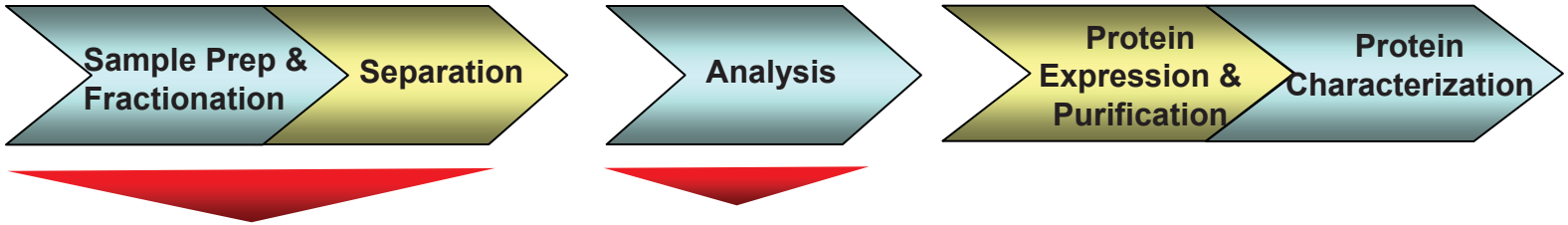
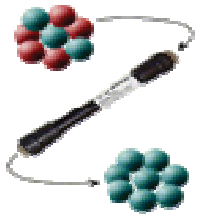
New Sample Preparation and Protein Fractionation Techniques

What is New in Protein Sample Preparation and Separation?



High Abundant Protein Removal



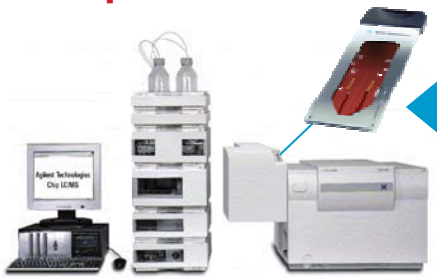
Multiple Affinity Removal System

Protein Fractionation

mRP Column

OGE

HPLC-Chip MS



Packing materials

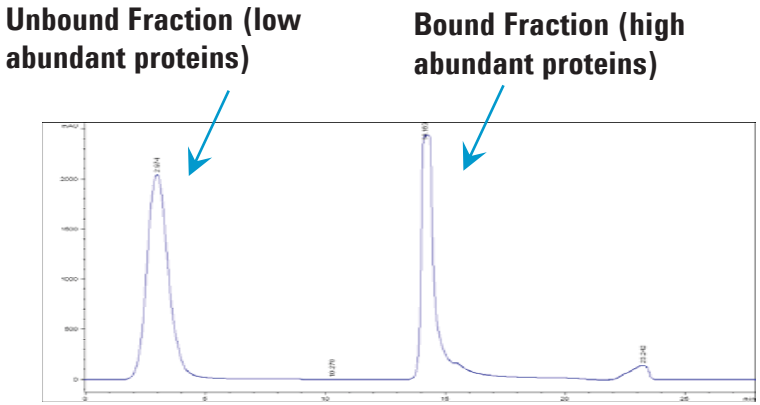
Focus:

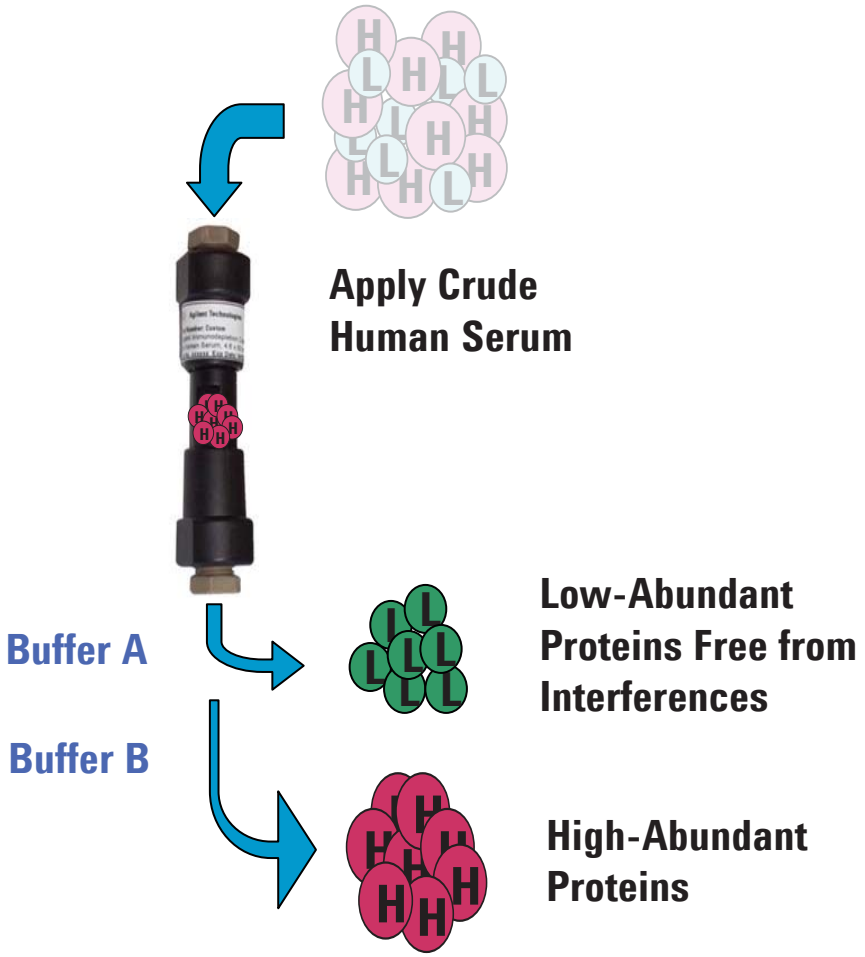
- Recovery of sample (fewest number of steps, return of sample)
- Selectivity
- Reproducibility (run to run, lot to lot of product)
- Reliability and increased productivity

The Multiple Affinity Removal System

A polyclonal antibody based system to rapidly deplete multiple high abundant proteins in serum/plasma/CSF.

