BIOTECH SUPPORT GROUP

NRicher™ C

Enrichment of Complement Cascade Related Proteins

- Consumable chemically derived beads, species agnostic as they are not derived from antibodies
- Enrich Complement Cascade related proteins from sera/plasma or cell lysates from both animals and humans, >90% Albumin removal
- Does not require any specialized instruments, just a standard microfuge
- Bead format suitable for automation compatibility, please inquire
- On-Bead digestion for LC-MS analysis, or optional elution for any functional, enzymatic, or immunoassay analysis

The complement cascade is a major component of the immune system that provides powerful host surveillance and protection from invading microbes. It also exerts an important influence on the adaptive immune response by acting synergistically with antibodies as well as promoting B- and T-cell stimulation. The intracellularly active complement system—the complosome, has been shown to play a critical function in regulating T cell responses, cell physiology (such as metabolism), and inflammatory disease processes. Complement dysregulation is implicated in chronic diseases such as age-related macular degeneration, paroxysmal nocturnal hemoglobinuria (PNH), along with neurologic, cardiovascular and oncology diseases. In acute inflammatory conditions, hyperactive complement may predispose individuals to adverse outcomes, as suspected in hospitalized Covid-19 patients.

The complement system consists of over 50 circulating and membrane proteins, comprising about 5% of the total protein mass in plasma. Most complement proteins circulate in blood as inactive precursors (zymogens), that when triggered, become activated through proteolytic cascades. Although textbooks describe three activation pathways (classical, lectin & alternative), leading to the protein aggregate C3-Convertase, Complement is also interconnected with Coagulation to eliminate damaged tissues; activating platelets and contributing to hemostasis in response to injury. Conversely, complement can be activated directly from proteolytic enzymes derived from coagulation (i.e., Thrombin, Kallikrein). As a result of complement activation, many outcomes are produced including: opsonization of pathogens or damaged-self cells to enhance phagocytosis; production of anaphylatoxins C3a & C5a; recruitment of leukocytes to the inflammatory site; and the terminating end of the cascade – assembly of the membrane attack complex (MAC) on the cell surface.

The terminating end of the complement cascade is derived from the C3 Convertase proteolytic product - C5b, which engages the sequential recruitment of C6, C7, C8, and C9, assembling the membrane attack complex (MAC). Also known as the "terminal complement complex", it results from the coordination of C5b-7 insertion in the membrane, which then captures C8, inducing polymerization of a C9 ring – to as many as 18, C9's per pore. Terminal MACs perforate the cell membrane of the invading pathogen or target cell, and when a sufficient number of MAC pores form, the cell dies by osmotic lysis. Sub-lytic doses of MAC however, induce dramatically different effects than lytic doses, including adherence, aggregation, chemotaxis, cell division, and extracellular vesicle release.

In such a critical juncture, the complement system must maintain a delicate balance between activation and inhibition to allow activation when necessary to counteract infectious agents or modified self/host tissues, while concurrently protecting healthy self/host tissue. This protection is achieved systemically through the concerted action of regulators and inhibitors ensuring cell and tissue integrity essential for normal and healthy well-being. Notwithstanding such importance in disease and well-being, there are limited biomarkers to help in clinical practice. Total C3 and C4 immunoassays are available, while complement activity (CH50 lytic assay) is measured if a deficiency is suspected.

