomics programs have yielded 50,000 well-defined structures deposited in the protein data bank (PDB, www.pdb.org), strongly reinforcing the tra with recent recognition that many proteins or regions of

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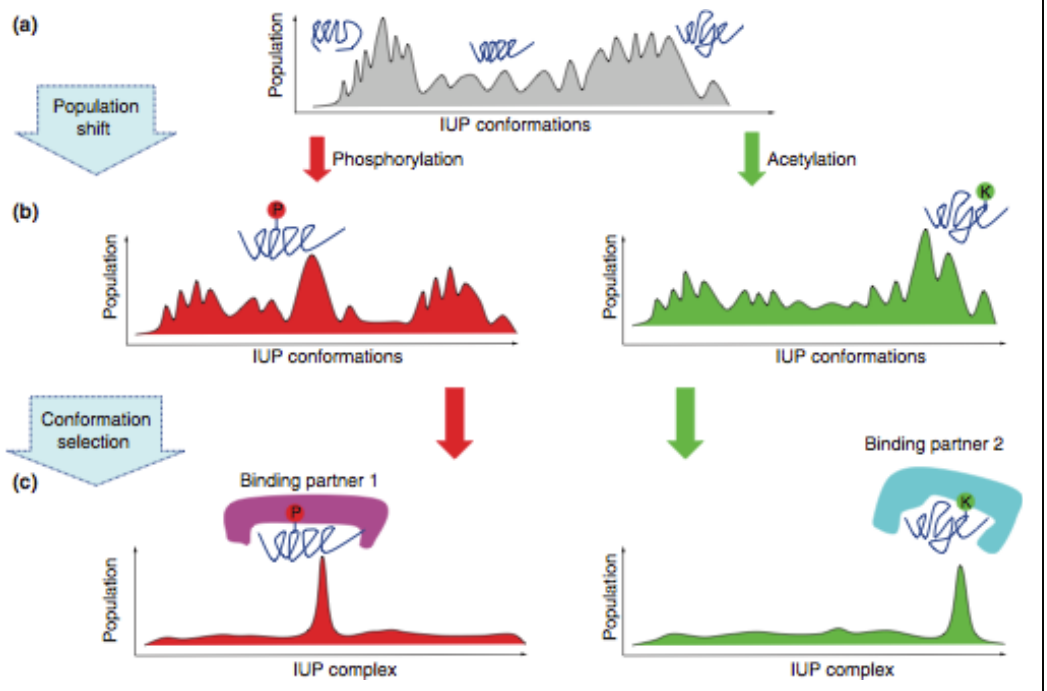
D.J. Rigden (ed.) *From Protein Structure to Function with Bioinformatics*,

From D.J. Rigden (ed.) *From Protein Structure to Function with Bioinformatics* ©Springer Science+Business Media B.V. 2009[15]

proteins lack a well-defined three-dimensional structure , physiological conditions, however, challenged the universality of this paradigm (Tompa 2002, 2 emerging alternative view of proteins, the need for a reassessment and extension of the structure-function paradigm became compelling (Wright and Dyson 1999).

Energy Landscape of Intrinsically Disordered Sequence
From one sequence, conformational selection tunes function[16]

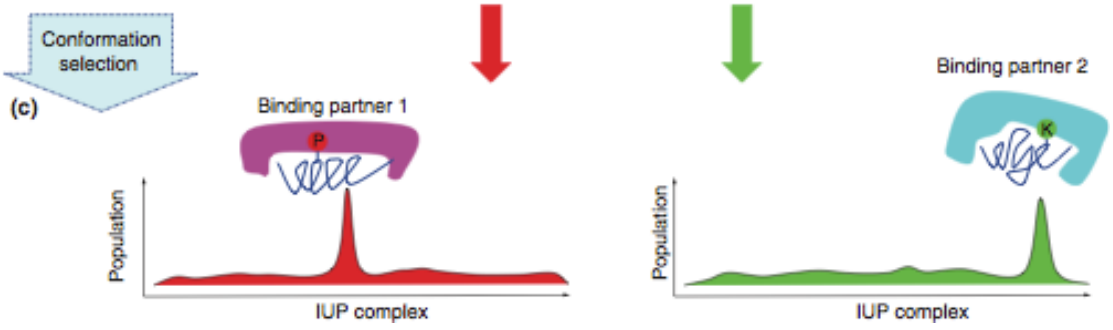


The discovery of intrinsically disordered proteins (IDPs) and hybrid proteins consisting of ordered domains and intrinsically disordered protein regions (IDPRs) challenged the protein structure paradigm stating that a protein must have a defined 3D-structure, based solely on amino acid sequence in order to perform a function[15]. Intrinsically disordered sequence cycles through a continuum of conformations. Mutations, post-translational modification, and non-covalent binding factors all play a role in fine tuning the polypeptide sequence to final function [16].

Because of this phenomena, populations of proteins annotated to the same sequence nevertheless can display multiple functions within tissues and disease states. α-enolase serves as an example of one such "moonlighting protein", one of a growing list of proteins that are recognized as identical gene products exhibiting multiple functions at distinct cellular sites[17]. Likewise, the same or similar function may be presented by multiple sequences, with subcellular control mechanisms regulating functional diversity. As a consequence, strictly abundance based biomarkers may lack the high dynamic range and greater specificity potentially provided by functional based biomarkers, to define the phenotype. Thus, Functional Proteomic techniques such as proposed here, support a top-down proteomic strategy starting with functional annotation of the structurally intact protein, and ending with sequence and structural annotation.

So…
Simply counting protein abundances is inadequate to establish function from sequence abundance, and to characterize phenotypes

Conventional Proteomics counts these two very different functions the same



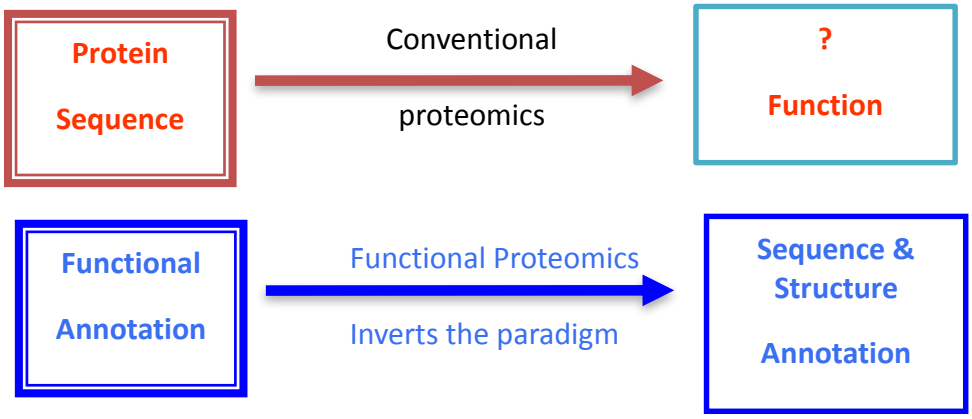
Functional Proteomics counts these differently

Such functional annotation at the protein level is crucial to assessing mechanism of action of drug candidates, particularly since even clinically successful drugs are often promiscuous, modulating multiple proteins at once. The challenge has been to overcome the analytical bias towards the most abundant proteins, and the complexities of mining the data to a manageable number of biomarker proteins that can be analyzed in more depth.