- **Launched in August 2003**
- **Individual Ab Materials are mixed in selected percentages and packed into a column format**
- **Agilent continues to innovate and lead this market**





Agilent Technologies

The Agilent Multiple Affinity Removal System

Selectivity

Only native human plasma proteins are used as antigens. This ensures highest selectivity for epitopes in "real samples". Our antibodies are so selective that species cross-reactivity is very low.

Our buffers are specifically formulated to minimize protein-protein interactions resulting in highest possible selectivity of binding (minimize any possible protein-protein interactions, such as with albumin)

Reproducibility

Run to run:

- Coupling chemistry of antibodies to column beads is designed for longest possible lifetime of Antibodies resulting in excellent run to run reproducibility. Only native protein antigen is used for affinity purification resulting in reproducible antibody selection.
- Buffers for affinity purification of our polyclonal antibodies are designed to disruption unwanted protein-protein interactions (such as with albumin) resulting in reproducible epitope selection.

Lot to lot: Manufacturing processes have been engineered to provide excellent lot to lot reproducibility

The Agilent Multiple Affinity Removal System

Ease of Use

LC column: Automated single pass, 2 buffer, 30 minute total run time to deplete 80 uL of human plasma/serum (4.6 x 100 mm column) at 98-99% efficiency. Larger column sizes available on request.

Spin tube: 2-step re-usable system, 10 minute total run time to deplete 15 uL of human serum/plasma

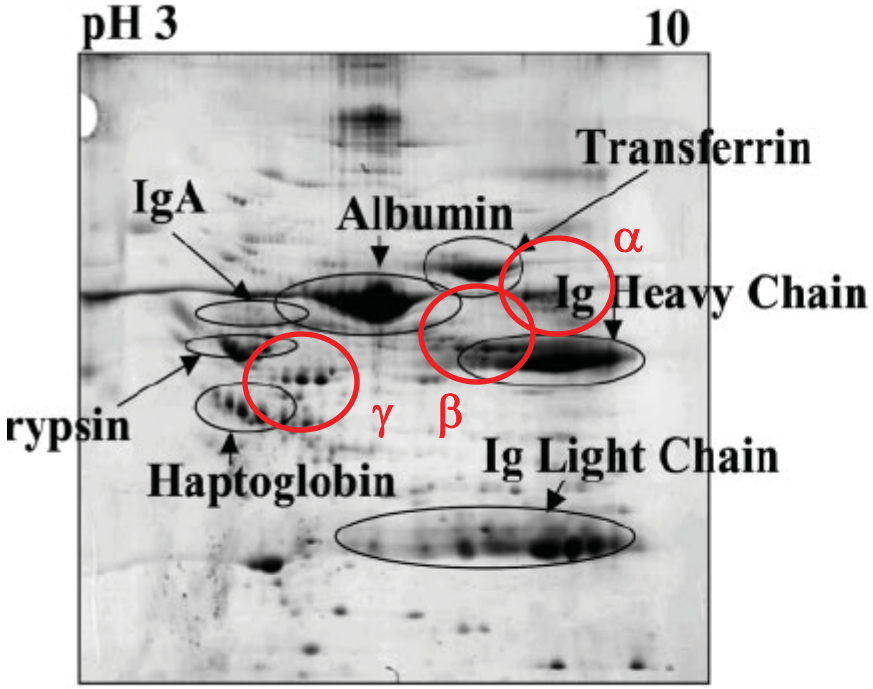
Compatibility with Downstream Analysis

1D gel: Proteins elute in buffer system immediately ready for application for 1DGE

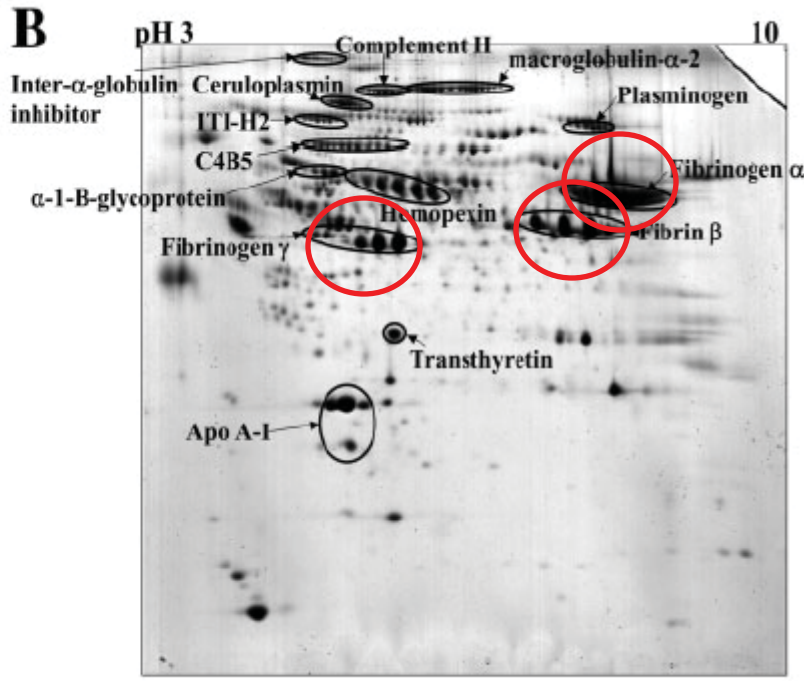
HPLC: Proteins can be simultaneously concentrated, desalted, and fractionated on our new mRP column

MS: There are no detergents present in our buffers

Why Multiple Affinity Removal System?



Plasma



Plasma after Top-6 Depletion

Data: Dr. Y.K. Paik

Agilent Multiple Affinity Removal System: Where Are We? & What is Next?