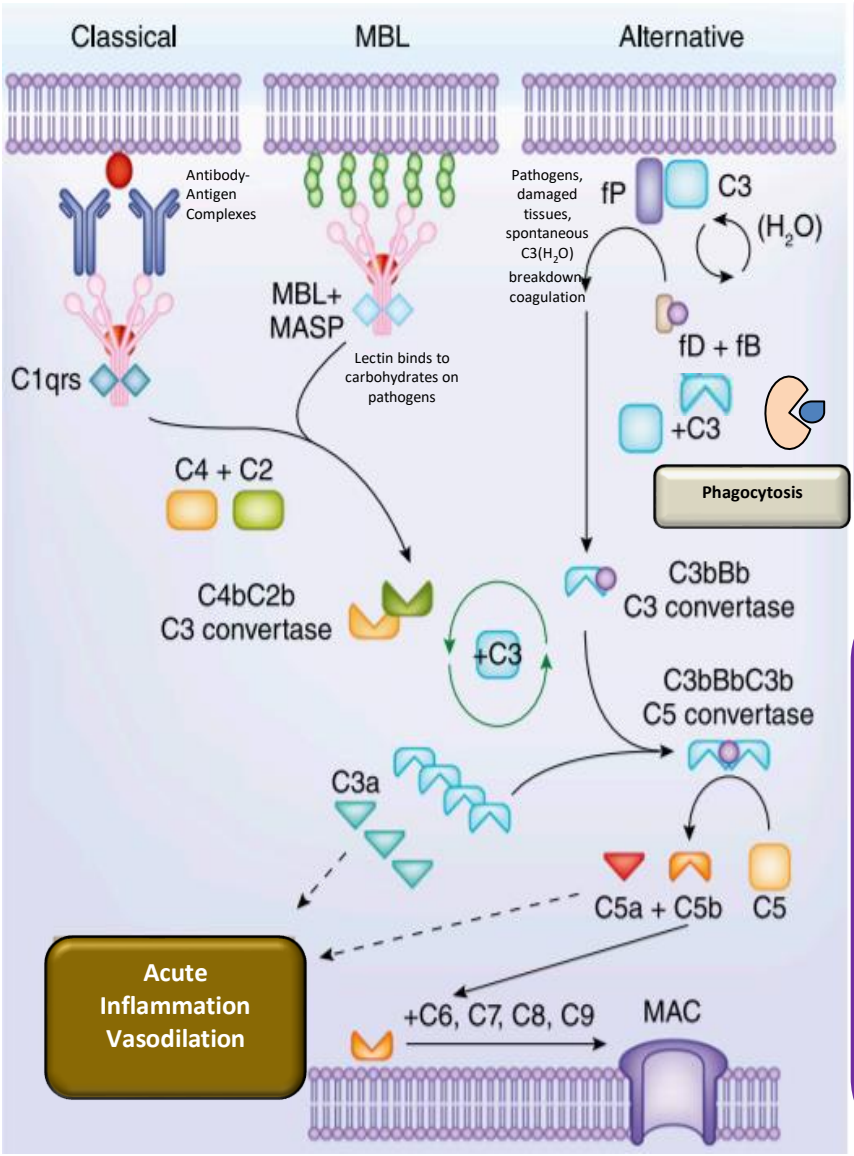
BIOTECH SUPPORT GROUP

For all these reasons, a more detailed proteomic characterization of the complement cascade and its related regulation is warranted. **NRicher™ C** can provide an approximate 2-4X enrichment for all the circulating complement and complement-regulating proteins, see Table. Using this, precision medicine biomarkers and therapeutic targets for modulating the innate immune response, in both acute and chronic disease, will be derived.