To support these challenges, Biotech Support Group has developed surface chemistries and separations strategies to untangle proteome complexity without compromising protein functional integrity. In the reference section at the end of this Handbook, we highlight some of the important research that was obtained from our products using this important feature of maintaining functional and structural integrity. The following sections describe two key related products, **NuGel™ NRicher™ 6** & ***Mx***, for such purposes.

The importance of assigning sequence to function on a proteome scale

- **Functional Biomarkers may offer better detectability and dynamic range**
- **Most drugs work by modulating protein function**
- **Many successful drugs modulate multiple proteins**
- **Understanding MOA and indications requires functional annotation**



New Functional Proteomic Technologies are now reliable and efficient!

The toolkit described in this Handbook includes:

- **NuGel™ NRicher™ 6 – for proteome separations of conformational variants**
- **NuGel™ NRicher™ Mx – for compound-centric displacement proteomics**
- **Array Bridge PEP Platform – for 1 & 2 DE resolution of functional proteins**

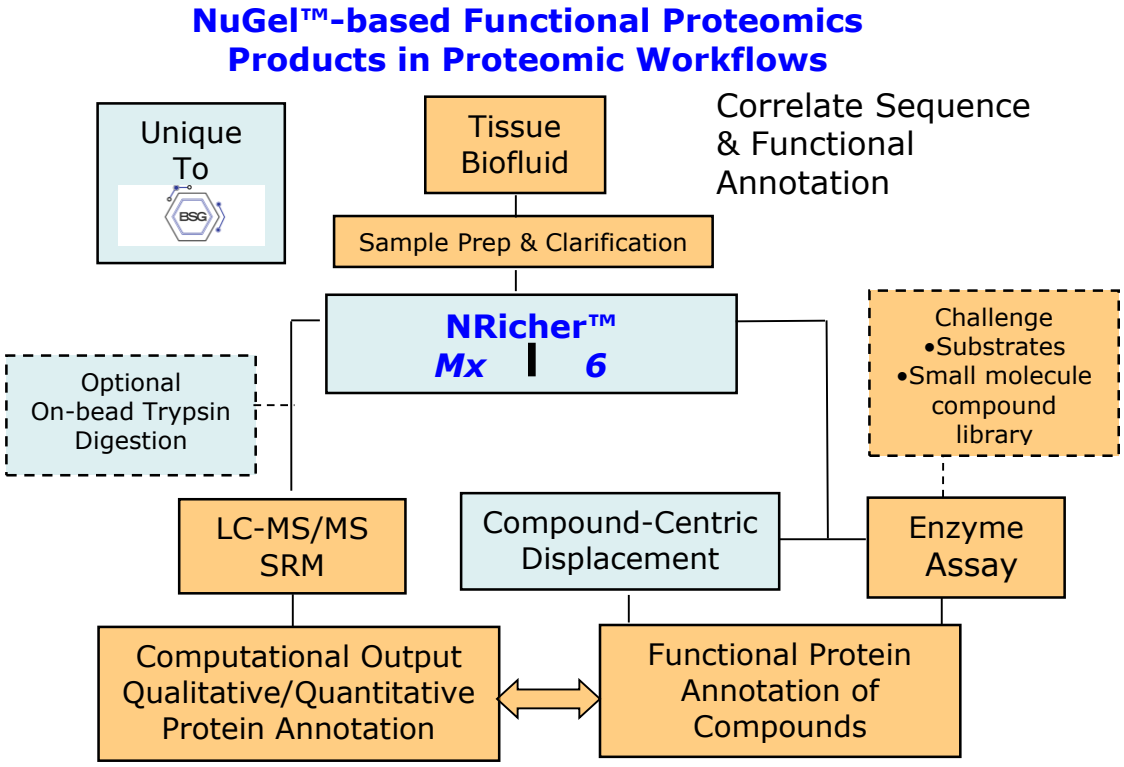
NuGel™ NRicher™

Small chemical compounds, coupled to the unique **NuGel™** porous silica architecture create novel mixed-mode binding interactions; each surface chemistry having a weak affinity bias towards its complement sub-proteome. Each mixed-mode interaction is different based on its combination of ionic, aliphatic, aromatic and polymeric character. Nevertheless, as they are not biologically derived, they are sufficiently economical for one-time consumable use, eliminating regeneration, contamination, and reduced performance. Such surface chemistries support a variety of the applications in the Biotech Support Group catalog.

The **NuGel™ NRicher™ 6** kit can be applied to functional proteomic applications. Included in the kit are 6 mixed mode surface chemistries to efficiently produce up to 12 differentiated sub-proteomes (6 eluates & 6 flowthroughs) with uncompromised functional and structural integrity.

The **NuGel™ NRicher™ Mx** is a composite mixture of the same 6 mixed mode architectures supplied with **NRicher™ 6**, designed to both compress protein concentrations from highly complex proteomes thereby enriching the low abundance content, and to immobilize protein content with weak affinity. The applications for these unique features are further described to:

- Optimize drug compounds
- Survey drug-interaction & protein promiscuity
- De-convolute drug targets & study mechanism of action
- Enrich & identify functional phenotypic biomarkers
- Annotate the multi-functional proteome



Functional proteomics can help optimize drug candidates to tissue-specific expression of isoforms, gauge promiscuity, elucidate mechanism of action and identify biomarkers

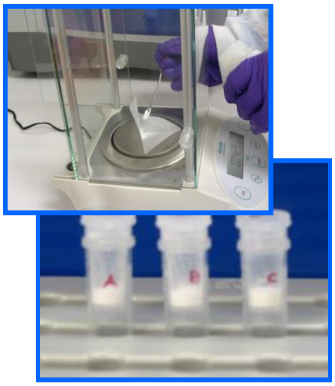
The choice of product will depend on the proteomics strategy, the primary and secondary reporting methods, and the efficiency of the workflow. As the products use the same surface chemistries but in different formats, one can consider for example, identifying an enriched biomarker starting with the composite **NRicher™ Mx** but optimizing enrichment with **NRicher™ 6**. Please contact us for technical guidance and recommendations for specific use and applications for each.