FY2004 → **Capacity** → **FY2005** → **Depth** → **FY2006**

"Original" Top-6 Human Serum

Spin Tube format

Mouse-3

"High Capacity" Top-6 Human Serum

Spin Tube format

(April 2006)

"High Capacity" Top-7 Human Plasma

\+

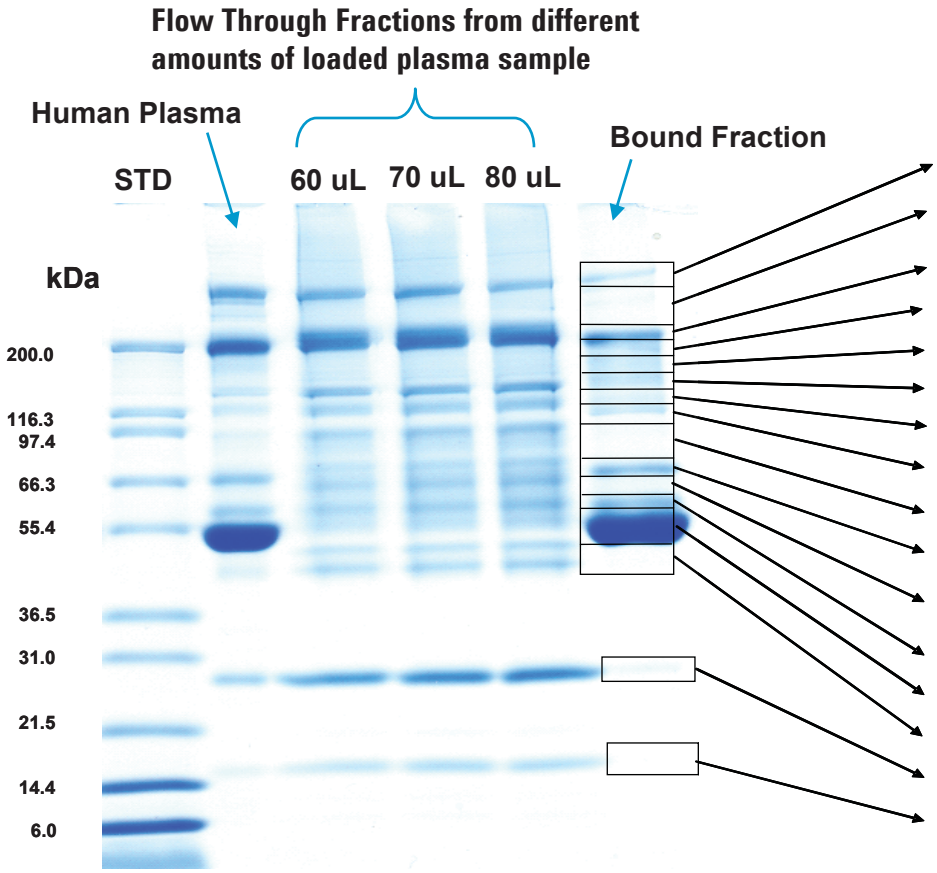
"High Capacity" Level-II human

While Maintaining our Focus:

- Recovery of sample (fewest number of steps, return of sample)
- Selectivity
- Reproducibility (run to run, lot to lot of product)
- Reliability and increased productivity

Selectivity of Plasma-7 Column

Proteins Identified in Bound Fraction by LC/MS/MS



Untargeted Proteins

Gel Band #	Proteins Identified
1	Fibrinogen
2	Fibrinogen, Haptoglobin , HSA
3	IgG, IgA, Complement C3
4	IgG, HSA, IgA
5	IgG, HSA
6	IgG, HSA, Ceruloplasmin
7	HSA, Haptoglobin, IgG
8	HSA, Complement B pre
9	IgG, HSA
10	Transferrin, HSA
11	HSA, Transferrin, Alpha-1-antichymotrypsin
12	Alpha1 -Antitrypsin, HSA
13	HSA
14	Apolipoprotein H
15	Apolipoprotein
16	Hemoglobin alpha chain

4-20% SDS -PAGE

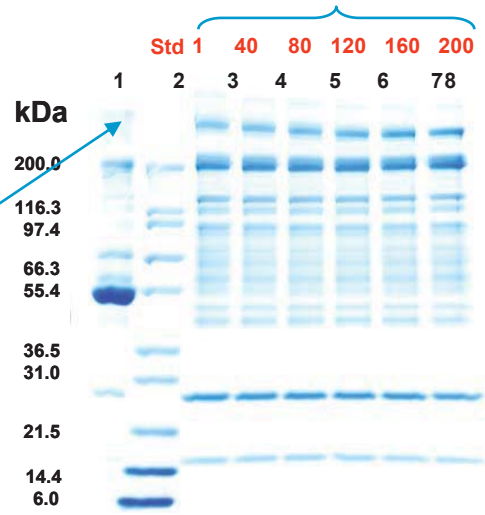
ELISA analysis indicate 99.4% depletion of Fibrinogen from 60, 70 and 80 µl of a plasma load on a 4.6x100mm column

Reproducibility from Run to Run

Comparison of runs 40, 80, 120, 160, & 200

SDS-PAGE analysis of the flow-through fractions from multiple runs on a Human Plasma 7 column

Bound Fraction (Bands excised for confirmation of ID by MS/MS)

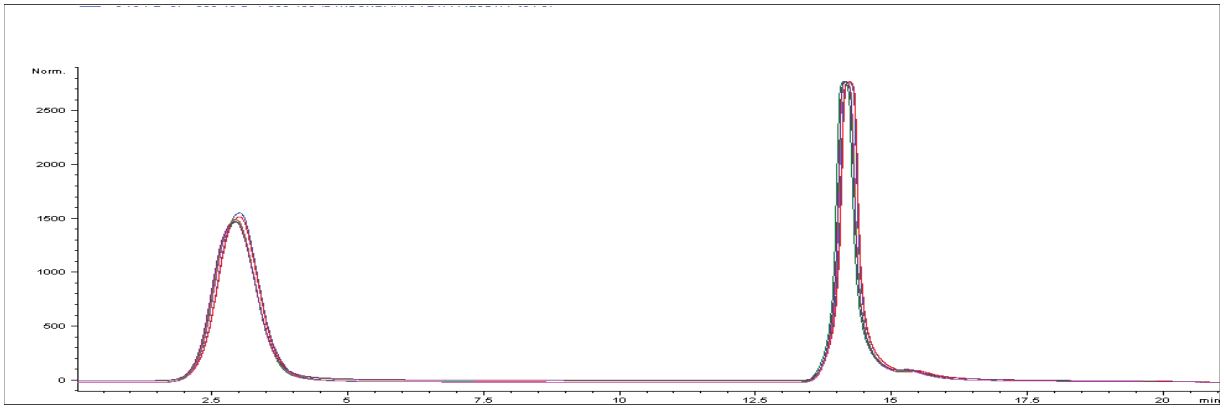


4-20% SDSPAGE

1- Human Plasma
2- Mark12 Standards
3- Flow-through Fraction, Run 1
4- Flow-through Fraction, Run 40
5- Flow-through Fraction, Run 80
6- Flow-through Fraction, Run 120
7- Flow-through Fraction, Run 160
8- Flow-through Fraction, Run 200

10 µg of protein/well

Column performs reproducibly for 200 runs!