Protein Name	Uniprot Identity	Apprx Conc. µg/ml	Action
C1R (Complement C1r subcomponent, Classical)	P00736	100	Serine protease that combines with C1q and C1s to form C1, the first component of the classical pathway of the complement system.
C1S (Complement C1s subcomponent, Classical)	P09871	80	Serine protease that combines with C1q and C1r to form C1, the first component of the classical pathway of the complement system. C1r activates C1s, to activate C2 and C4.
Complement C1q subcomponent subunit A	P02745	60	C1q associates with the proenzymes C1r and C1s to yield C1, the first component of the serum complement system.
Complement C1q subcomponent subunit B	P02746	55	C1q associates with the proenzymes C1r and C1s to yield C1, the first component of the serum complement system.
Complement C1q subcomponent subunit C	P02747	50	C1q associates with the proenzymes C1r and C1s to yield C1, the first component of the serum complement system.
C2 (Complement C2)	P06681	20	Part of the classical pathway, cleaved by activated factor C1 into two fragments: C2b and C2a. C2a combines with C4b to form C3 convertase (classical, lectin)
Complement factor D (fD)	P00746	3	Cleaves Factor B when the latter is complexed with factor C3b, assists to activate C3 convertase of the alternate pathway
Complement factor B (fB)	P00751	320	Cleavage Product Bb combines with C3b to form C3 Convertase (Alternative). Fragment Bb forms complex with Properdin.
Complement C3	P01024	1,500, 000	Central role in the activation of the complement system, multi-functional sub-unit C3b triangulates complex with Properdin and Complement Factor B. Many different proteolytic sub-forms exist in the circulation.
Complement Factor H	P08603	500	Accelerates the decay of the complement alternative pathway C3 convertase (C3bBb), cofactor of the serine protease factor I
Complement Factor H related protein 1 (CFHR1)	Q03591	40	The dimerized forms have avidity for tissue-bound complement fragments and efficiently competes with the physiological complement inhibitor CFH.
Complement Factor H related protein 2 (CFHR2)	P36980	60	The dimerized forms have avidity for tissue-bound complement fragments and efficiently competes with the physiological complement inhibitor CFH
Complement Factor H related protein 4 (CFHR4)	Q92496		Might be involved in complement regulation
Properdin (fP)	P27918	25	Properdin is present in plasma, and released from neutrophil granules upon stimulation. It is a positive regulator of the alternate pathway of complement, stabilizing the C3- and C5-convertase enzyme complexes.
Complement C4-A	P0C0L4	100	Non-enzymatic component of the C3 and C5 convertases, propagates the classical complement pathway, derived from proteolytic degradation of complement C4. The C4 protein derives from a simple two-locus allelic model, the C4A-C4B genes, that allows for an abundant variation in the levels of their respective proteins within a population.
Complement C4-B	P0C0L5	365	Non-enzymatic component of the C3 and C5 convertases, propagates the classical complement pathway, derived from proteolytic degradation of complement C4. The C4 protein derives from a simple two-locus allelic model, the C4A-C4B genes, that allows for an abundant variation in the levels of their respective proteins within a population.
Complement C4b-binding protein alpha chain	P04003	300	Cofactor for Factor I, accelerates decay of classical pathway C3 convertase. Also, a cofactor for Protein S in the coagulation pathway. In plasma, 60–70% of Protein S is bound to C4b-binding protein.
Complement C4b-binding protein beta chain	P20851	0.5	The beta chain of C4b-binding protein binds protein S.
Factor I (Complement factor I)	P05156	35	Serine protease controls complement by cleaving three peptide bonds in the α-chain of C3b and two bonds in the alpha-chain of C4b thereby inactivating these proteins. Essential cofactors for these reactions include factor H and C4BP in the fluid phase and membrane cofactor protein/CD46 and CR1 on cell surfaces.
MASP 1 (Mannan-binding lectin serine protease 1)	P48740		Activates MASP2 or C2 or C3
MASP 2 (Mannan-binding lectin serine protease 2)	O00187		Cleaves/Activates C2 and C4
MASP 3 (Mannan-binding lectin serine protease 3)	P48740		Cleaves/Activates Complement Factor D
MBL2 (Mannose-binding protein C)	P11226		Binds mannose, fucose and N-acetylglucosamine, activates the lectin complement pathway.
Complement C5	P01031	60	C5 convertase initiates the assembly of the membrane attack complex (MAC).
Complement C6	P13671	60	Constituent of the membrane attack complex (MAC)
Complement C7	P10643	90	Constituent of the membrane attack complex (MAC)
Complement component C8 alpha chain	P07357	50	Constituent of the membrane attack complex (MAC)
Complement component C8 beta chain	P07358	50	Constituent of the membrane attack complex (MAC)
Complement component C8 gamma chain	P07360	20	Constituent of the membrane attack complex (MAC)
Complement C9	P02748	50	Constituent of the membrane attack complex (MAC)
Clusterin	P10909	60	A multi-functional protein, Clusterin along with Factor H, C4BP, CFHR1, and Vitronectin limit formation of complement activation products at cell surfaces.
Vitronectin	P04004	115	Inhibits terminal MAC.

BIOTECH SUPPORT GROUP

Complement Activation can come from three textbook pathways, and directly from proteolytic enzymes derived from coagulation (i.e., Thrombin, Kallikrein)



Membrane Attack Complex (MAC) Perforates the cell membrane

Benevolent Effects

Lytic quantities
Cell Death

- **Microbial Pathogens**
- **remove damaged or modified host cells**

# of pores sufficient for lysis varies with cell type. Enucleated cells, red cells, platelets require as little as one MAC, nucleated cells much more

Malevolent Effects

Sub-Lytic quantities
Nucleated cells adapt, through variety of membrane and soluble proteins
Cellular stress response:
adherence,
aggregation,
chemotaxis,
cell division,
Ca++ flux,
exosome release

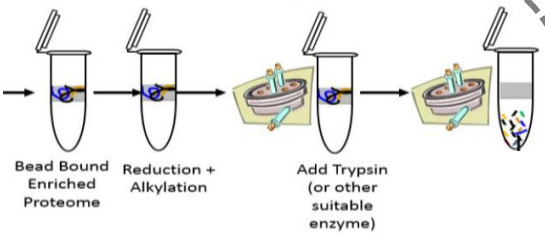
BIOTECH SUPPORT GROUP

NRicher™ workflow

High abundance (i.e., Albumin) proteins selectively passes or voids through

the beads, concentrating and enriching sub-proteomes on the beads

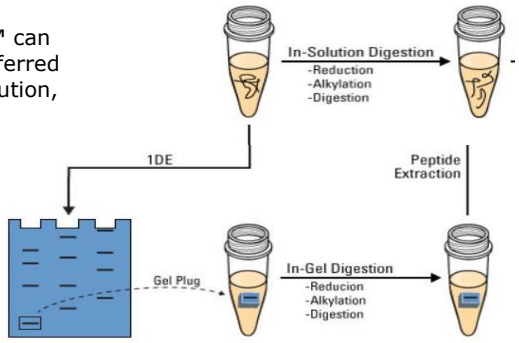
Enriched sub-proteomes remain on the bead and digested using Bead-Assisted Sample Prep (BASP); protocols provided with the **NRicher™** products