NuGel™ NRicher™ 6 - Applications and Protocols

NuGel™ NRicher™ 6 is a functional proteomics and enrichment kit, containing 6 separate mixed mode separation **NuGel™** surfaces, and all necessary buffers for use.

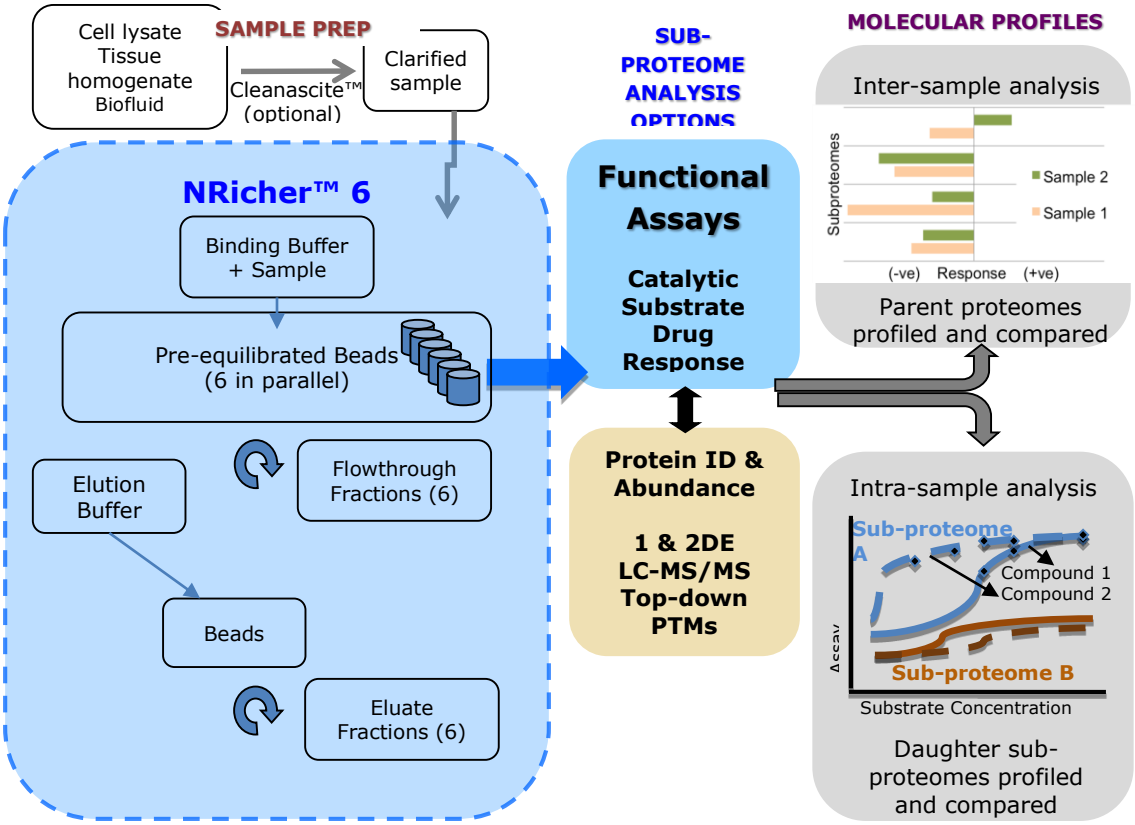
- 12 differentiated subproteomes, 6 flow-through fractions, and 6 elution fractions
- Uncompromised functional and structural attributes
- Compare function molecular profiles for biomarker discovery
- Enrich low abundance functional biomarkers for sequence and structural annotation
- Kit includes 6 mixed mode bead chemistries per prep

The **NuGel™ NRicher™ 6** protocol is extremely simple and does not require any specialized instruments or HPLC.



Simply, weigh the **NuGel™** powder, place in spin-filters, mix and centrifuge. The 6 flowthrough fractions and 6 eluate fractions fit into existing proteomic workflows, usually without any further treatment. No harsh buffers are used for elution, preserving the functional and structural integrity of the derivative sub-proteomes.

Here is how **NuGel™ NRicher™ 6** can be used in assorted proteomic workflows. The sub-proteome analysis is driven by the needs of the investigation with the separated sub-proteomes integrated with the functional reporter assay. Any functional reporter assay can be used, including enzymatic, structure based interrogation (i.e., activity-probes), even cellular response. In this way, the parent proteomes generate unique molecular profiles which can be compared on an inter-sample, or sample to sample basis, to uncover sub-proteomes that differ in their functional characteristics. In the same way, the daughter sub-proteomes can be indexed and compared within the same parent sample, to compartmentalize a particular functional characteristic of interest. Consequently, relationships between function, sequence, abundance and structure can be built.


Cell lysate
Tissue homogenate
Biofluid
SAMPLE PREP
Cleanascite™ (optional)
Clarified sample
NRicher™ 6
Binding Buffer + Sample
Pre-equilibrated Beads (6 in parallel)
Elution Buffer
Flowthrough Fractions (6)
Beads
Eluate Fractions (6)
SUB-PROTEOME ANALYSIS OPTIONS
Functional Assays
Catalytic
Substrate
Drug
Response
Protein ID & Abundance
1 & 2DE
LC-MS/MS
Top-down
PTMs
MOLECULAR PROFILES
Inter-sample analysis
Subproteomes
Sample 2
Sample 1
(-ve) Response (+ve)
Parent proteomes profiled and compared
Intra-sample analysis
Sub-proteome A
Compound 1
Compound 2
Sub-proteome B
Substrate Concentration
Daughter sub-proteomes profiled and compared

Key Reference

A research article published in Nature, describes the simplicity and efficiency of the **NRicher™ 6** proteomic enrichment technology to observe and annotate soluble multiprotein complexes common to metazoa.

The citation is:
C Wan, B Borgeson, S Phanse, F Tu, K Drew, G Clark, et al. **Panorama of ancient metazoan macromolecular complexes**. *Nature* Volume:525, Pages:339–344 Date published:(17 September 2015). doi:10.1038/nature14877

In brief, the article's authors aimed at elucidating the components of multiprotein complexes on a proteome-wide scale. The authors identify protein complexes from nine species in parallel, based on biochemical fractionation of native soluble macromolecular complexes followed by tandem mass spectrometry to identify components. The article states "…clarified heart and liver homogenates were frozen, and subsequently pooled, depleted of hemoglobin using HemogloBind™ according to manufacturer's (Biotech Support Group) instructions…prior to sucrose gradient fractionation. A second Biotech Support Group product, formerly called SeraFILE PROspector and now trademarked as **NRicher™ 6**, when used for sub-proteome enrichment, generated about twice the number of observations and annotations then without such pre-separations. The article states "Affinity bead based sample pre-separations were performed for *C. elegans* lysates as per manufacturer's instructions…fractions (SeraFILE bead eluates) were subjected to ion exchange fractionation…". The authors conclude that many complexes are conserved across species, and by correlating the results with genome sequence information, are able to predict more than one million interactions in 122 eukaryotes.