Agilent Technologies

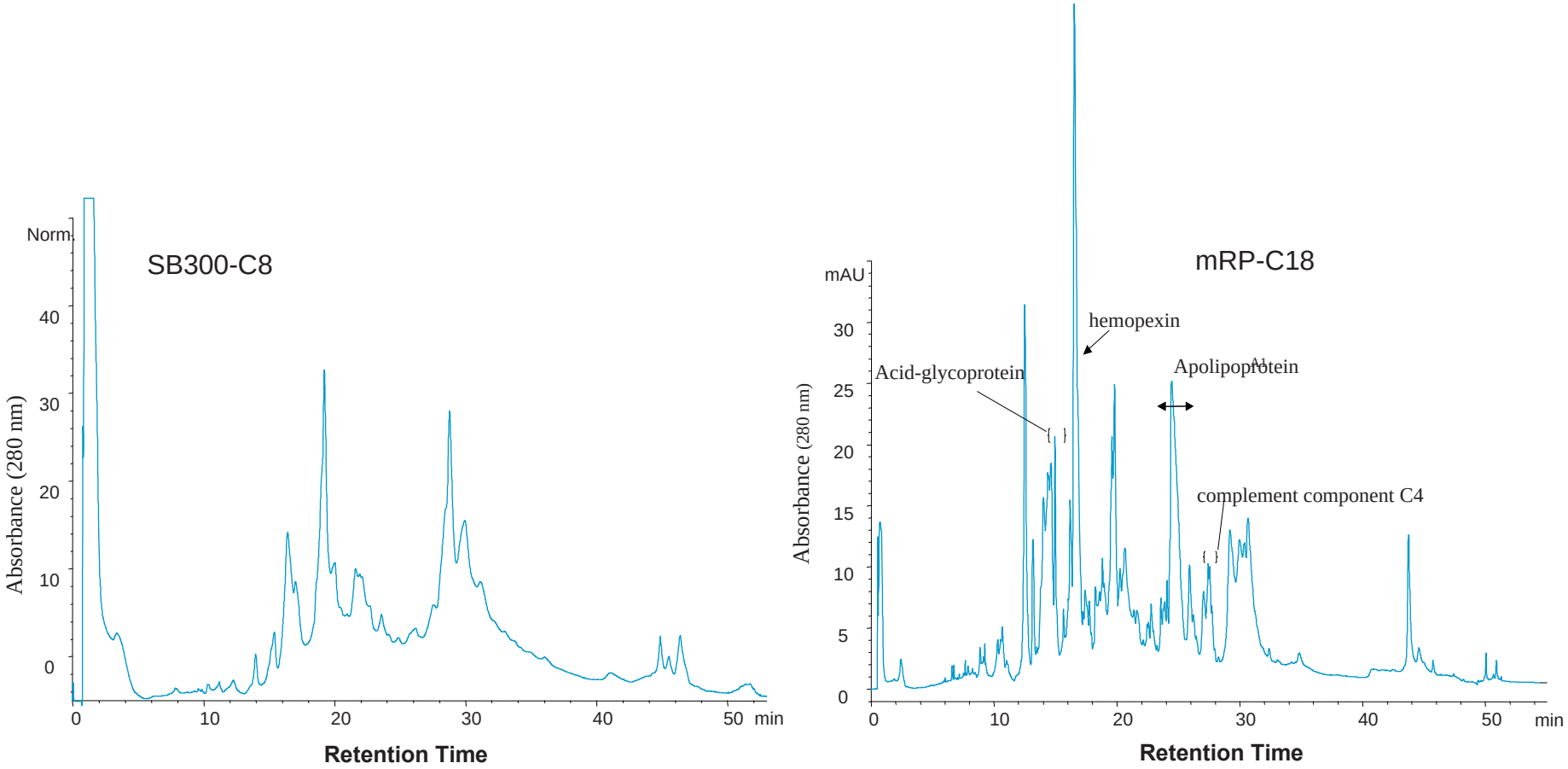
mRP-C18 High-Recovery Protein Fractionation Column

mRP (macroporous reverse-phase)

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

Comparison of mRP with Zorbax SB300-C8

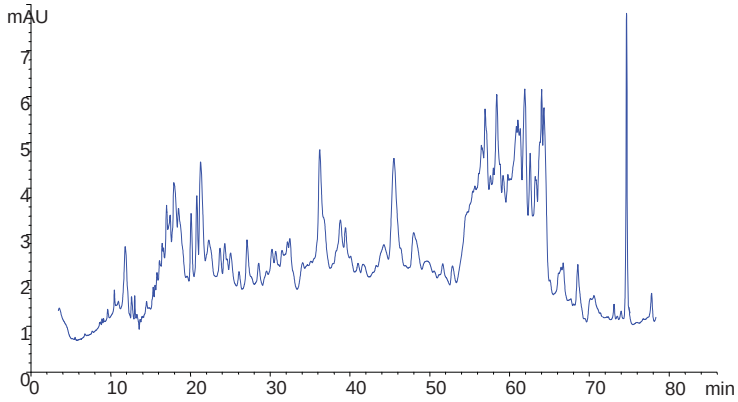


Sample: 270ug flow-through (6M urea/5.0% AcOH) of immunodepleted human serum from Multiple Affinity Removal System column
Columns: Panel A – Zorbax SB300-C18 (300 A, 5.0um), 4.6 mm x 50 mm i.d., SS; Panel B – mRP-C18 (macroporous, 5um), 4.6 mm x 50 mm i.d., PEEK, 0.75mL/min., DAD 280nm
Mobile Phase & Conditions: A-0.1% TFA/water, B-0.08%TFA/ACN, Temp 80° C, gradient:5-30%B in 5min., 30-55%B in 33min., 55-100%B in 4min.
1D SDS PAGE: Collected 36 fractions (1.0 min. time slices) from immunodepleted human serum RP separation

