FRACTIONATE

Protein Fractionation Techniques, HPLC and OFFGEL Fractionation

Tobias Preckel and Taegen Clary

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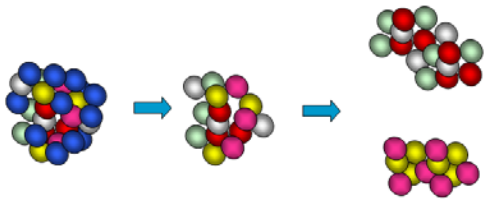
Introduction

OFFGEL electrophoresis

- separates proteins or peptides according to *pI*
- based on the resolving power of immobilized pH-gradient (IPG) gels
- performs traditional in-gel IEF and OFFGEL electrophoresis
- OFFGEL mode provides analytes in solution
- compatible with LC-MS and upfront sample preparation techniques



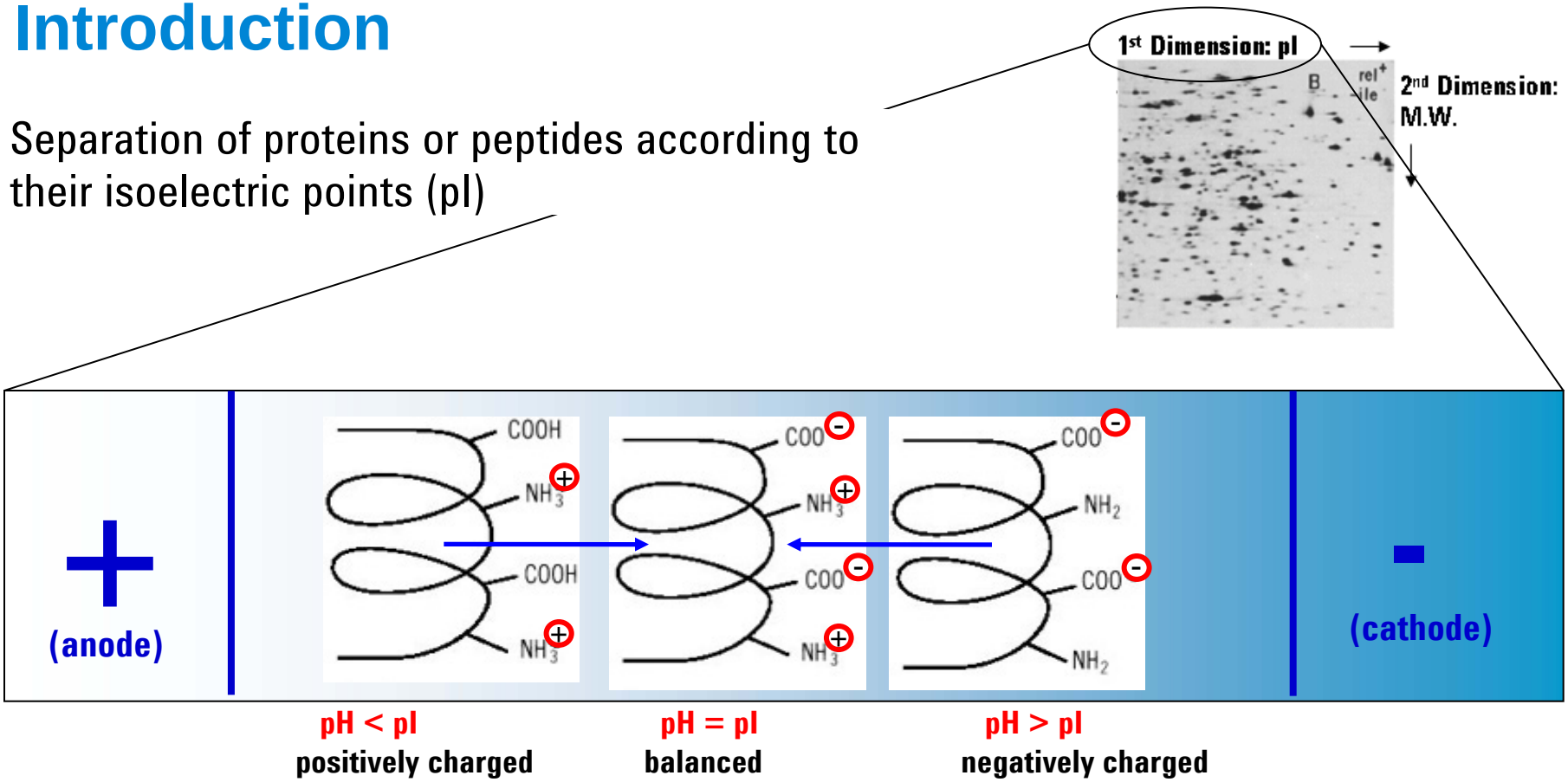
Target Applications

Biomarker discovery, protein ID, differential expression and PTM (post-translational modifications) analysis, phosphopeptide analysis, protein characterization

Analysis of recombinant protein isoform impurities

Introduction

Separation of proteins or peptides according to their isoelectric points (pI)



pI-based Fractionation:
OFFGEL principle

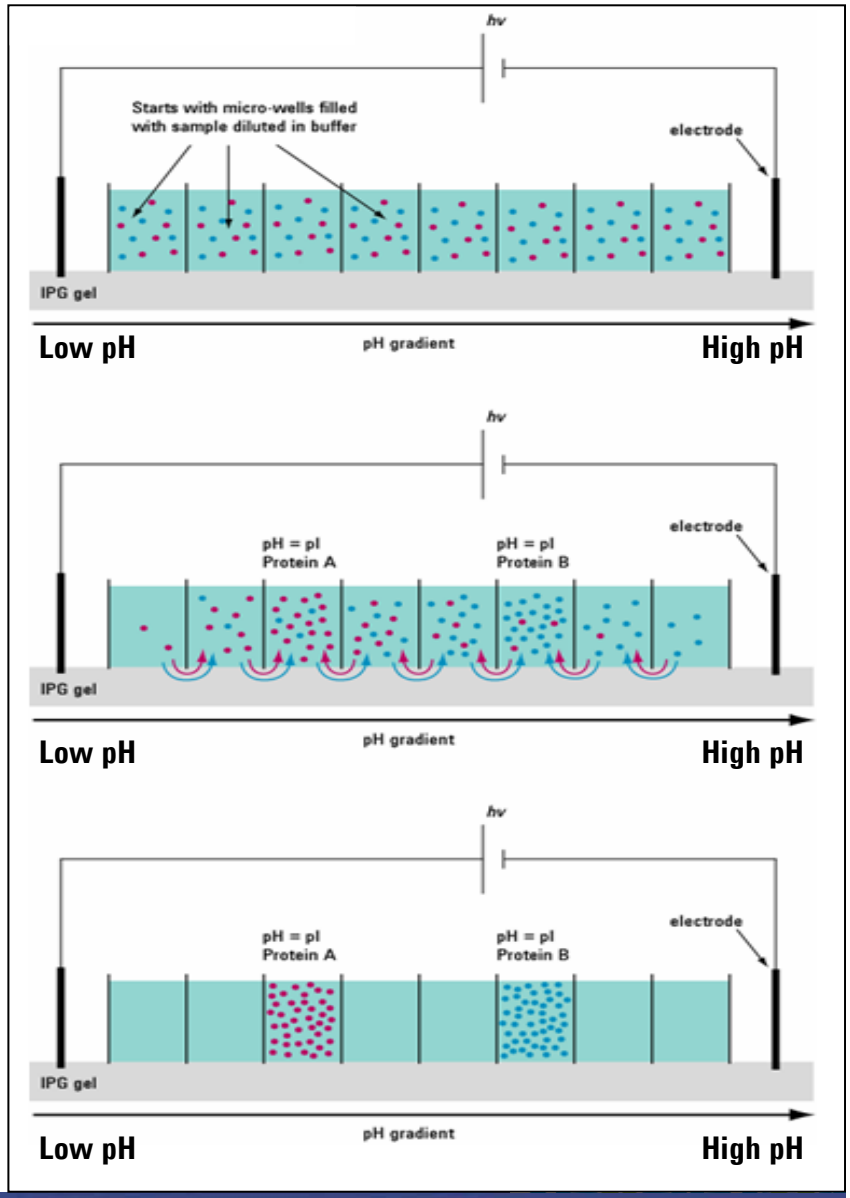
- after rehydration the IPG gel seals against the frame
- diluted sample is distributed across all wells
- liquid fractions can be removed with a pipette

Number of fractions: 12 or 24

Fraction volume: 150 µl

Fractionation time: 8 - 36 h

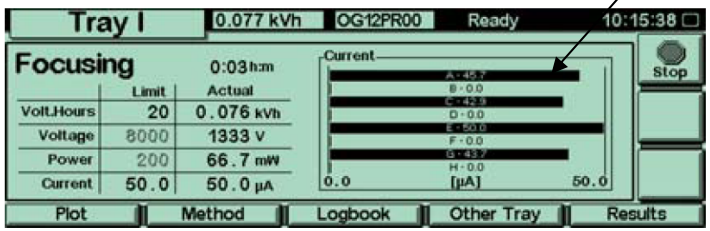
Recovery: 70% proteins
>80% peptides

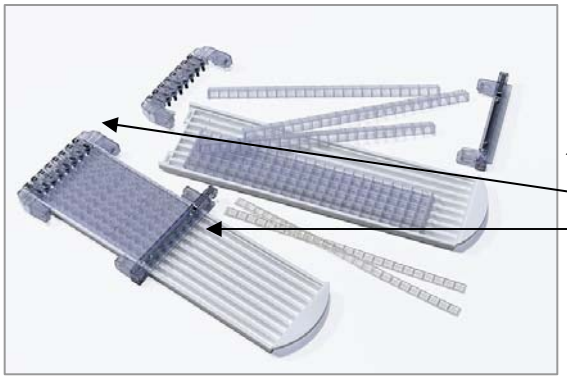


Instrumentation

- Local controller with preinstalled software
- Validated methods
- Online view and storage of run parameters (voltage, current, temp.)
- Runs 16 samples in parallel
- Current measurement for each sample

- Reagent and consumable kits containing plastic materials, reagents and IPG-gels





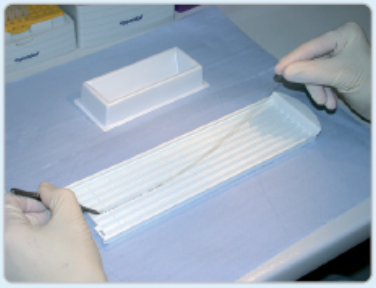
Gel strips are placed in tray grooves (8 per tray)

Fixed and movable electrode

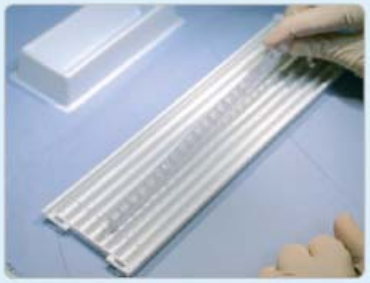
OFFGEL Workflow

overview

Five easy steps to set up and run a fractionation



1. Place a dry IPG gel strip in the tray.



2. Place a well frame over the IPG gel strip, pipette 20 µl rehydration solution into each well and allow the IPG gel to swell.