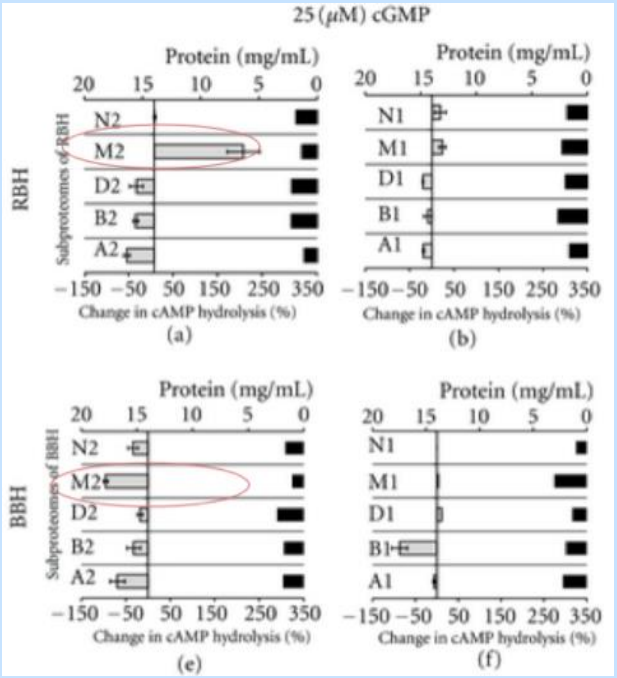
Functional Biomarker Response After NRicher™ Separations

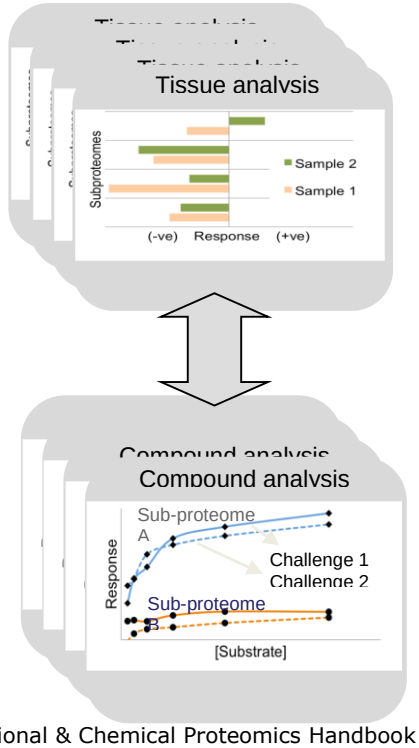
NRicher™ 6 can distinguish between closely related conformational variants that could otherwise not be separated by conventional size or pI characteristics. As an example below, Oka, et al, describes how it is used to efficiently produce unique sub-proteomes of cyclic nucleotide phosphodiesterase activity, with resulting enzyme measurements (cyclic AMP hydrolysis) integrated into molecular profiles. The results demonstrate that the proposed methods provide a means to simplify inter-sample differences, and to enrich protein markers attributable to sample-specific phosphodiesterase response to substrate challenge[6].

Molecular profiles differ under a competitive small compound challenge. Plotted are the relative changes in in cAMP hydrolysis activity of each derivative subproteome in the presence of the competitive substrate cGMP.

Encircled are the enriched sample-specific catalytic responses that which distinguish the two samples; RBH (rat brain) & and BBH (bovine brain).

From: *International Journal of Proteomics, vol. 2012, Article ID 515372, 8 pages, 2012. doi:10.1155/2012/515372.*





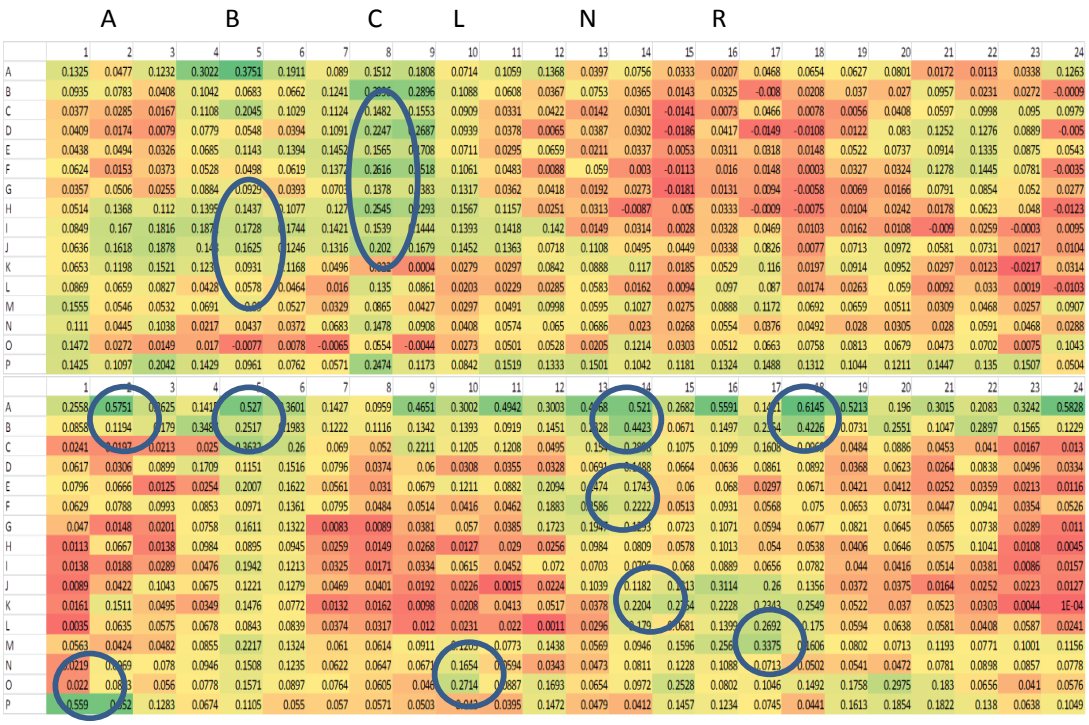
- **Sub-fractionate conformational variants**
- **Conformational Variant Molecular Profiles can be distilled, and correlated to tissues and compounds**

Bullseye
Right Protein Conformation Target
Right Tissue Type
Right Compound

NRicher™ 6 can also support a first dimension separation in the Array Bridge PEP functional proteomics platform, described in Section 3 of this Handbook.