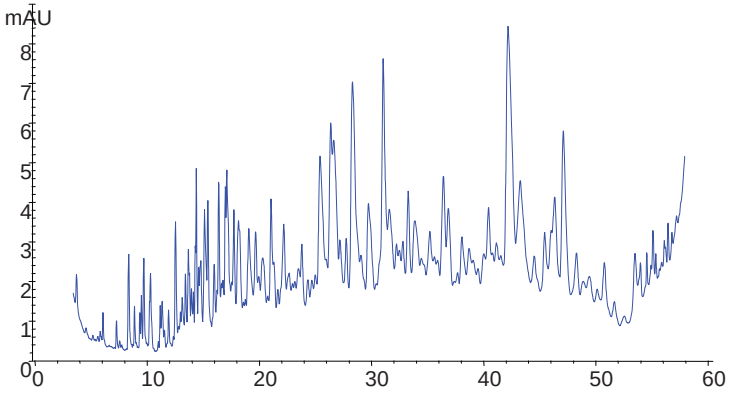
Agilent Technologies

Protein Fractionation on mRP

(4.6 x 50mm mRP-C18)

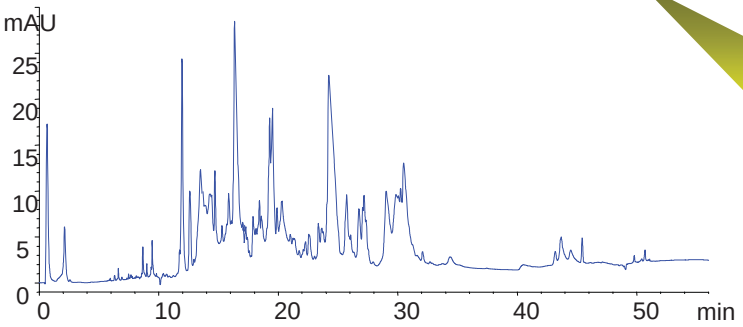


Hela Membrane Prep

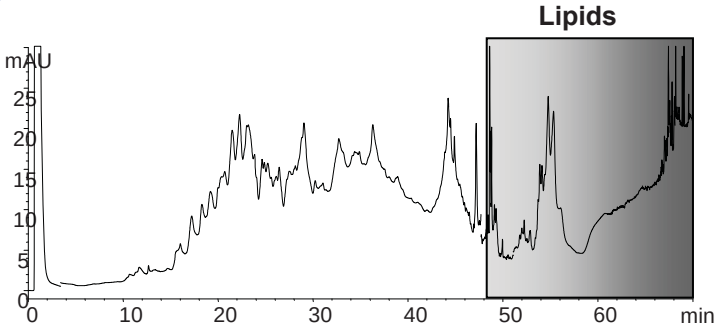


HeLa cell lysate (352ug)