3. Pipette 150 µl of the diluted sample into each well and close the frame with a cover seal.

4. Attach the electrodes to the tray.

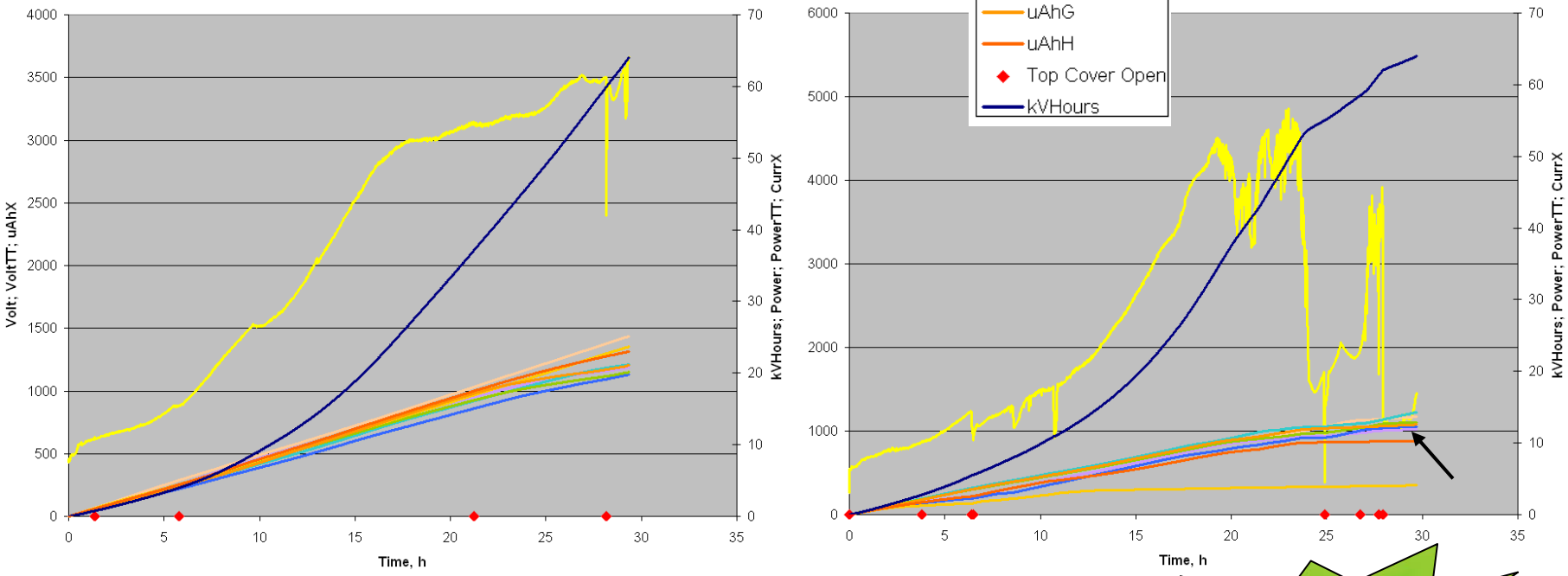
5. Place the loaded tray into the fractionator and press "Start".

3100 Features

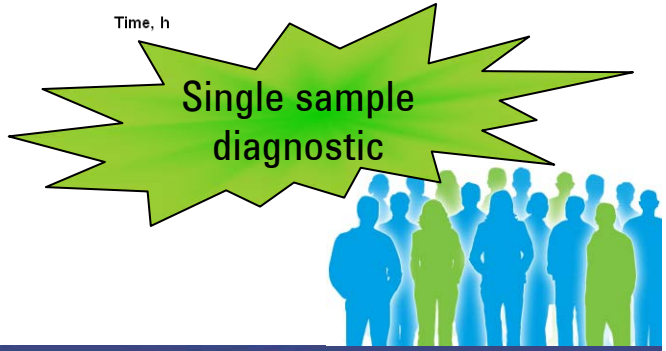
- **Protein/peptide fractionation with 0.1 – 0.6 *pI*** resolution
- **µg to mg load** capacity **(50 µg – 5 mg per sample)**
- **Possible to run conventional** in-gel **IEF as well as** OFFGEL **fractionation**
- Diagnostics**: Online current control <u>for each individual sample</u> allow to check fractionation quality & progress of each individual sample**

OFFGEL Fractionation:

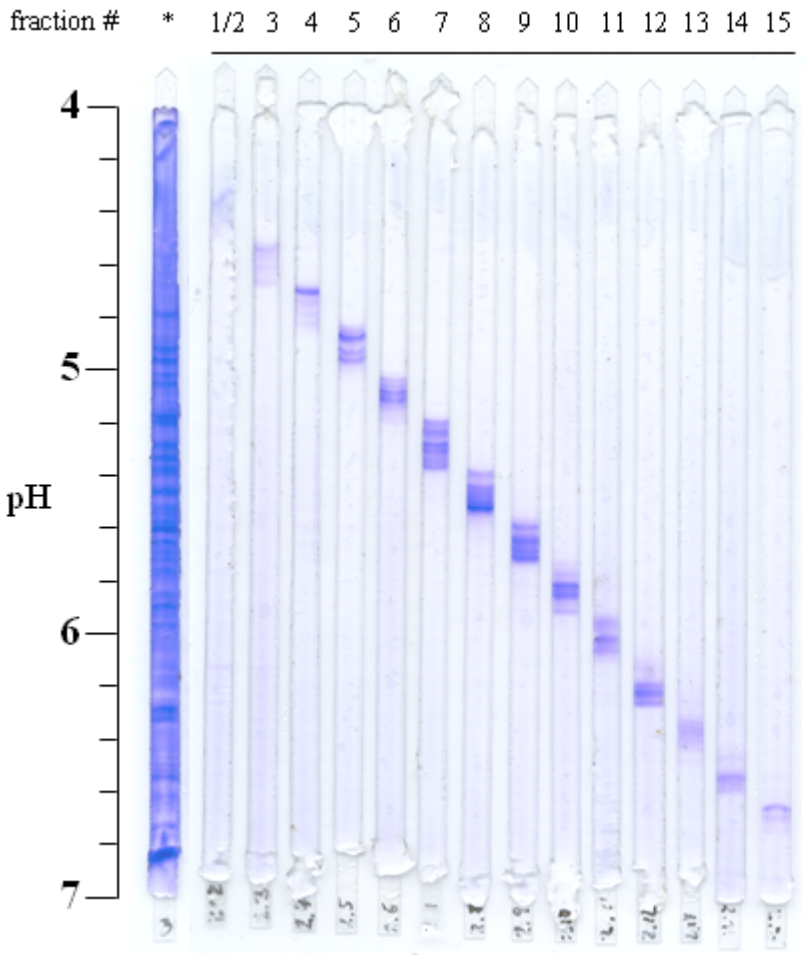
Current/Voltage Logfile



Logging of voltage and current for every sample allows quality control of fractionation prior to expensive LC-MS !