NRicher™ 6

Beads A, B, C, L, N, R Functional Activity Profile
Normal Serum (upper panel) vs.
Colon Cancer Serum (Lower Panel)



PEP Functional Activity Analysis, courtesy of ArrayBridge (St. Louis, MO). 25 µl serum was load volume for all bead separations. Modified SDS-PAGE was employed for size separation and electro-eluted into the wells. After addition of refolding solution, each well was monitored for Hexokinase activity using beef extract for cascade enzymes; NADP reduction being the final spectrophotometric reporting measurement. The circled regions are activities up-regulated after subtraction of background. The observed activities show a different pattern for each of the 6 NRicher™ beads -designated A,B,C,L,N,R, and generally a feature pattern distinguishing the normal (upper panel) from the colon cancer (lower panel) sera.

NuGel™ NRicher™ Mx - Applications and Protocols

The **NuGel™ NRicher™ Mx** supports two key applications:

1. **Protein Compression** – reduces the dynamic range of protein concentrations and enriches for the low abundance proteome
2. **Compound-centric Displacement Proteomics (CCDP)** – a new chemical proteomics method to characterize small molecule compound, protein interactions.

NuGel™ NRicher™ Mx Protein Compression

Many methods have been commercialized to reduce the dynamic range of protein concentrations within complex mixtures. **NuGel™ NRicher™ Mx** is the only one that is universal as it can be applied to any tissue from any biological source, and is suitable for small sample and protein loads; only about 1 mg total protein being required. It also preserves the functional and structural integrity of the enriched proteomes as no harsh denaturants are used for elution. The **NuGel™ NRicher™ Mx** material is a composite mixture of the 6 **NRicher™ 6** mixed mode interaction surface characteristics, each with its own complement bias. The highest abundance proteins bias towards some but not all surface chemistries. Under saturation overload conditions, the highest protein concentrations are then reduced due to competitive displacement effects – a consequence of weak affinity binding. As each of the 6 surface architectures has sufficiently different bias in its complement proteome, together they work to compress the overall protein concentrations relative to their starting concentrations, the low abundance protein content being enriched relative to the high abundance protein content.

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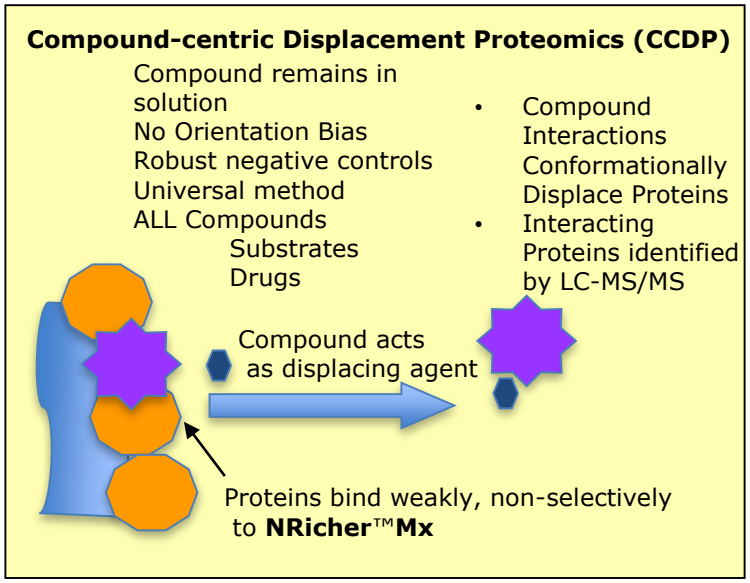
NuGel™ NRicher ™ Mx - Compound-centric Displacement Proteomics

The key application for **NuGel™ NRicher™ Mx** is in chemical proteomics, in a method we call compound-centric displacement proteomics or CCDP.

Compound-centric Displacement Proteomics (CCDP) is an advantaged alternative to other methos; no covalent modifications are necessary. The **NuGel™ NRicher™ Mx** product supports the CCDP method as the full complement of proteins bind non-covalently and non-selectively.

Small compound interactions – a process sometimes called affinity elution, can conformationally displace proteins that can then be identified by LC-MS/MS. The advantages of this method are that the compounds remain in solution so there is no orientation bias and it is universal to all compounds and sample types.



Label (i.e., iTRAQ) or label-free quantitation (MS2 spectral counts or XIC-based) LC-MS/MS analysis can then be applied to identify/quantify the CCDP-derived proteins. In the example below, MS2 spectral counts are the number of peptides identified and associated with the gene designation. These counts can serve to monitor the relative abundance of proteins. As the background proteins are always the same, regardless of the challenge compound, many different compounds can be run in parallel, with good quantitative metrics of comparison. Such is not the case when comparing different immobilized compounds, all derived from different immobilization reactions and efficiencies.

MS2 Spectral Counts of CCDP-derived Sub-proteomes

Protein Description	Caffeine	Imatinib	Neg.Cont.
Hemoglobin subunit beta-1	87	550	53
Glucose-6-phosphate isomerase	192	459	76
Malate dehydrogenase	117	356	35
transketolase	72	160	24
Cytochrome c, somatic	47	123	3
Succinyl-CoA:3-ketoacid transferase	69	122	19
Transgelin	0	84	0
Annexin A2	26	66	0
fumarate hydratase	17	42	2
annexin A3	5	36	0
glutathione reductase	9	38	0

A partial list of LC-MS/MS identification and spectral counts demonstrate Imatinib interaction proteins from a common tissue homogenate, using CCDP. Caffeine was employed as a non-specific control compound, negative control was the final wash buffer.

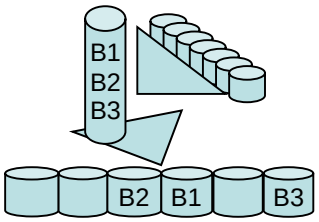
The advantage of the CCDP method is that solution phase compounds are not subject to orientation biases. Negative controls are robust. Non-specific challenge can be evaluated and compared to specific challenge compounds. Furthermore, it can be applied towards any soluble compound as it does not require substitution or tag modifications. As compound concentrations can be varied, some measure of relative binding affinities between compounds

can be characterized. Finally, protein compression can help minimize bias towards the high abundance protein content.