Highest Recovery



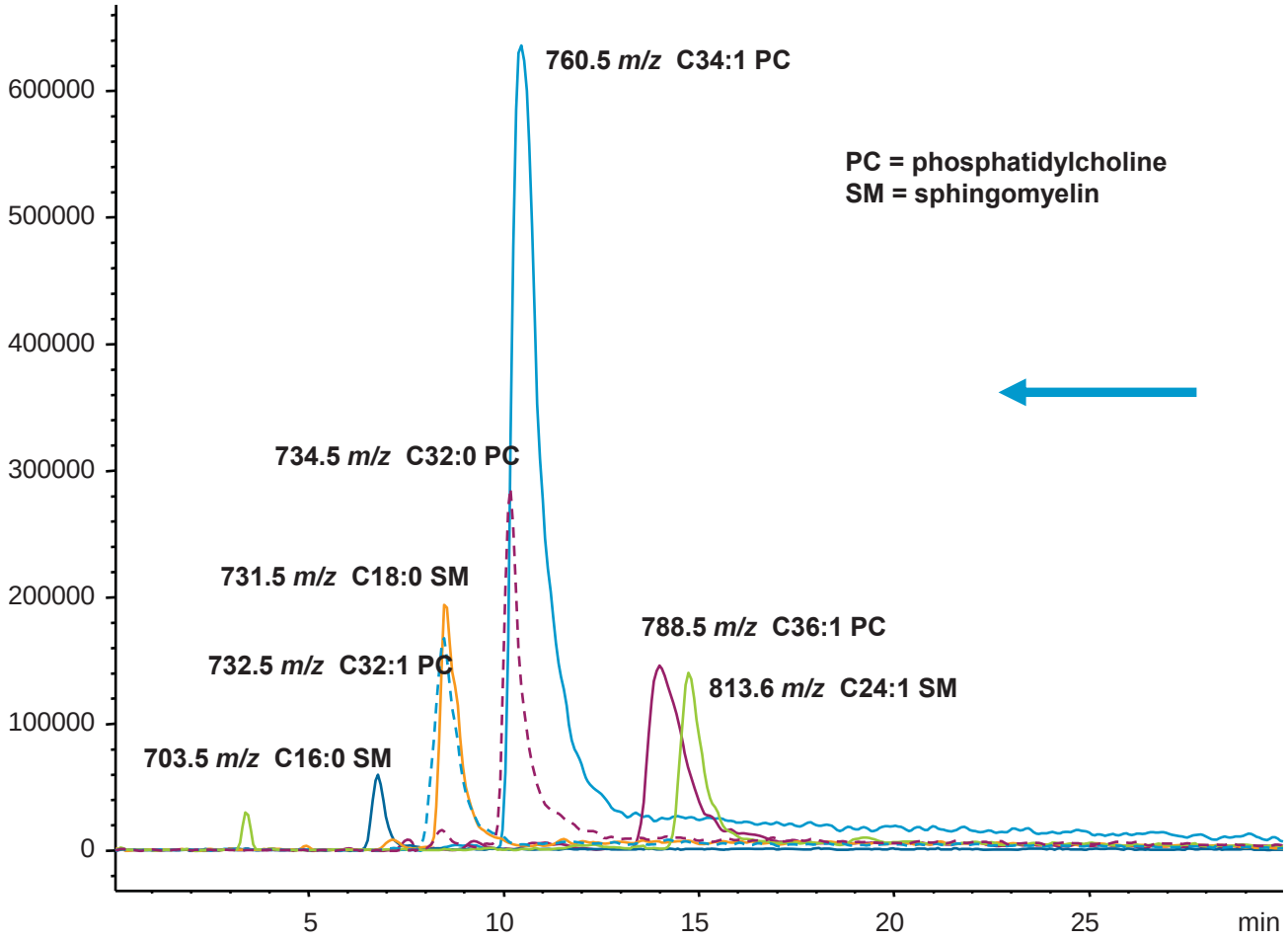
"Top-6" depleted human serum

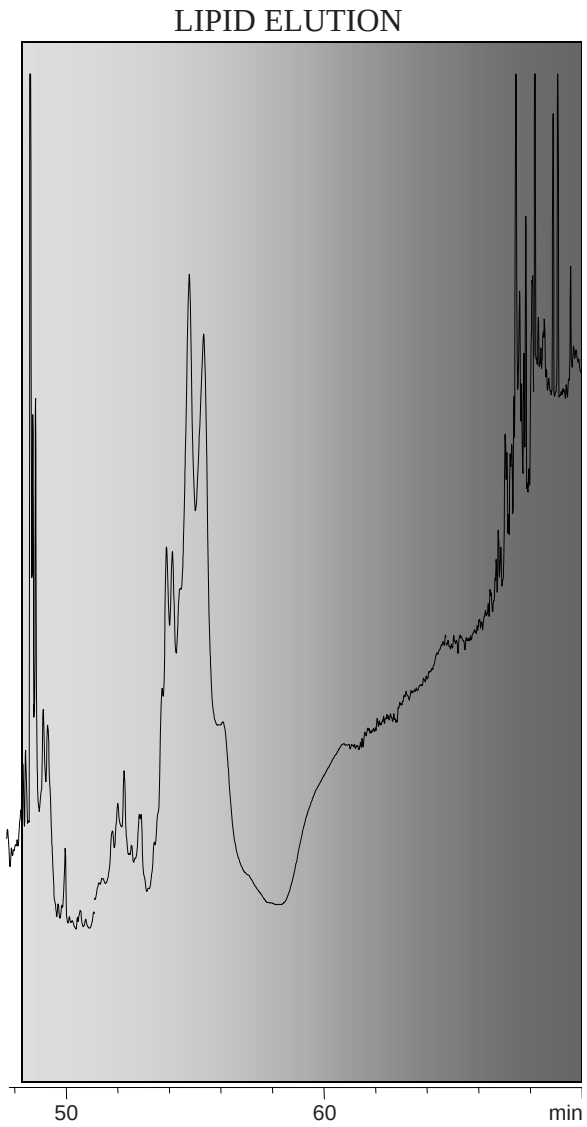


Human Brain membrane lipid Raft prep (500ug)

Agilent Technologies

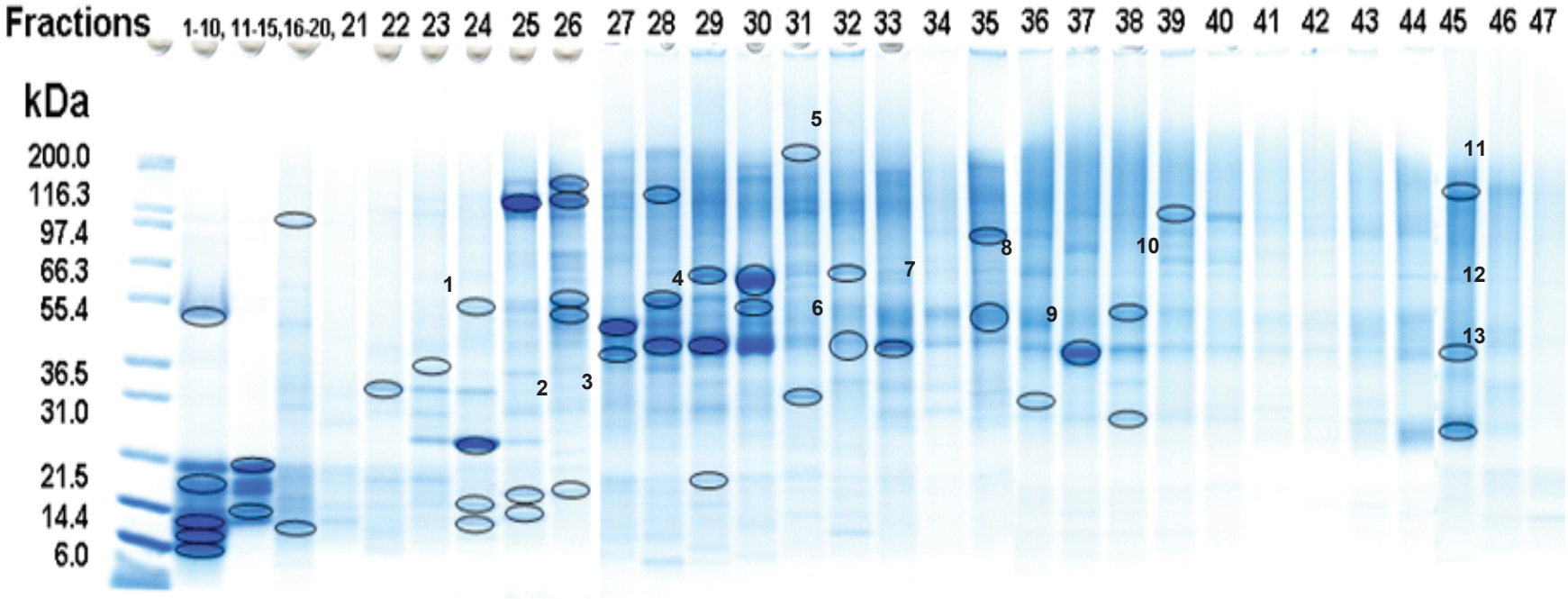
Preliminary LC-MS data of the lipid fraction isolated from the human brain membrane rafts sample