Analysis of OFFGEL Fractions by IEF



E. coli cell extract

Coomassie Brillant Blue stain

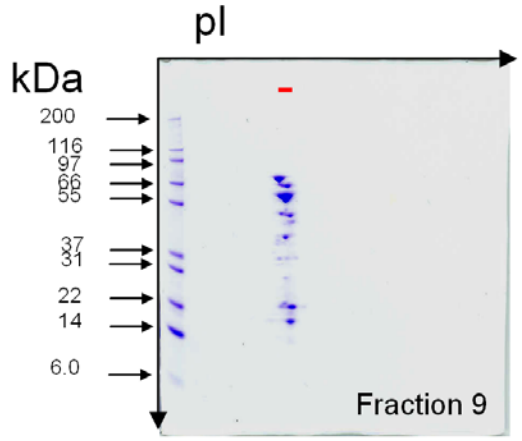
* unfractionated sample

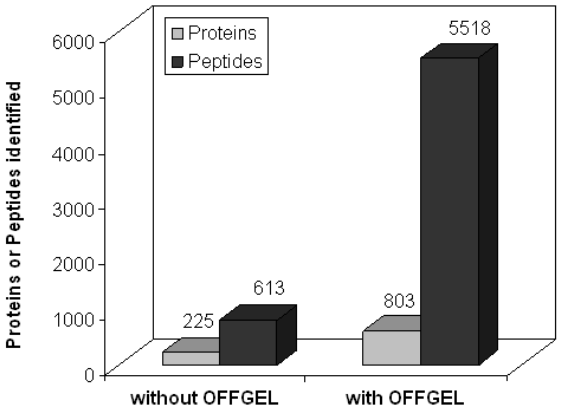
E.coli lysate

Standard in-gel IEF and staining

OFFGEL Protein Fractionation

4 times more proteins detected with OFFGEL





E.coli lysate

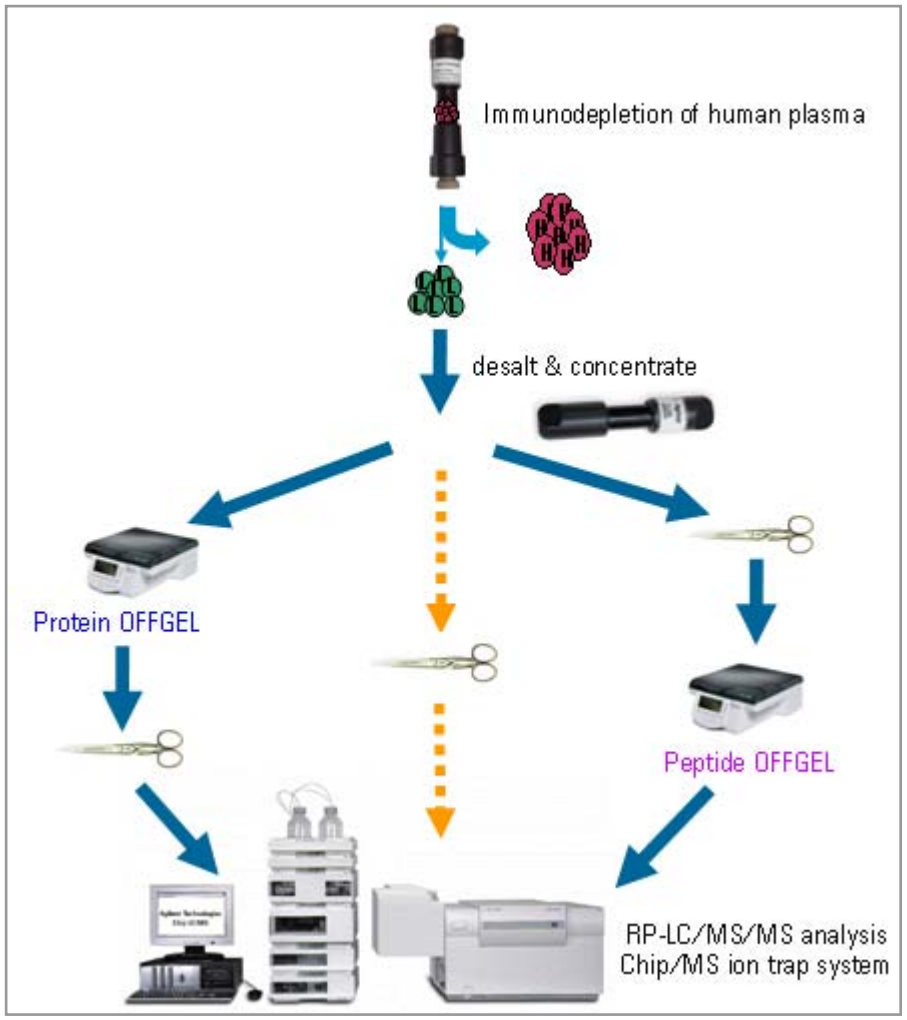
pH 4-7, 24 fractions

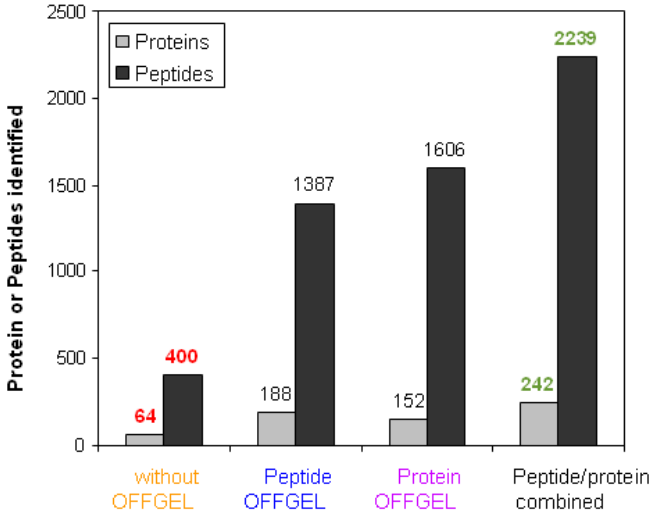
digest

HPLC/MS/MS

OFFGEL Increases MS Sensitivity

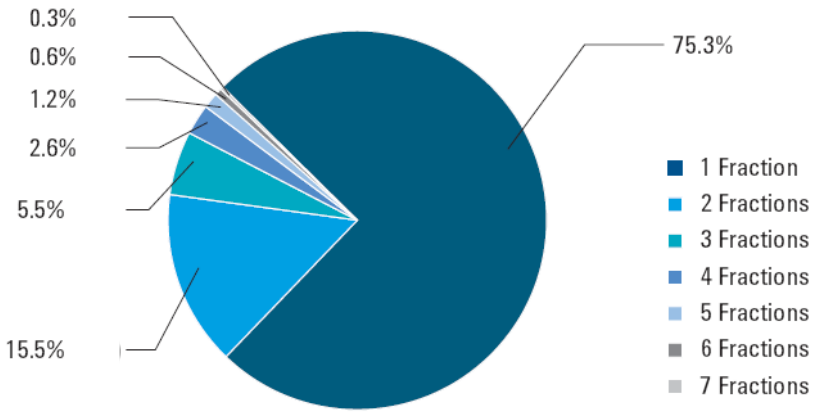
example workflow





4-fold increase of detected proteins due to OFFGEL fractionation

OFFGEL Peptide Fractionation



=> Minimal overlap: 90% of peptides are found in 1 or 2 fractions!

Number of OFFGEL fractions containing each individual peptide (absolute numbers of peptides in parenthesis)

Combination of IEF with SDS-PAGE

Agilent 3100 OFFGel Fractionator + 2100 bioanalyzer

Complex protein mixture

↓

Sample clean up
(chemicals are removed that interfere with labeling reaction)

↓

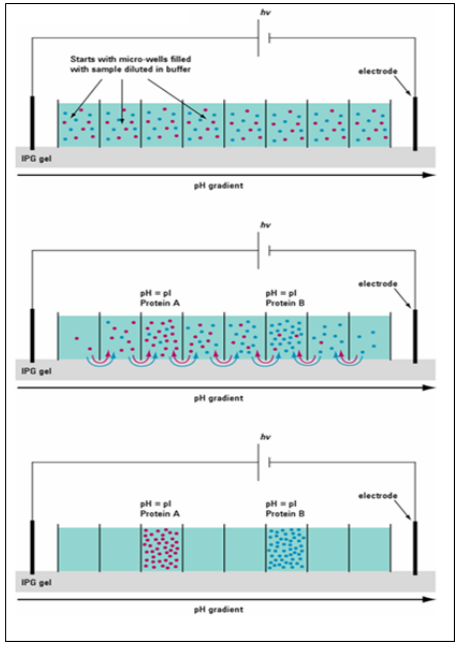
Labeling reaction with fluorescent dye

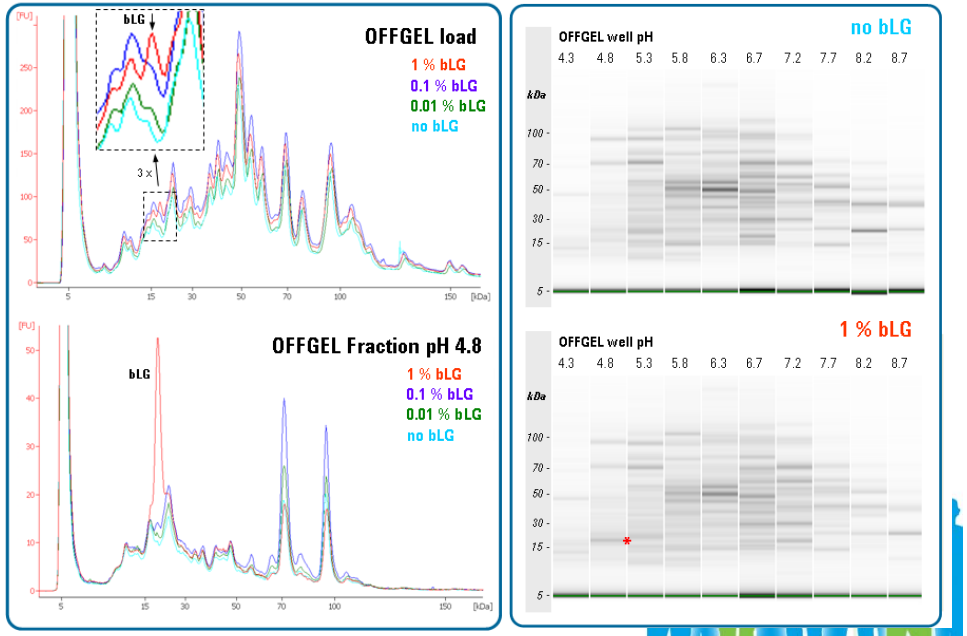
↓

OFFGEL electrophoresis

↓

High sensitivity protein sizing on the Bioanalyzer





Summary

- OFFGEL reduces sample complexity of protein or peptide samples by providing fractions in liquid phase
- OFFGEL electrophoresis provides pI-information as an additional identification marker which may be used to validate MS results
- special features:
    - ability to run in-gel & OFFGEL mode
    - 16 samples on two separate power supplies
    - online diagnostic check of fractionation quality
    - specifically for OFFGEL mode
        - highest resolution (0.1 *pI*)
        - µg to mg load capacity

www.agilent.com/chem/offgel

Agilent's Proteomics Sample Preparation Workflow

MARS

High Abundant Protein Removal

Multiple Affinity Removal System

OFFGEL

pI-based Protein Fractionation

OFFGEL Electrophoresis System

mRP

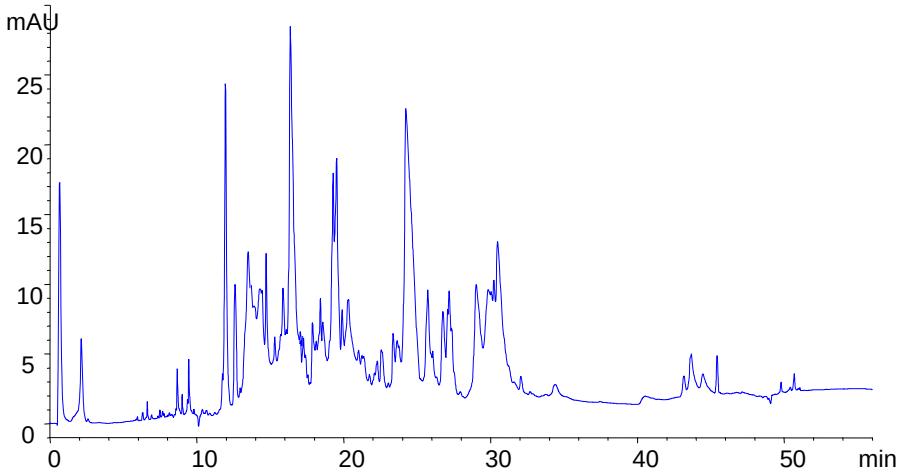
Protein Fractionation

Macroporous Reverse Phase Column

mRP (Macroporous Reverse Phase) Column

High Recovery Protein Fractionation

mRP Column



mRP-C18 Protein Fractionation Column

What is it?