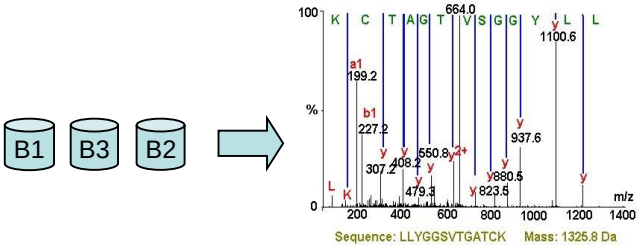
As consumables, both **NuGel™ NRicher™ 6** and ***Mx*** products feature:

- Enrichments readily compatible with virtually all proteomic interrogations
- Microtube kit formats, simple bind/wash/elute protocols
- No specialized instruments, or HPLC required
- Disposable, no column regeneration
- Tryptic digestion or enzyme assay can be 'on-bead'
- Universal, species and tissue type agnostic
- No scale or molecular weight bias

Furthermore, any biomarkers discovered using the composite materials of **NRicher™ Mx** can be deconstructed to **NRicher™ 6**, and optimized to enrich for the biomarkers of interest, as represented here.



Biomarkers (B#) in the composite **NRicher™ Mx** reagents, can be deconstructed and optimized for targeted peptide or protein assays using individual **NRicher™ 6** reagents.



Optimized protein enrichment can be used in targeted MRM/SRM assays

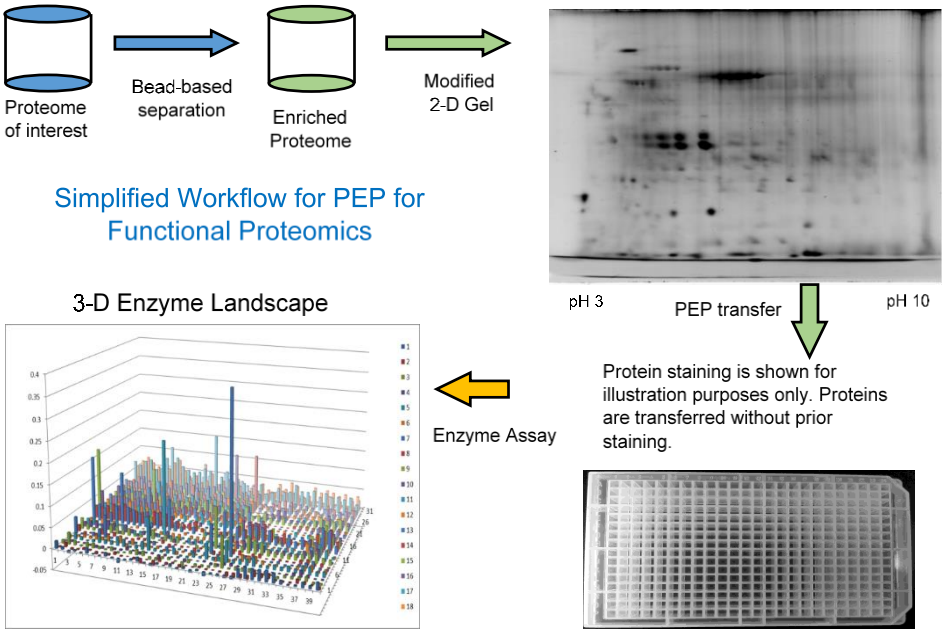
Section 3

Functional Proteomics Using the Protein Elution Plate (PEP)

A novel functional proteomics platform technology called PEP (Protein Elution Plate) was developed to separate complex proteomes from natural sources and analyze protein functions systematically. The technology takes advantage of the powerful resolution of one and two-dimensional gel electrophoresis. The modification of electrophoretic conditions in combination with a high-resolution protein elution plate supports the recovery of functionally active proteins.

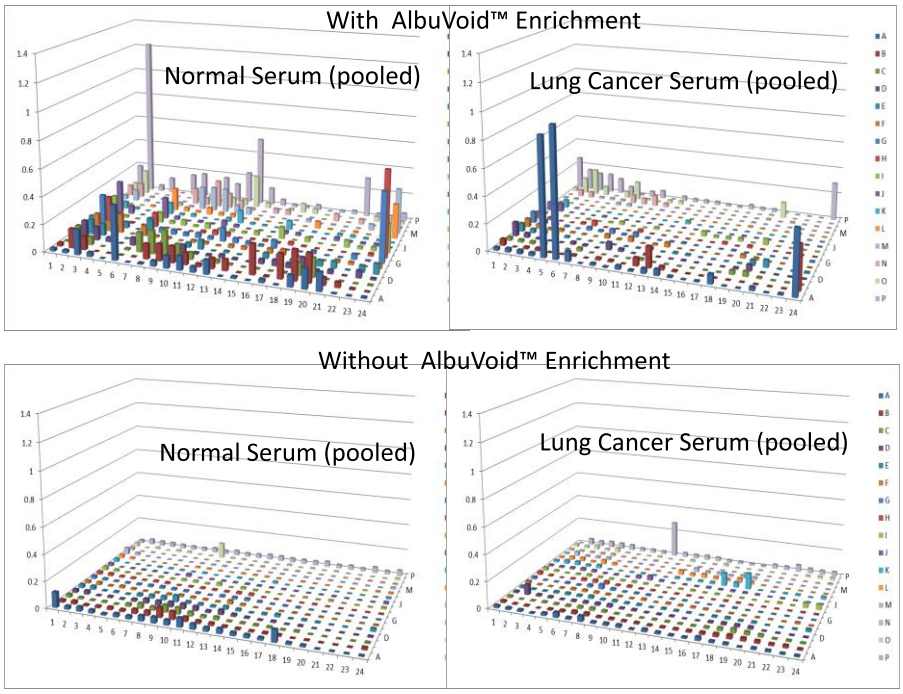
Since most functional proteins or enzymes exist at relatively low levels and there is a limited loading capacity on a 2-DE gel, it can be beneficial to first enrich the low abundance proteins before 2-DE and PEP analysis. In Figure below, **AlbuVoid**™ and **KinaSorb**™ have both been shown to effectively enrich low abundance proteins while depleting the high abundance proteins, albumin in the case of serum[18]. As stated previously, **NuGel™ NRicher™ 6** can also support a first dimension separation in the Array Bridge PEP functional proteomics platform.

It is important to note that the proteins obtained after treatment with all BSG products, are functional (not denatured) and suitable for the modified 1 & 2DE and final enzyme activities to be analyzed.



The selection of the activity to be monitored is an important choice in the functional proteomic investigation. The methods require protein refolding, a phenomena usually obtained for structurally simple enzymes. Non-denaturing modifications to the first dimension separation may be necessary for complex, multi-subunit enzymes like Proteasome; an application that may be suitable for **NuGel™ NRicher™ 6**. That is an area for future investigation. While such refolding challenges are acknowledged, the functional analysis does not need a complete refolding back to wild-type activities, for the described methods to be productive. Ultimately, it is the constellation of enzyme activity features that drives the investigation. Because many enzyme assays are very sensitive—with detection as low as picogram amounts of protein, even just a residual amount of activity will allow sufficient feature development to compare and contrast biological samples.