OR

Digest Options

Eluate from **NRicher™** can be digested by any preferred method, In-Gel, In-Solution, FASP, etc.



LC-MS

Eluates from **NRicher™** beads can be applied to other common analyses:

- Enzymatic/Functional assays
- 2DE
- ELISA/immunoassay

The NRicher™ Workflow. All **NRicher**™ beads are processed the same, using buffers and spin-filters provided with the kits. The beads are supplied as a dry powder, weighed and dispensed into the top of a spin-filter, and follows a bind/wash protocol using a standard microfuge to separate the buffer solutions from the beads. Once the **NRicher™**-derived sub-proteome (different for each application) is bound to the beads, a variety of options are available to the user including:

>Bead-Assisted Sample Prep (BASP™), whereby reduction, alkylation and digestion are performed on the bead-bound proteome, without the use of detergents, seamlessly integrating to LC-MS analysis,

OR

>Optional Elution to off-bead digestion (i.e., FASP), or other common functional or immunoassay analyses

BIOTECH SUPPORT GROUP

Product	Size	Total serum/plasma samples processed	Item No.
NRicher™ C	10 Preps	10 x (25-50) µl samples	NRCO-10
NRicher™ C	50 Preps	50 x (25-50) µl samples	NRCO-50

Processes 25-50 µl serum per prep. It is recommended that the volume be optimized for the application. For example, when recovery is paramount for quantitative targeted SRM/MRM enrichments investigations, smaller volumes may be better.

For best results – the lysate should be clear and free of colloidal material. We recommend first filtering through a 0.45 µm syringe, or microfuge-type filter before beginning the prep.

The centrifugation time may vary, adjust as necessary to get complete filtration through the beads.

The protocol can be scaled up or down proportionally to adjust for different volumes. The bead amount can be adjusted to accommodate more or less Complement capture.

In bold are the **NRicher™** kit components.

Items Required	10 Prep	50 Prep	Reagent
NRicher™ C Beads	0.25 gram	1.25 gram	Supplied
Binding Buffer NRBB (0.05M HEPES, pH 6.0)	5 ml	25 ml	Supplied
Wash Buffer NRWB (0.05M HEPES, pH 7.0)	12 ml	60 ml	Supplied
Elution Buffer NREB (0.25M Tris + 0.5M NaCl, pH 9-10)	3 ml	15 ml	Supplied
Spin-filter & tube assemblies*	10	50	Supplied
DTT, Iodoacetamide, Trypsin and Formic Acid, 50% Acetonitrile (ACN)			Not Supplied

***Additional Spin-Filters (low protein binding, 0.45 µm filter element) can be purchased separately, please inquire.**

If there are any questions about compatibility or substitution with other buffers, please contact us.

Protocol For Enrichment of Complement Proteins from Serum/Plasma, & On bead Digestion For LC-MS Analysis

Optional Elution Protocol is included for Off-bead digestion or any functional, enzymatic, or immunoassay analysis

BIOTECH SUPPORT GROUP

1. **Bead Conditioning.** Weigh out 25 mg of **NRicher™ C** beads in a spin-filter. Add 150 µl of **Binding Buffer NRBB.** Vortex for 5 minutes at room temperature followed by centrifugation for 2 minutes at 5,000-10,000 rpm (2,000-8,000xg). Discard the filtrate. Repeat step-1.
2. **Sample Processing.** Add 200 µl of **Binding Buffer NRBB** to beads followed by (25 to 50) µl of the Serum to the beads. Vortex or mix thoroughly for 10 min and then centrifuge for 4 minutes at 5,000-10,000 rpm (2,000-8,000xg).
3. To the beads, add 500 µl of **Wash Buffer NRWB.** Vortex for 5 min and centrifuge for 4 minutes at 5,000-10,000 rpm (2,000-8,000xg). Discard the **Wash** filtrate.
4. **After discarding the wash from step 3, the NRicher™ beads contain the enriched sub-proteome. As an option for LC-MS sample preparation, the bead assisted on-bead digestion protocol (BASP™) is provided starting on step 6, see box below.**

   Optional Bead Elution. To the beads, add 300 µl of **Elution Buffer NREB.** Vortex or mix thoroughly for 10 min and centrifuge for 4 minutes at 5,000-10,000 rpm (2,000-8,000xg). Recover the filtrate as the eluted sub-proteome (0.25M Tris + 0.5M NaCl, pH 9.0-10.0), suitable for further analysis.