Reverse Phase column for protein separation and fractionation. The silica based particles and recommended LC methods have been optimized for:

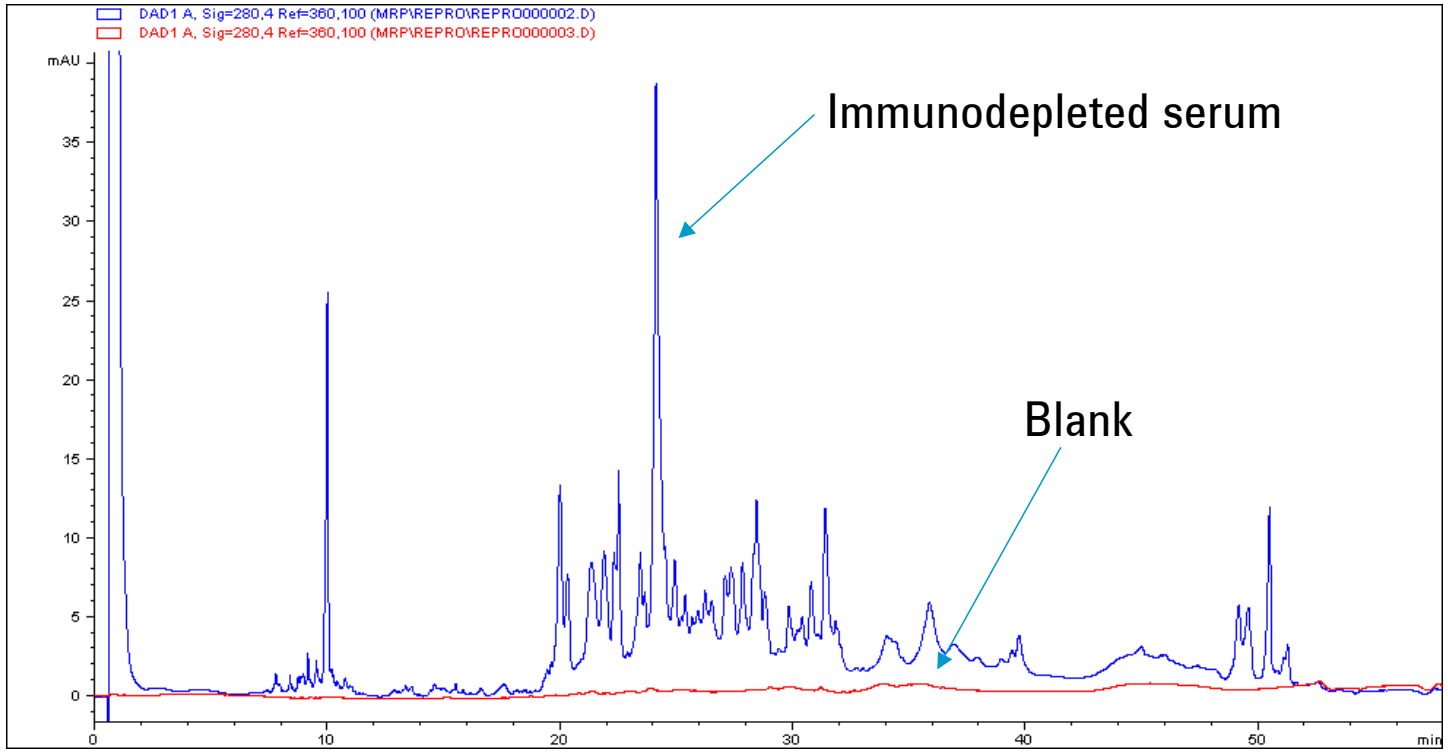
- Highest recoveries of protein samples (95% - 99% of loaded sample)
- Highest resolution separations
- Reproducibility
- High sample loading capacity (3X higher than most standard RP columns)
- Lifetime

mRP-C18 Protein Fractionation Column

Key Applications:

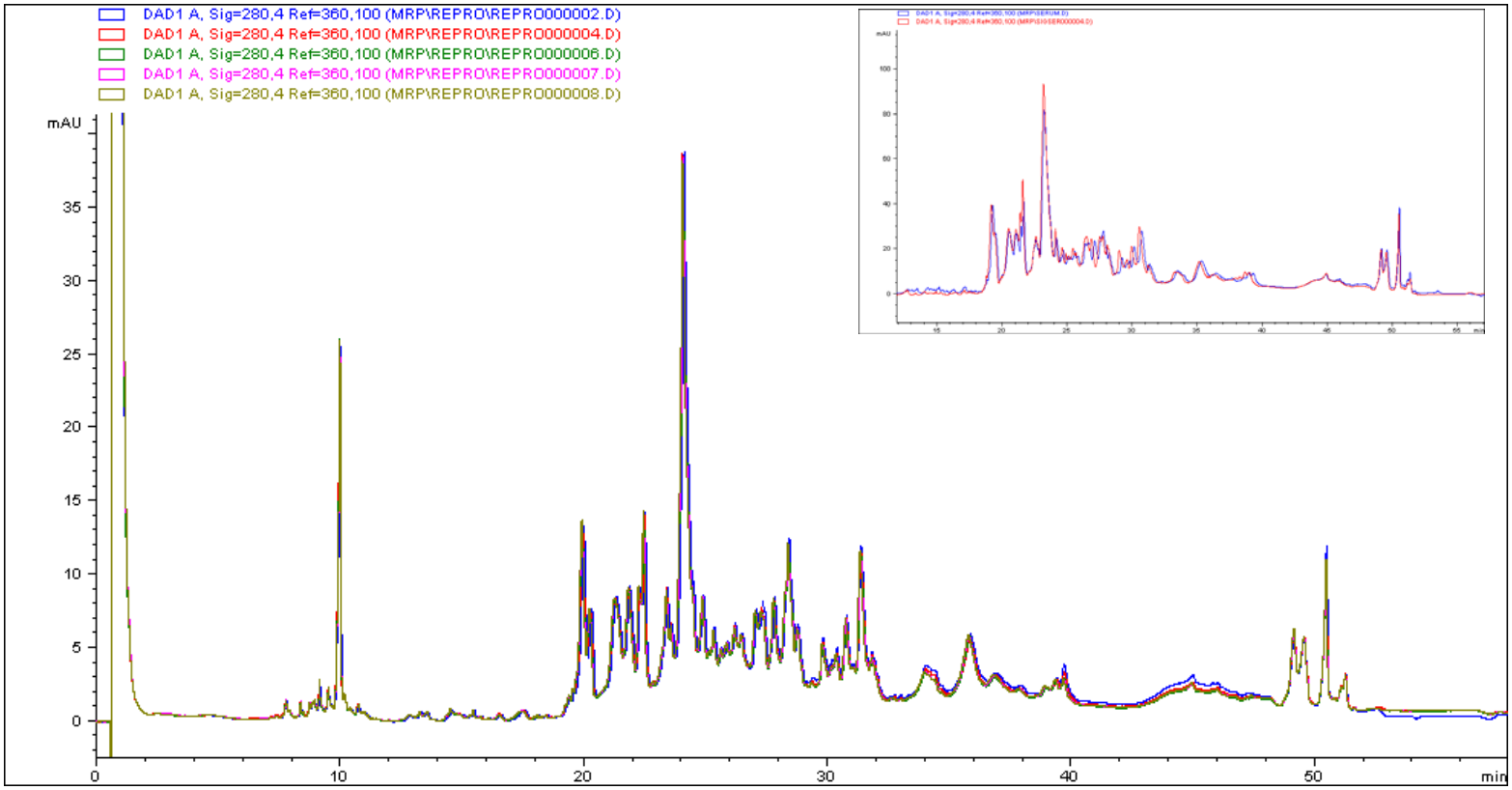
- Positioned to be used after MARS protein depletion for further fractionation (eliminates need to concentrate & de-salt)
- Can be used for a wide variety of sample types for protein prefractionation, desalting and concentrating applications, including:
  - Whole cell lysates
  - High recovery membrane protein fractionation

mRP-C18 Recovery using Immunodepleted Serum

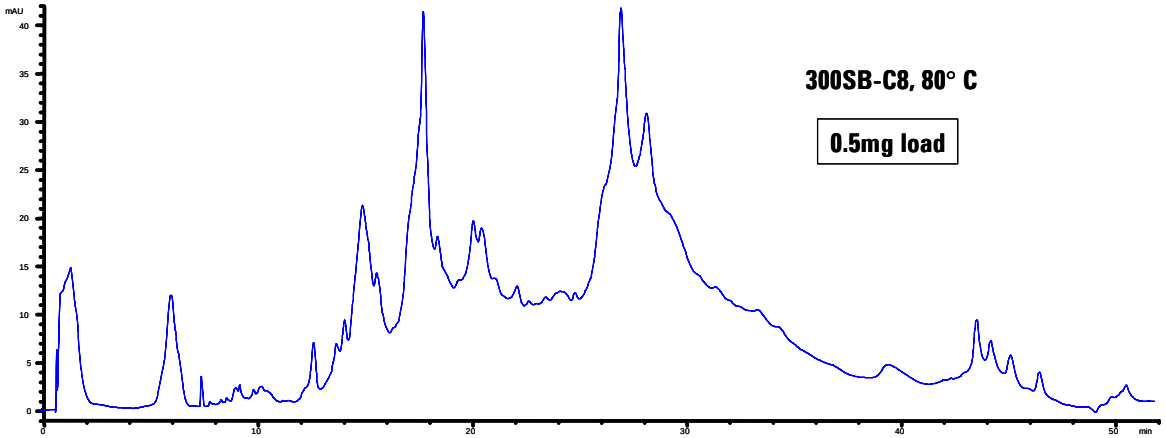


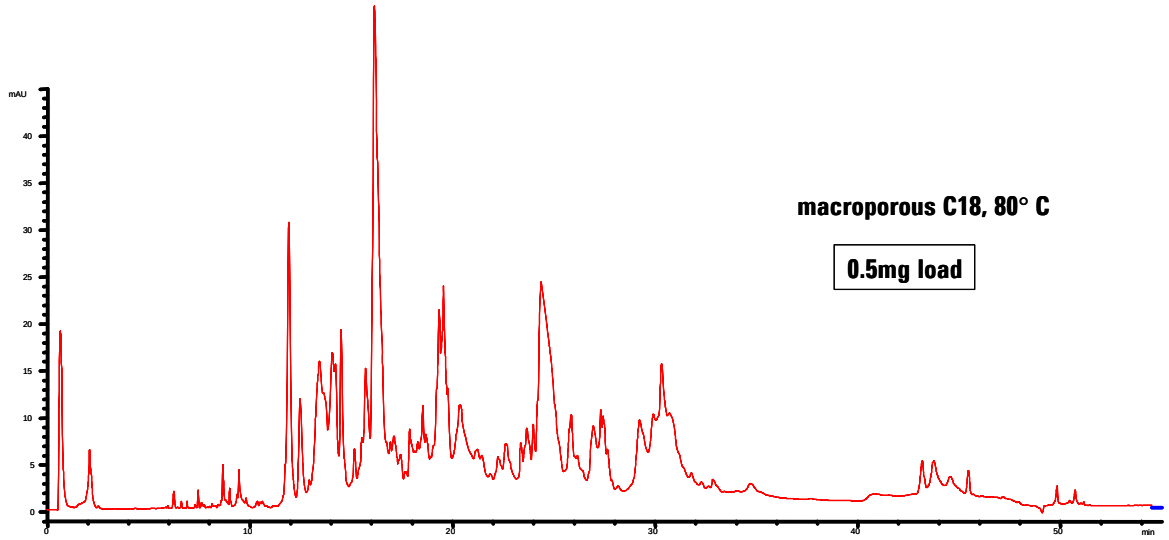
Protein Conc. Pre-mRP*	Protein Conc. No column	Protein Conc. mRP recovery	% recovery
47 µg	49.8 µg	49.3 µg	99%