Thus, subject to the limits of refolding, and depending upon the goals of the investigation, the PEP technology allows for an open and exceedingly diverse selection of enzyme substrates. It can be adapted to most common quantitative instruments to monitor either spectrophotometric, fluorimetric, or chemiluminescent reporting signals generated by product formation. It is important to note that the choice of substrate will drive the application and goals of the investigation: Broad spectrum substrates will produce more profile features, and may be best suitable for pure data driven biomarker discovery applications. Narrow spectrum substrates produce less features, and this may prove advantageous when cataloging or annotating subset proteomes with similar functional attributes, or any hypothesis driven biomarker discovery project.



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Article

Bead Based Proteome Enrichment Enhances Features of the Protein Elution Plate (PEP) for Functional Proteomic Profiling

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In this short case study, we demonstrate that enrichment of select sub-populations of proteins is beneficial to systematically analyze protein functions of a whole enzyme family from an entire proteome. In Figure on previous page, **AlbuVoid™** was used to remove Albumin and enrich the low abundance proteome, noting that distinguishable features are presented from the lung cancer *vs.* the normal sera. **KinaSorb™** was used to enrich for both a narrow spectrum substrate profile—Hexokinase activity, and a broad-spectrum protein kinase activity. The number of observable features was consistent with such narrow and broad-spectrum activities. **AlbuVoid™** enrichment and PEP processing proved suitable for profiling the functional activities of Hexokinase, Protease and Alkaline Phosphatase. These enzyme

feature profiles are indicative of the functional diversity that can be generated, annotated and compared within and between sample phenotypes.

As a result, such functional annotation will be complementary to that acquired through gene expression and protein abundance analyses. For drug development, the functional landscape of a proteome will likely improve compound specificity while minimizing promiscuous interference with other protein functions. This can be a confounding problem when post-translational modifications activate protein function, of which would not otherwise be observable if only protein abundance was monitored.

In addition, because of gene editing and post-translational modification, almost all functional proteins including enzymes have isoforms. Therefore, these technologies will be valuable tools in the analysis of the function of enzyme isoforms to further understand their biochemical functions, compartmentalization, sequence regulation, and potentially therapeutic modulation in different tissues and diseases.

This methods development study demonstrates the beneficial elements of combining bead-based enrichment products upfront to PEP, generating many detectable features within the derived functional profiles. These methods support LC-MS protein identification, and confirm a previous report demonstrating that the proteins isolated from the PEP process are sufficiently pure and can be identified by mass spectrometry. Consequently, future studies with these combined methods will include protein identification and assignments as part of the study. Such gene identifications will supplement the functional activities to provide a comprehensive proteome characterization of the disease or tissue phenotype of interest. These new methods thus enhance the study of functional diversity now open to any proteome investigation, limited only by the availability of substrates and sensitive ways to monitor, *in vitro*, the conversion of substrates to products.

References for The BSG Functional Integrity Advantage:

- Mild buffer conditions maintains native structure with retained enzymatic, functional & bio-activities
- Supports enzyme biomarker assays
- Functional & Chemical Proteomics
- Structural & activity-probe Proteomics
- Top-down & ArrayBridge PEP Proteomics

We examine key user references where the **functional integrity** provided by our products was particularly advantageous in the investigation.

C Wan, B Borgeson, S Phanse, F Tu, K Drew, G Clark, et al. **Panorama of ancient metazoan macromolecular complexes**. *Nature* Volume:525, Pages:339–344 Date published:(17 September 2015). doi:10.1038/nature14877
Two of BSG products were able to contribute to this rigorous examination of protein complexes. When our products were used as a pretreatment step in the overall workflow, about twice the number of observations and annotations became possible. This further validates that the sub-proteome bias characteristics of NRicher™ 6 can simplify complex proteomes into less complex sub-proteomes with efficiencies suitable for deep functional proteome characterization. Furthermore, this study demonstrated the importance of a key feature implicit to all of our products; that is the maintenance of functional and structural integrity after separations. Without that particular feature, these additional observations would not have been possible.

Hauser-Davis RA, Lima AA, Ziolli RL, Campos RC.First-time report of metalloproteinases in fish bile and their potential as bioindicators regarding environmental contamination. Aquatic Toxicology.2012;110-111:99-106
Authors RA Hauser-Davis and team cites **Cleanascite™** as an ideal lipid clarification reagent during sample preparation of fish bile containing matrix metalloproteinases (MMPs). Researchers identified matrix metalloproteinases in the bile of mullets (Mugil liza) and tilapias (Tilapia rendalli) which required clarification and purification studies prior to performing gel electrophoresis and zymography analysis.

McGarry, Kevin G., et al. "Evaluation of HemogloBind treatment for preparation of samples for cholinesterase analysis." (2013). Advances in Bioscience and Biotechnology, 2013, 4, 1020-1023.
In this article, measurement of cholinesterase activity prior to depletion and after removing hemoglobin is performed. A comparison of total cholinesterase activity with Ellman method and after **HemogloBind™** treatment prior to Ellman method, did not display a statistical difference in mean ChE activity. Total cholinesterase activity of whole blood samples with HemogloBind™ treatment is also consistent. Moreover, the HemogloBind™ protocol is simple with one incubation and short, low speed centrifugation.

Mizukawa, B., George, A., Pushkaran, S. et al. Cooperating G6PD mutations associated with severe neonatal hyperbilirubinemia and cholestasis. Pediatric Blood Cancer.2011;56:Â 840-842.
Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common erythrocyte enzyme disorder, estimated to affect more than 300 million people worldwide. The authors report a novel G6PD mutation in an infant who presented with neonatal cholestasis. The article states "Blood samples from the patient and control subjects were lysed and depleted of hemoglobin using **HemoVoid™**". Such depletion was necessary to allow immunoblotting at an area otherwise overwhelmed by an excessive amount of hemoglobin tetramer (M.W. 68 kDa). The hemoglobin depleted fraction was analyzed by native gel electrophoresis in

polyacrylamide gradient gels of 4-15%, in the absence of SDS, followed by western blotting and immunoblotting for G6PD. All three oligomers of G6PD were observed indicating that the functional and structural integrity of the enzymes were preserved after **HemoVoid™** enrichment.

Personal Correspondence.
"We used **HemogloBind™** successfully to remove much of the visual interference within a casein zymogram to monitor calpain activity in the Sickle red blood cells". Personal Correspondence. Athar Chishti, PhD, Professor, Department of Developmental, Molecular & Chemical Biology, Tufts University School of Medicine.