Agilent Technologies

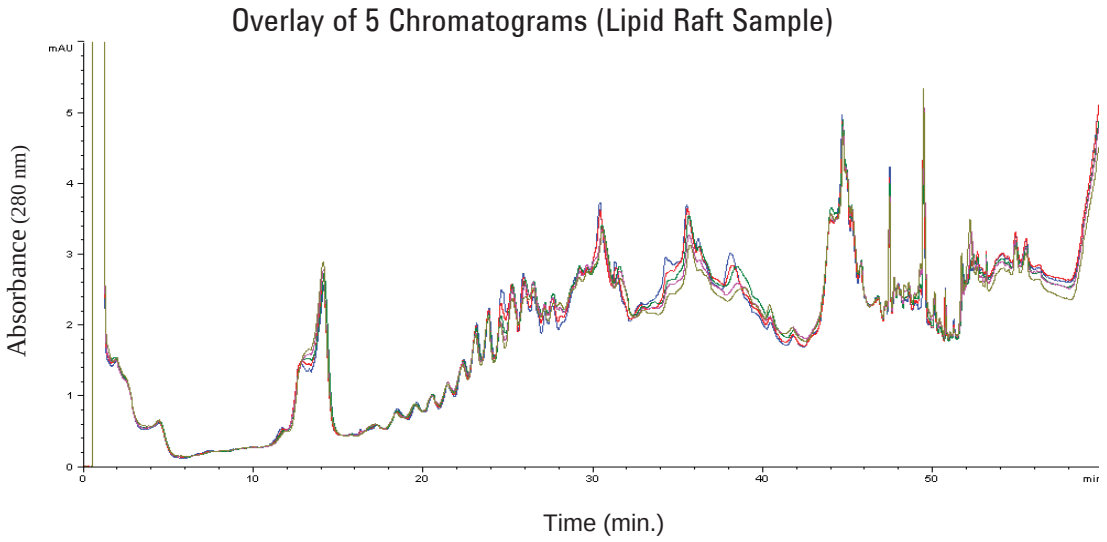
Lipid Raft Sample: mRP Fractionation followed by 1D-SDS PAGE Fractionation

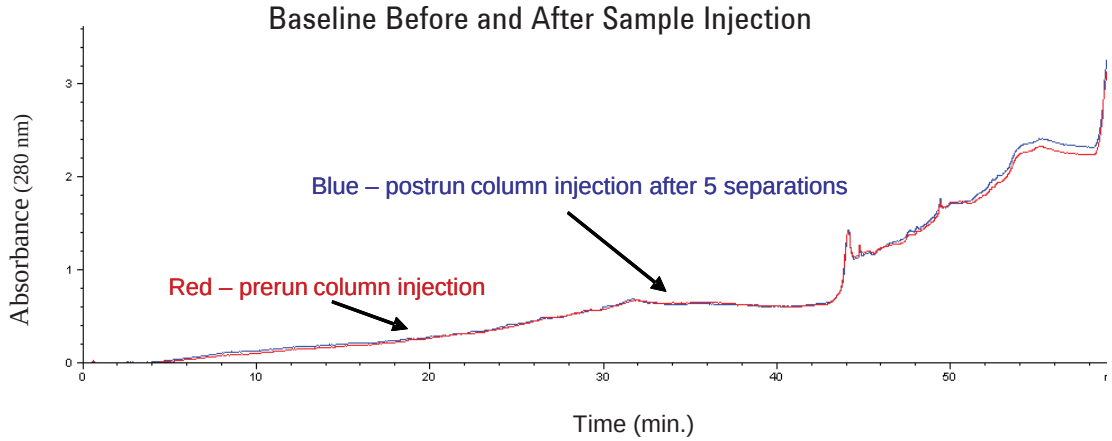


Selected Excised Bands Which are Intregal Membrane Proteins

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)