mRP-C18 Reproducibility



RP Load Tolerance Comparisons



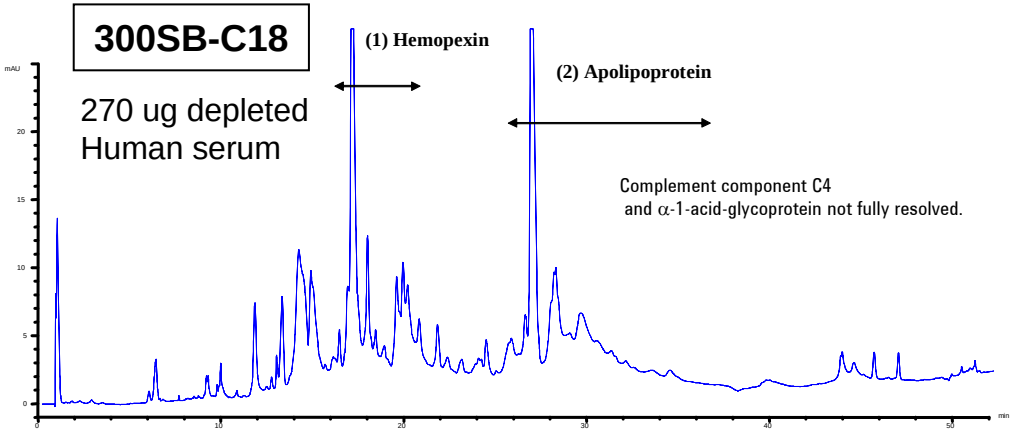


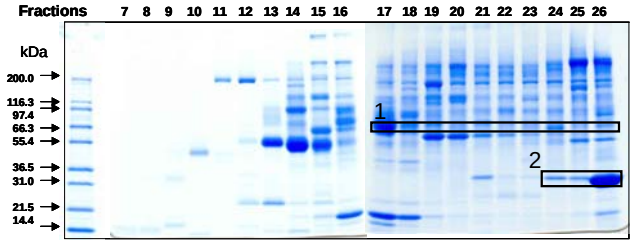
Conditions: mRP-C18, 4.6 mm ID x 50 mm; 0.75 mL/min.
Sample: Immunodepleted Human serum (500 ug Protein) in 6M urea/1% HOAc
A – 0.1% TFA in water, B – 0.08% TFA in AcN
3-30%B in 6 min, 30-55%B in 33 min, 55-100%B in 10 min

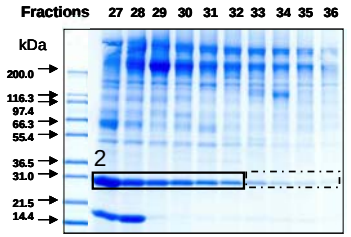
Column Comparison of Separation Efficiency

300SB-C18

270 ug depleted Human serum

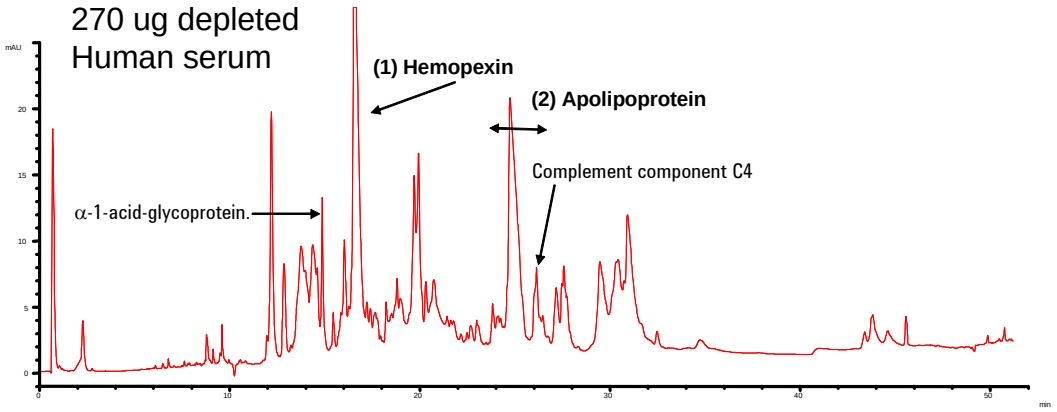


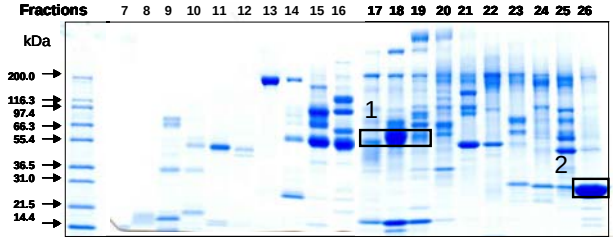


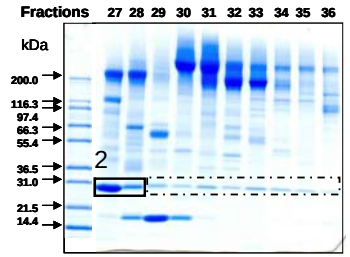


Macroporous Reverse Phase C18

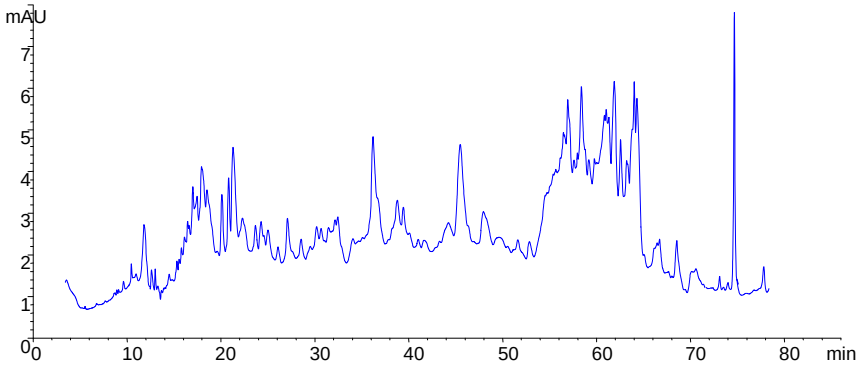
270 ug depleted Human serum



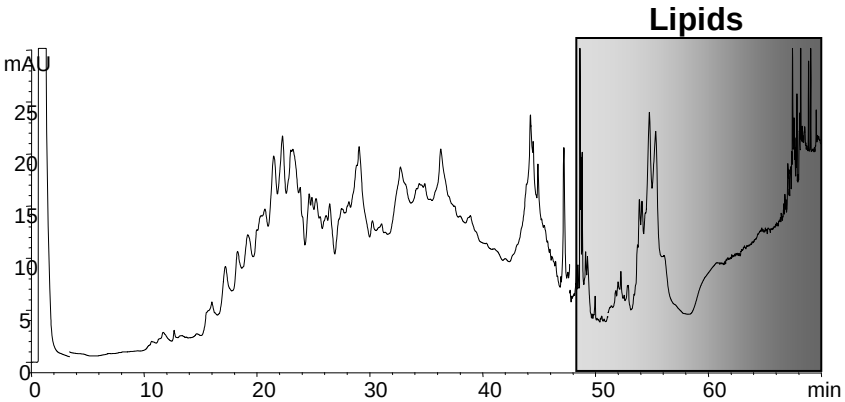




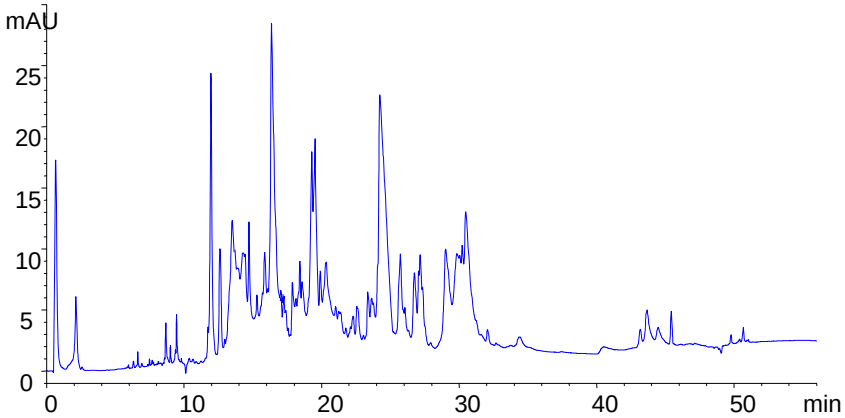
Applications for mRP-C18: Protein Fractionation



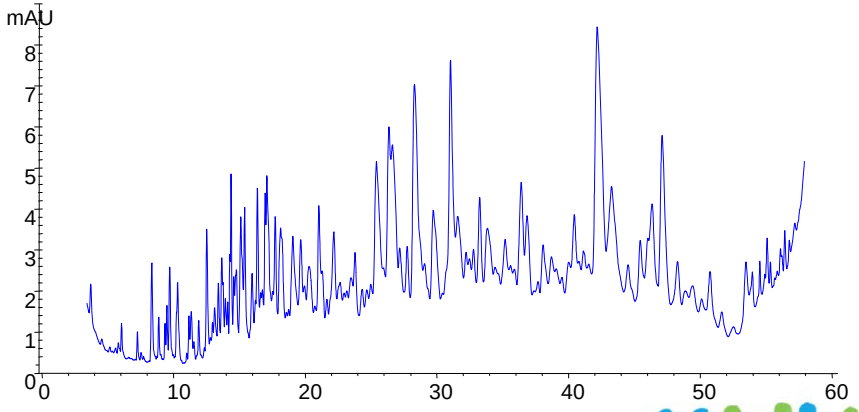
Hela Membrane Prep



Human Brain membrane lipid Raft prep(500µg)