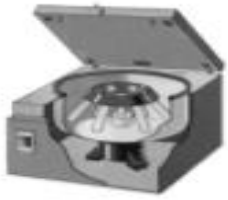
The bead assisted on-bead digestion protocol (BASP™) is provided below. The digest buffer is **Wash Buffer NRWB** (0.05M HEPES, pH 7.0). Comparable buffers (0.02-0.10M, pH 6-7) can be used. Higher pH buffers are not recommended.

5. Using **Wash Buffer NRWB**, prepare to 10mM of DTT concentration, and add 100 µl to the **NRicher™** beads and vortex for 10 minutes and incubate for 30 minutes at 60C.
6. Cool the samples to RT, add suitable volume of Iodoacetamide to 20mM and incubate in the dark for 45 minutes.
7. Centrifuge at 5,000-10,000 rpm (2,000-8,000xg) for 5 minutes, and discard filtrate. Rinse the bottoms of the spin-filter tubes with 500 µl of 50% ACN, **Wash Buffer NRWB** twice, to remove any traces of the filtrate.
8. Add 8 µg trypsin in 100 µl **Wash Buffer NRWB** to the **NRicher™** beads and keep at 37°C for a minimum 4 hours to maximum overnight. Overnight is recommended to start with. In select targeted circumstances, 2 hours may be sufficient.
9. Centrifuge at 5,000-10,000 rpm (2,000-8,000xg) for 5 minutes, and retain digested peptides filtrate.
10. To further extract remaining peptides, add 150 µL 10% formic acid, vortex 10 min, centrifuge at 5,000-10,000 rpm (2,000-8,000xg) for 5 mins., and combine this volume with volume from step 10.
11. Total is about 250µl. Prepare to desired final concentration. Store at -80°C until LC-MS/MS.

BIOTECH SUPPORT GROUP

NRicher™ Beads Are Versatile to A Variety of Bead Processing Formats

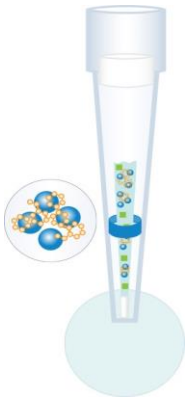
Microfuge Spin-filter is our standard



Other formats compatible with the 50 µm NRicher™ beads are:

High Throughput Automation Compatible INTip™ SPE (DPX Technologies) Format

Aspirate and dispense cycles mix NRicher™ beads and solutions



The INTip™ SPE tip format improves ease of use and scalability to process multiple samples in parallel, utilizing 96-well plates and automated liquid handlers. The tip-based formats have been proven to be compatible with most automation platforms, i.e., Integra, Hamilton, etc. Please inquire for more information, as these formats are customized to the application and automation platform.

96-Well Vacuum or Pressure Filter Format

The NRicher™ beads can be readily processed in 96-well filter formats. Please inquire.

BIOTECH SUPPORT GROUP

Related Separations, Enrichment/Depletion & Sample Prep - All Product Categories
(https://www.biotechsupportgroup.com/Products-a-z_a/258.htm)

Albumin & IgG Removal
(https://www.biotechsupportgroup.com/Articles.asp?ID=451)

Lipid Removal and Clarification
(https://www.biotechsupportgroup.com/Articles.asp?ID=456)

Hemoglobin Removal
(https://www.biotechsupportgroup.com/Articles.asp?ID=452)

Sample Prep – Liquid Biopsy
(https://www.biotechsupportgroup.com/Articles.asp?ID=457)

Sample Prep – Glyco, Virus, Kinase, Aqueous Protein Crash/Metabolomics
(https://www.biotechsupportgroup.com/Articles.asp?ID=453)

Sample Prep – Mass Spectrometry
(https://www.biotechsupportgroup.com/Articles.asp?ID=432)

Sample Prep – Genomics
(https://www.biotechsupportgroup.com/Articles.asp?ID=455)

CONTACT US

We welcome your questions and comments regarding our products.

Tel: 732-274-2866, 800-935-0628 (North America) Mon – Fri 9am-6pm EST.
Email sales@biotechsupportgroup.com
Web: www.biotechsupportgroup.com