NuGel NRicher Mx: Compound-centric Displacement Proteomics- An advantaged method to survey small molecule-protein interactions
Poster reprint first presented at US HUPO April 6-8, 2014.
Note: NuGel™ NRicher™ Mx was formerly called NuGel™ PROfessor
Functional proteomics relies in part, on the functional or structural features of intact, non-denatured proteins. As such, chemical and affinity-based proteomics can be considered a subset of functional proteomics. Regardless of the evolving terminology, the subject of chemical proteomics is to identify, characterize and quantify the binding interactions of small compounds to proteomes. The consideration of those interactions as functional modulators within the cell is paramount to understanding a potential therapeutic compound's mechanism of action. These same tools and methods also can help survey the promiscuous behavior of compounds towards multiple proteins and posit such behavior as deterministic of either toxicity or efficacy. We describe herein, new tools and methods for this purpose, called Compound-centric Displacement Proteomics (CCDP). Employing a new product - **NuGel NRicher Mx** which can non-covalently bind proteins, a subset of proteins can be displaced upon introduction of soluble small compounds.

Functional Proteomic Profiling of Phosphodiesterases
International Journal of Proteomics. Volume 2012, Article ID 515372, 8 pages. doi:10.1155/2012/515372.
Note: The SeraFILE features disclosed in this article are the basis for NuGel™ NRicher™ 6
Functional proteomic profiling can help identify targets for disease diagnosis and therapy. Available methods are limited by the inability to profile many functional properties measured by enzymes kinetics. The functional proteomic profiling approach proposed here seeks to overcome such limitations. It begins with surface-based proteome separations of tissue/cell line extracts, using SeraFILE, a proprietary protein separations platform. Enzyme kinetic properties of resulting subproteomes are then characterized, and the data integrated into proteomic profiles. As a model, SeraFILE-derived subproteomes of cyclic nucleotide-hydrolyzing phosphodiesterases (PDEs) from bovine brain homogenate (BBH) and rat brain homogenate (RBH) were characterized for cAMP hydrolysis activity in the presence (challenge condition) and absence of cGMP. Functional profiles of RBH and BBH were compiled from the enzyme activity response to the challenge condition in each of the respective subproteomes. Intersample analysis showed that comparable profiles differed in only a few data points, and that distinctive subproteomes can be generated from comparable tissue samples from different animals. These results demonstrate that the methods provide a means to simplify intersample differences, and to localize proteins attributable to sample-specific responses. It can be potentially applied for disease and non-disease sample comparison in biomarker discovery and drug discovery profiling.

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BIOTECH SUPPORT GROUP

Functional & Top-down Proteomics

Building sequence/structure/function relationships

The continuum of protein conformations attributable to post-translational modification and non-covalent interactions produces important functions that cannot be directly or linearly correlated to protein abundance. Thus, functional annotation complements sequence annotation, but relies in part, on the functional or structural features of intact, non-denatured proteins. While the terminology can often overlap, chemical, and activity or structure-based proteomics can be considered a subset of functional proteomics.

The **NuGel™** based **NRicher™** product line supports all functional, chemical and top-down proteomic applications. The functional and structural integrity are always preserved upon separations with **NRicher™** products. So functional protein attributes, as when the same or similar underlying sequence can have multiple conformations and functions, or when different sequences cross-over in function, are now open to investigation. Those aspiring to sift through these biological complexities can apply **NRicher™** products to:

- Annotate multi-functional subproteomes
- Survey drug-interaction protein promiscuity
- Elucidate conformational variants
- Identify phenotypic biomarkers

NuGel™ NRicher™ Mx

Chemical Displacement Proteomics

- Enrich proteomes with weak binding
- Displace bound proteins with small compounds or substrates
- Identify compound interacting proteomes with LC-MS
- Composite of the **NRicher™ 6** mixed mode beads

Protein Description	Caffeine	Imatinib	Neg.Cont.
Hemoglobin subunit beta-1	87	550	53
Glucose-6-phosphate isomerase	192	459	76
Malate dehydrogenase	117	356	35
transketolase	72	160	24
Cytochrome c, somatic	47	123	3
Succinyl-CoA:3-ketoacid coenzyme A transferase	69	122	19
Transgelin	0	84	0
Annexin A2	26	66	0
fumarate hydratase	17	42	2
annexin A3	5	36	0
glutathione reductase	9	36	0

A partial list of LC-MS/MS identification and spectral counts demonstrate Imatinib interaction (displaced) proteins from a common tissue homogenate, using CCDP. Caffeine was employed as a non-specific control compound, negative control was the final wash buffer.

Product	# of preps*	Item No.
NuGel™ NRicher™ Mx	5	SR610-5
NuGel™ NRicher™ Mx	25	SR610-25

*Based on processing 0.5-1.0 mg total protein

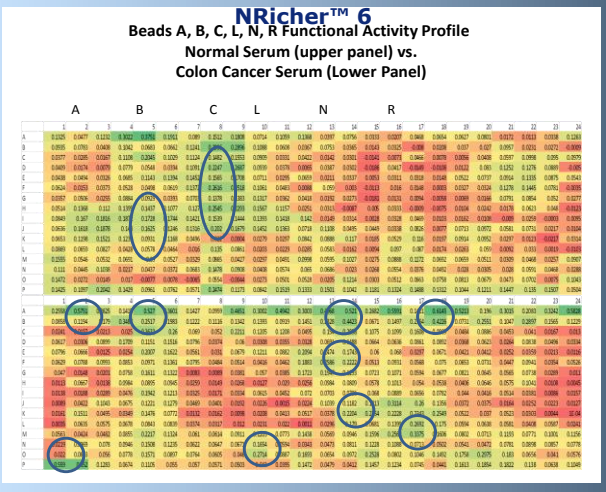
NuGel™ NRicher™ 6

Functional proteomics and enrichment kit

- 12 differentiated subproteomes, 6 flow-through fractions, and 6 elution fractions
- Uncompromised functional and structural attributes
- Compare functional molecular profiles for biomarker discovery
- Enrich low abundance functional biomarkers for sequence and structural annotation
- Kit includes 6 mixed mode bead chemistries per prep
- Top-down proteomics

Product	# of preps*	Item No.
NuGel™ NRicher™ 6	10	SRPRO-10
NuGel™ NRicher™ 6	50	SRPRO-50

*Based on processing ~1.0 mg total protein.



PEP Functional Activity Analysis, courtesy of ArrayBridge (St. Louis, MO). 25 µl serum was load volume for all bead separations. Modified SDS-PAGE was employed for size separation and electro-eluted into the wells. After addition of refolding solution, each well was monitored for Hexokinase activity using beef extract for cascade enzymes; NADP reduction being the final reporting measurement. The circled regions are activities up-regulated after subtraction of background. The observed activities show a different pattern for each of the 6 NRicher™ beads- designated A,B,C,L,N,R, and generally a feature pattern distinguishing the normal (upper panel) from the colon cancer (lower panel) sera.