Depleted human serum



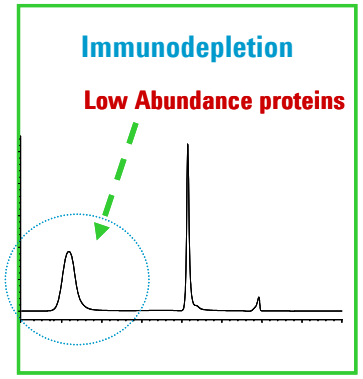
HeLa cell lysate (352µg)

(4.6 x 50mm mRP-C18)

mRP Fractionation of Depleted Human Serum

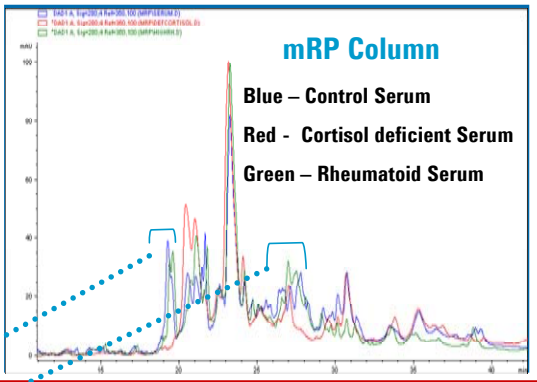
1D

A. Removal of 6 most abundant Proteins



2D

B. Fractionation of the low abundant proteins

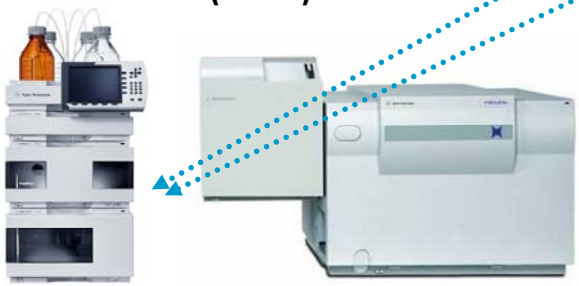


LC/MS system

2 Fractions Analyzed by MS

Tryptic digestion

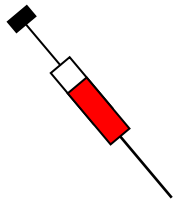
3D(+ 4D)



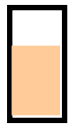
Data Analysis

serum # spectra total intensity	def. Cort. # spectra total intensity	High Rheumatoid # spectra total intensity	# Unique Peptides	Score	Protein
14 3.09E+07	0 0.00E+00	4 8.15E+06	12	178.37	H factor 1 (complement)
8 1.94E+07	0 0.00E+00	9 2.26E+07	8	115	apolipoprotein H (beta-2-glycoprotein I)
0 0.00E+00	3 4.65E+06	1 1.60E+06	3	39.47	ceruloplasmin
0 0.00E+00	0 0.00E+00	2 3.54E+06	2	30.34	complement component 1 inhibitor precursor
2 8.65E+06	0 0.00E+00	0 0.00E+00	2	28.61	apolipoprotein C-III precursor
1 1.84E+06	0 0.00E+00	2 3.13E+06	2	27.34	complement factor B preproprotein
0 0.00E+00	0 0.00E+00	2 3.40E+06	2	24.99	hemopexin
0 0.00E+00	0 0.00E+00	2 4.13E+06	2	24.77	alpha-1-acid glycoprotein 2 precursor

Serum Proteomics Workflow



Blood Sample from Patient

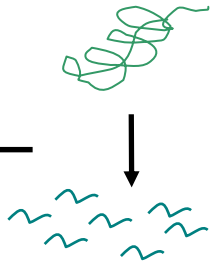


Serum/Plasma sent to lab

Remove High Abundant Protein

Fractionation of Proteins

Fractionation of Proteins and/or Peptides

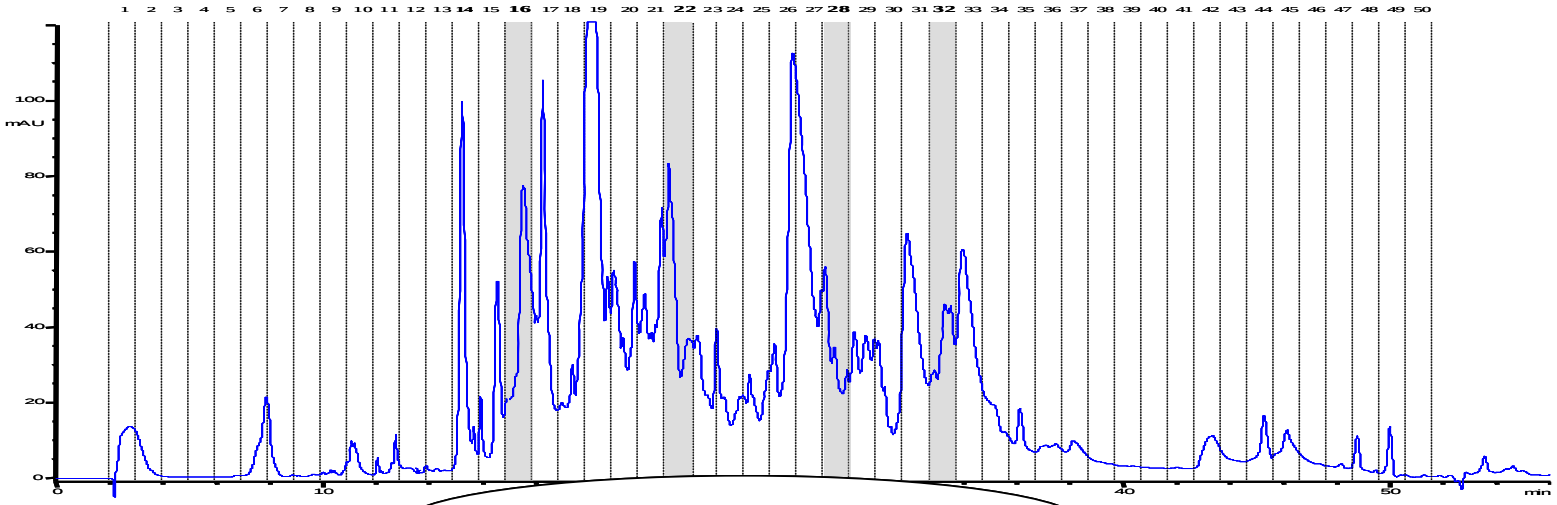


Trypsin enzyme digestion of proteins into peptides

1-D (Reverse Phase) or 2-D (Ion Exchange + Reverse Phase) Chromatography

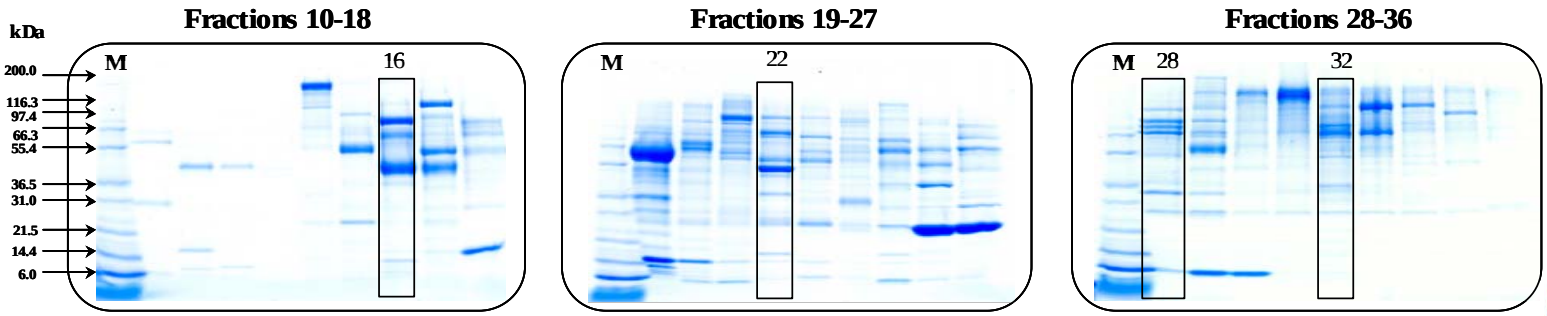
Mass Spectrometer + Software ID peptides and verify presence of protein (quantitation)

mRP Fractionation of Depleted Human Serum

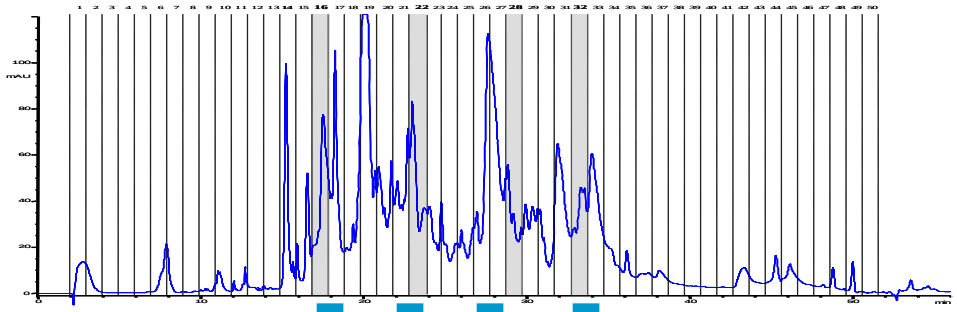


4 – 20% SDS PAGE (reducing)

M = Mark 12 Standards



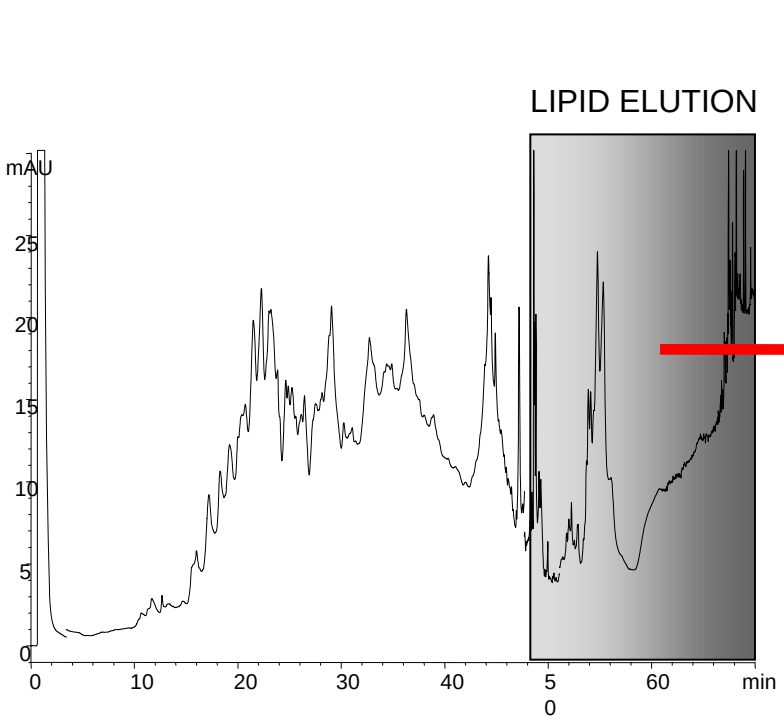
mRP Fractionation of Depleted Human Serum

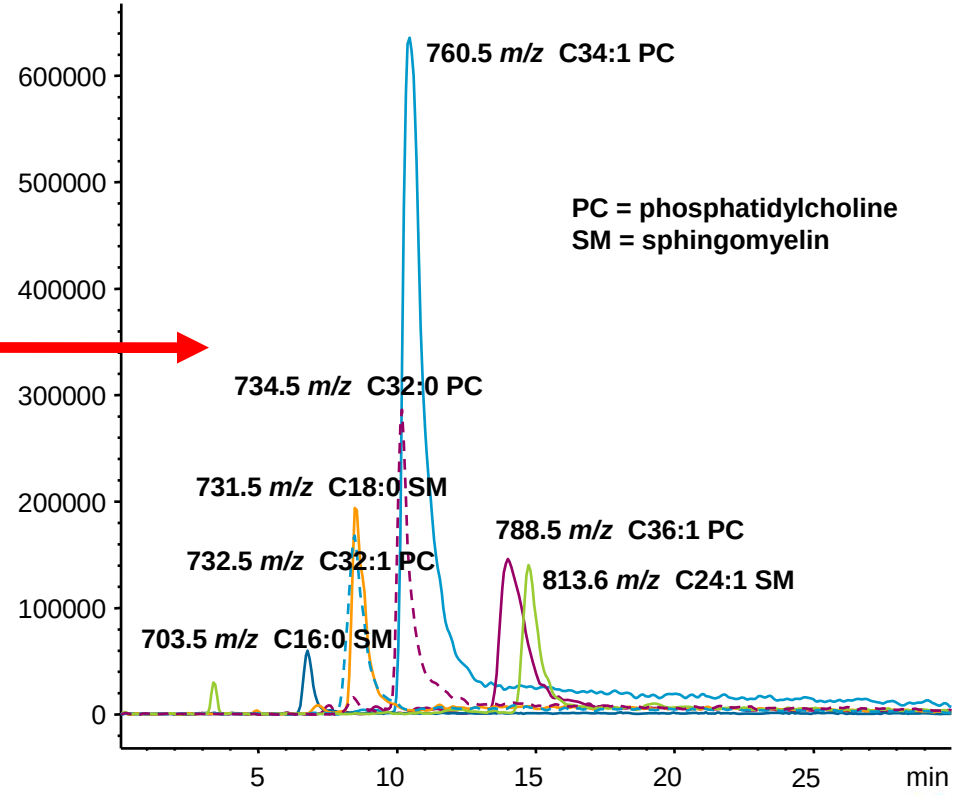


In-solution tryptic digest of each fraction

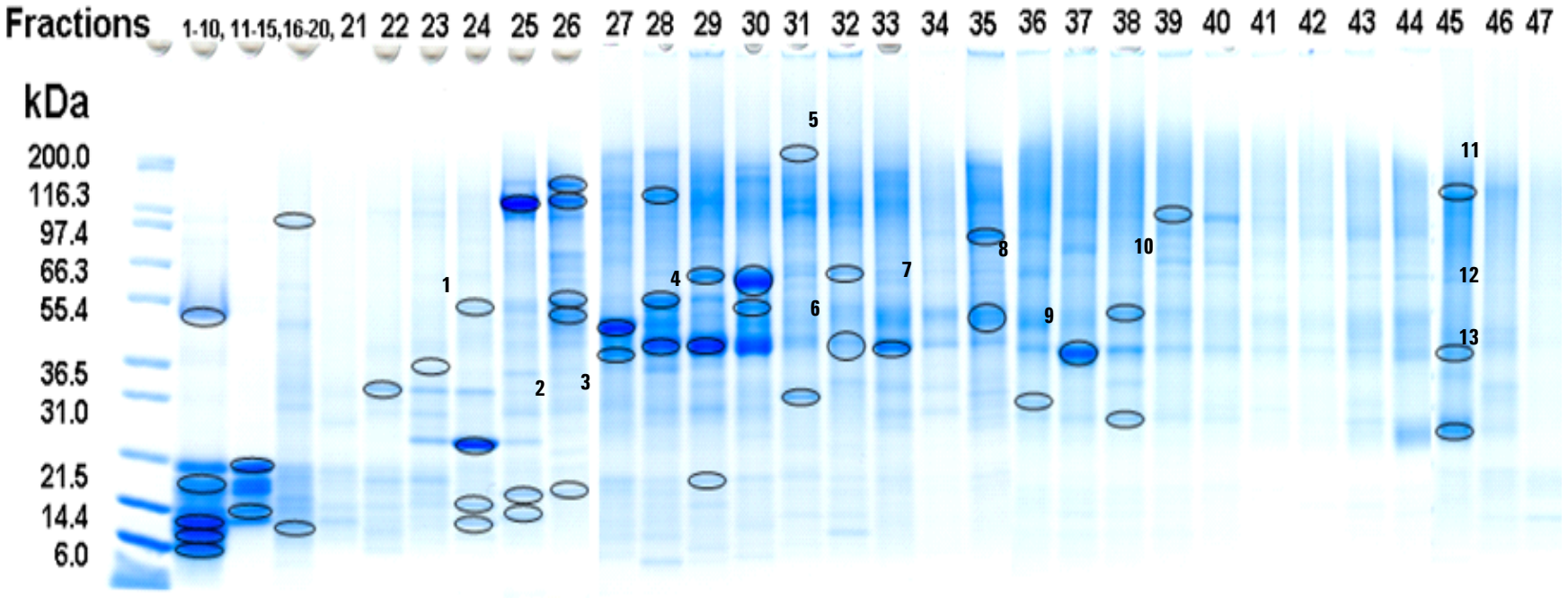
SAMPLE	Total Acquisition Time (hours)	# MS/MS Collected	# Proteins Identified
Human Serum**	36	67,997	40
Immunodepleted Human Serum	24	46,808	170
Fraction #16	22	59,767	144
Fraction #22	22	59,598	114
Fraction #28	22	58,473	96
Fraction #32	22	54,758	107
Combined Fractions	88	232,596	461

Human Brain Membrane Lipid Raft Prep (500µg)





Human Brain Membrane Lipid Raft Prep

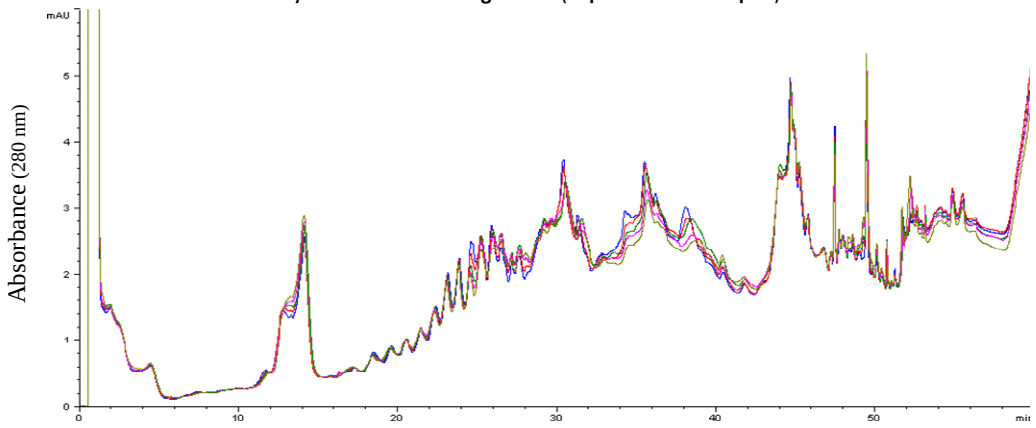


Selected Excised Bands Which are Intregal Membrane Proteins

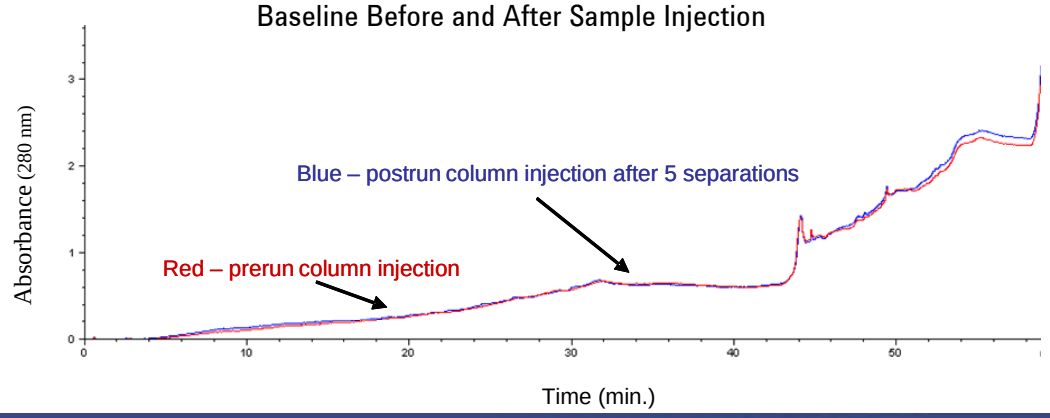
1. Voltage-Dependent Anion Selective Channel Protein 1
2. Cytochrome C Oxidase subunit IV (COX IV)
3. Cytochrome C Oxidase subunit IV (COX IV)
4. 2',3'-Cyclic-Nucleotide 3"-Phosphodiesterase (CNP)
5. Spectrin Alpha Chain, Brain (Alpha-II Spectrin)
6. Vacuolar ATP Synthase Subunit E
7. Creatine Kinase, B Chain
8. ATP Synthase alpha chain
9. Vacuolar ATP Synthase Subunit D
10. Vacuolar ATP Synthase Subunit B
11. Contactin Associated Protein
12. Vacuolar ATP Synthase Subunit C
13. ATP Synthase Chain B
14. Thy-1 Membrane Glycoprotein Precursor (Thy1)

Human Brain Membrane Lipid Raft Prep: Reproducibility and Baseline Stability

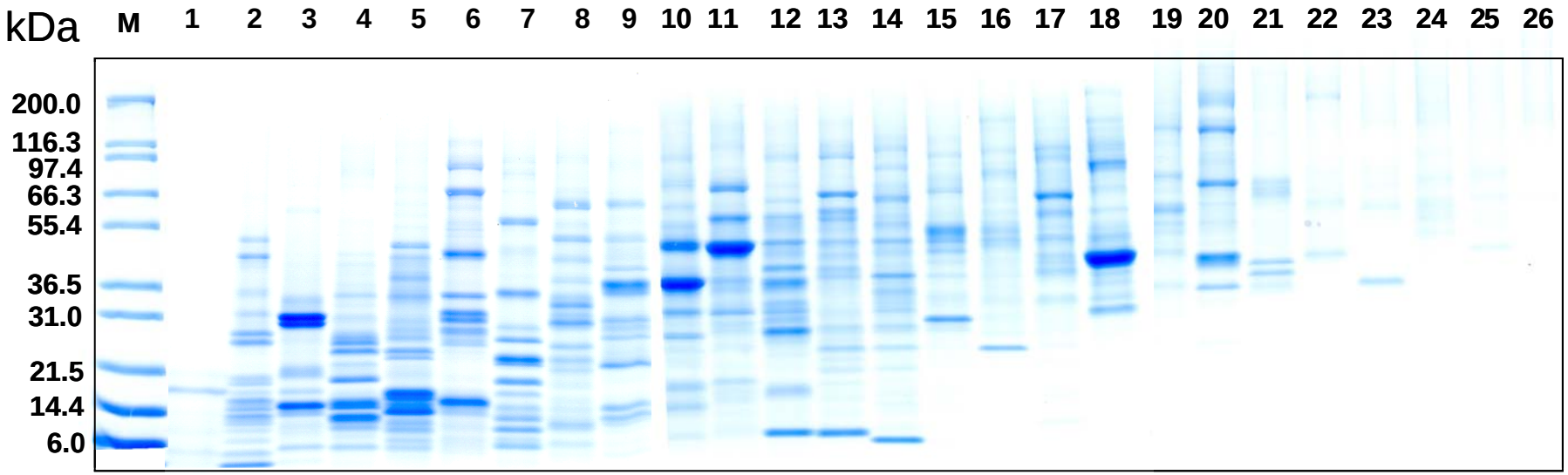
Overlay of 5 Chromatograms (Lipid Raft Sample)



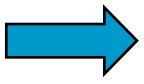
Baseline Before and After Sample Injection



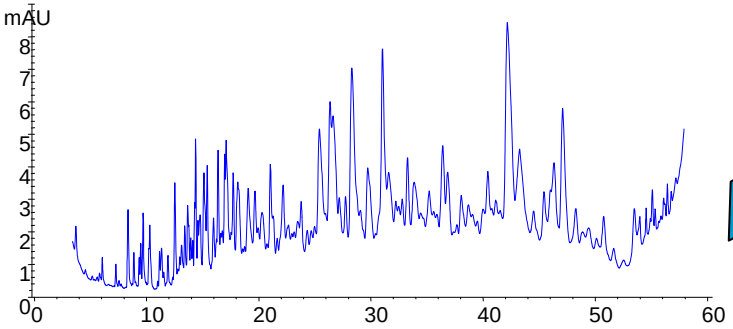
Hela Cell Lysate mRP Fractionation

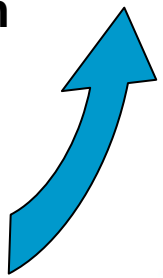


mRP Column

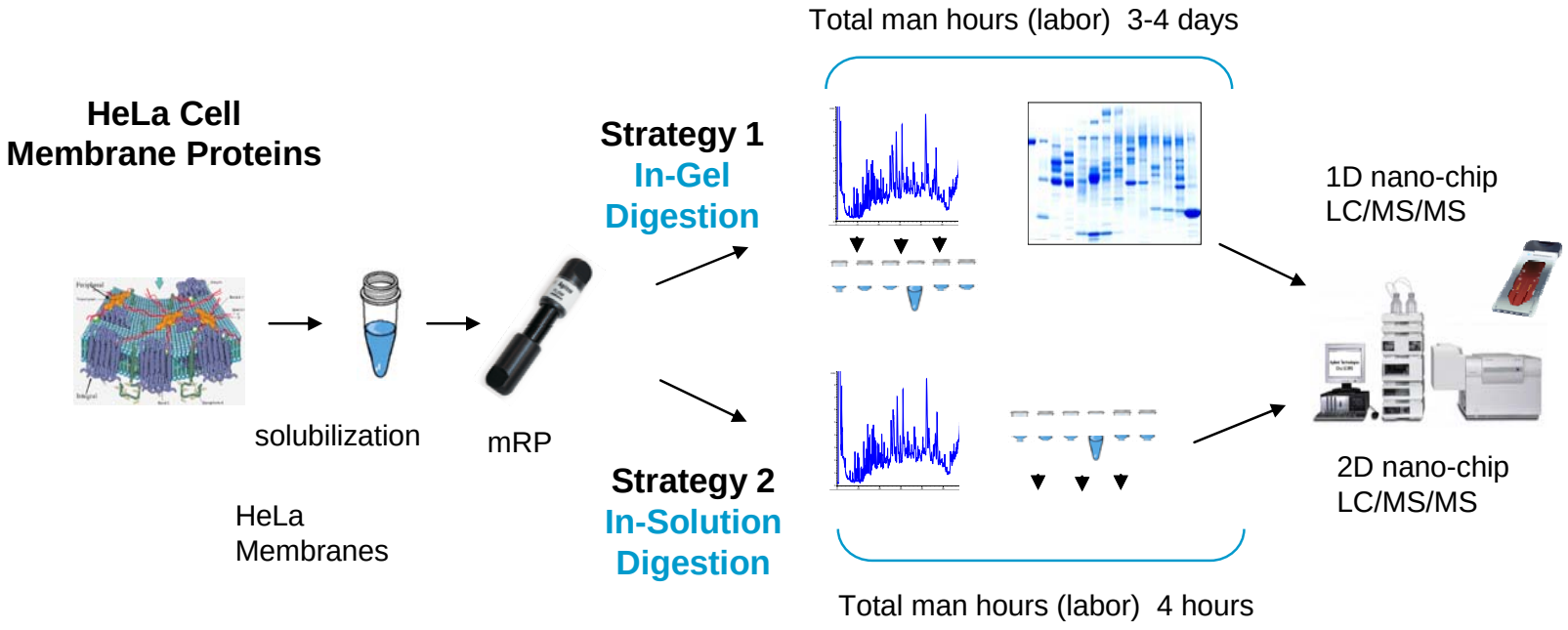


4.6 x 50mm mRP C18 column





Hela Cell Membrane Protein Fractionation and ID



		Total Acquisition Time (hrs)	# MS/MS Spectra Collected	# Distinct Peptides Matched	# Total Proteins Identified	# Membrane Proteins Identified	# Integral Membrane Proteins Identified
Strategy 1	**216 gel bands**	108	486,700	3841	688	364	286
Strategy 2	**17 mRP fractions**	102	412,741	5383	954	470	337

Summary

mRP is a Reverse Phase column for protein separation and fractionation, offering:

- Highest recoveries of protein samples (95% - 99% of loaded sample)
- Highest resolution separations
- Reproducibility
- High sample loading capacity (3X higher than most standard RP columns)
- Lifetime

Key Applications:

- Positioned to be used after MARS protein depletion for further fractionation (eliminates need to concentrate & de-salt)
- Fractionation of protein in a wide variety of sample types including:
  - Whole cell lysates
  - High recovery membrane protein fractionation

mRP-OFFGEL Selection Guide

	Fractionation of complex samples in-liquid phase for maximum resolution and sensitivity	
	Macroporous Reverse Phase (mRP)	**OFFGEL Electrophoresis**
Brief Description	HPLC Column for protein fractionation	Instrumentation and consumable kits for protein and peptide fractionation
Target Applications	Protein Discovery & Characterization, Biomarker Validation	Protein Discovery, Protein Characterization
sample types	proteins	proteins or peptides
Operating conditions	70-80C, reverse phase	cooled samples, aqueous buffer
Dowstream Compatibility	LC-MS*, LC-UV, MALDI*, 2-D gels	
loadable sample amount	2µg-380 µg	50µg-5mg
fractionation principle	**reverse phase (hydrophobicity)**	**isoelectric point (*pI*)-based**
run-time	60 min/gradient	8 hours-36 hours, up to 16 samples in parallel
recovery	95-99%	protein: 70%, peptides: >80%
Knowledge Transfer	no need for additional HPLC training, similar to any other reverse phase separation	2-D gel users can easily transfer IEF parameters to OFFGEL fractionations or validate OFFGEL vs. in-gel IEF
How to use mRP and OGE together	OFFGEL protein fractionation followed by mRP for second dimension fractionation and desalting	

* *pI* of fractions can be used as an additional validation parameter for MS results

Thank you for attending!

To learn more about the sample simplification with the Multiple Affinity Removal System, visit the NEW **Solution Source** for BioSeparations at **www.agilent.com/chem/fractionate1**

To reserve your space for the next e-Seminar, register today.

Upcoming e-Seminars

SEPARATE - Reverse-Phase Separation of Proteins, Peptide, and Other Bio-Molecules

IDENTIFY - Identify, Characterize and Measure Bio-molecules in a Variety of Sample Sources

Upcoming Proteomics e-Seminars

"Integration of MassProfiler and Metlin ID software into the MassHunter QUAL Software" – David Weil, Application Engineer, Agilent Technologies
April 10, 2008 – 11:00 am EDT

"Biomarker Discovery by Targeted and Profiling Proteomics" - Professor Rainer Bischoff, Analytical Biochemistry, University of Groningen
April 24, 2008 – 11:00 am EDT

"Protein Analysis Using CAD/ETD Ion Trap Tandem Mass Spectrometry" - Professor Ole Norregard Jensen, Protein Research Group at University of Southern Denmark
May 8, 2008 – 11:00 am EDT

"Peptide Quantitation With An Agilent 6410 QQQ System" - Ning Tang, Application Scientist, Agilent Technologies, Inc.
June 26, 2008 – 11:00 am EDT

Register for these eSeminars at

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