Lipid Raft Sample: Reproducibility and Baseline Stability

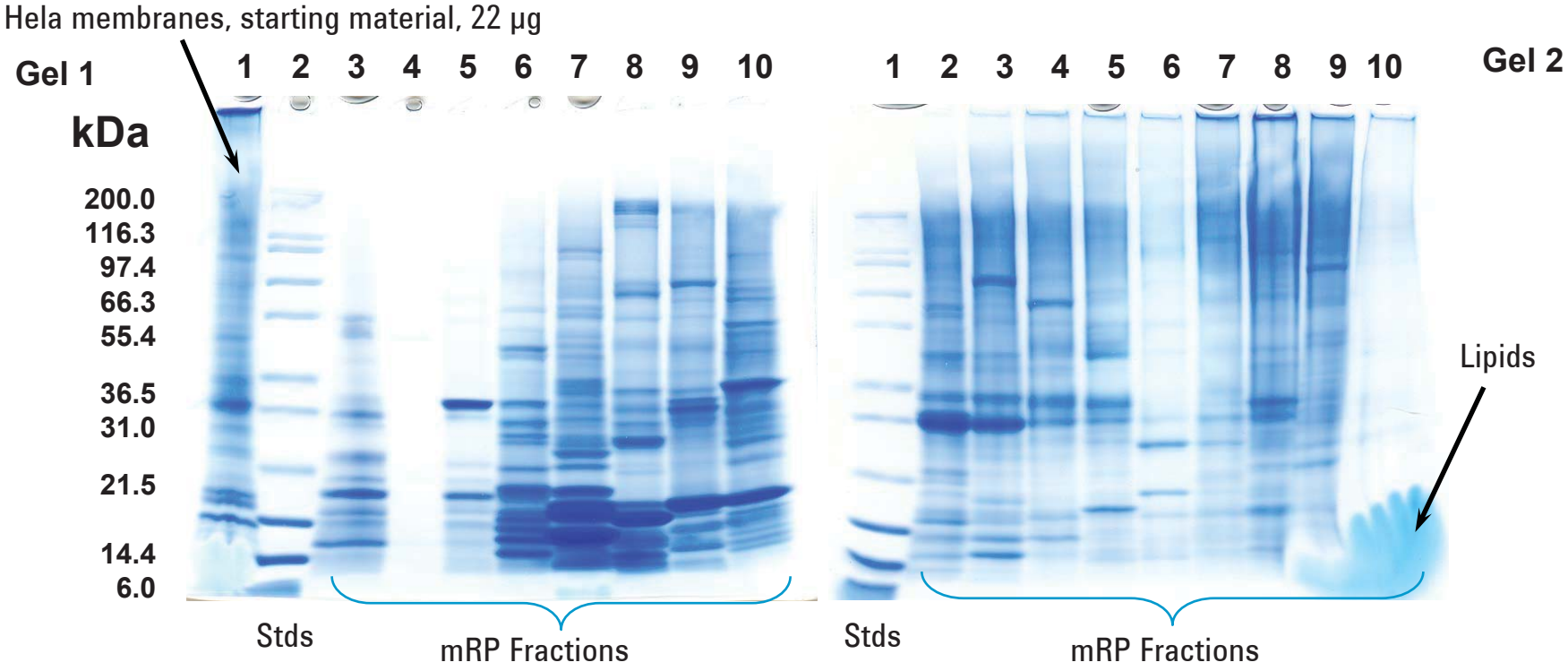




Agilent Technologies

Hela Cell Membrane mRP Fractions followed by 1D-SDS PAGE Fractionation

4.6 x 50mm mRP



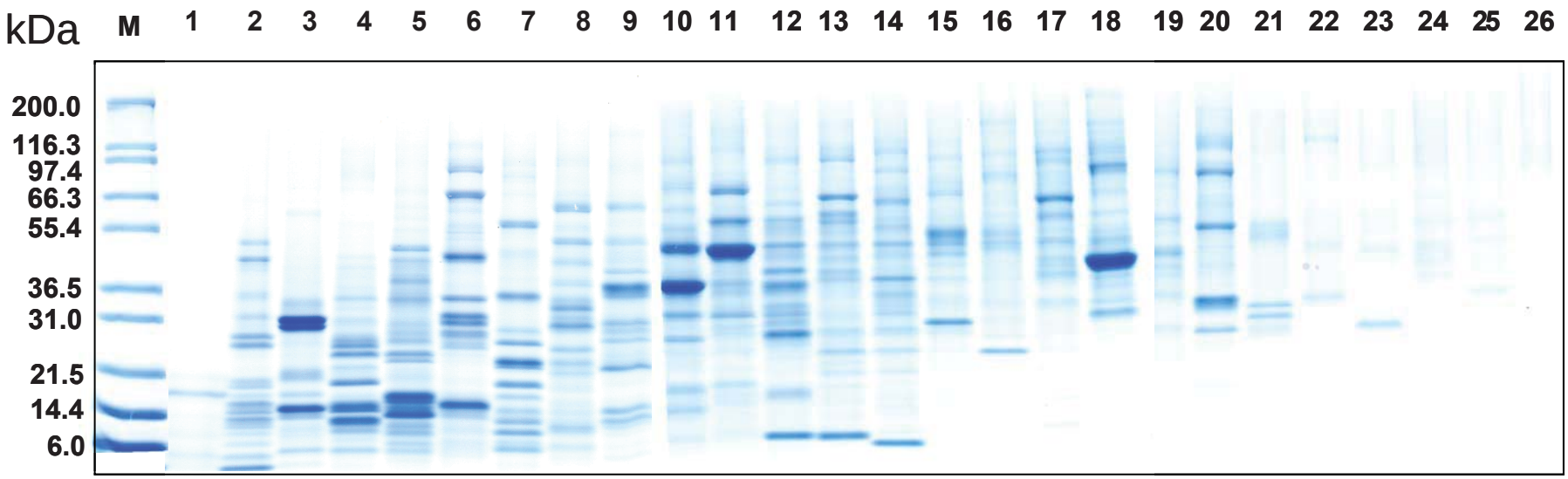
Identification performed by chip-based nano LC/MS/MS from excised gel bands

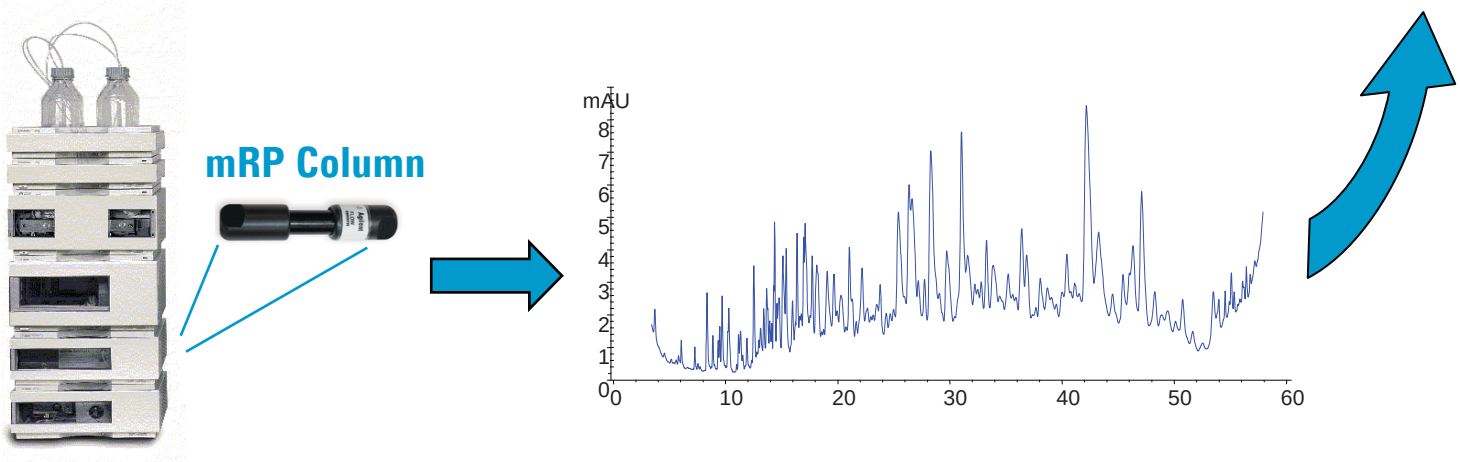
Sample (216 gel bands from mRP)	Total Acquisition Time (hrs)	# MS/MS Spectra Collected	# Distinct Peptides Matched	# Total Proteins Identified	# Membrane Proteins Identified	# Integral Membrane Proteins Identified
HeLa Membranes	108	486,700	3841	688	364	286

Agilent Technologies

Hela Cell Lysate mRP Fractionation followed by 1D-SDS PAGE Fractionation

4.6 x 50mm mRP C18 column

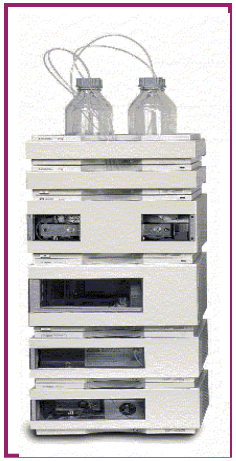




Agilent Technologies

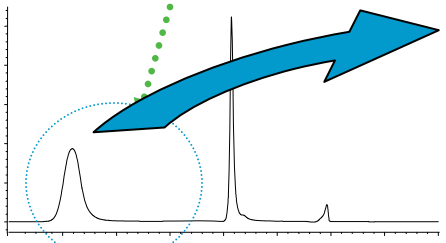
For Plasma/Serum Biomarker Discovery: Combine "Top-6" and mRP Fractionation

Removal of 6 most abundant proteins ⟶ **Fractionation of lower abundance proteins**



MARS Immunodepletion

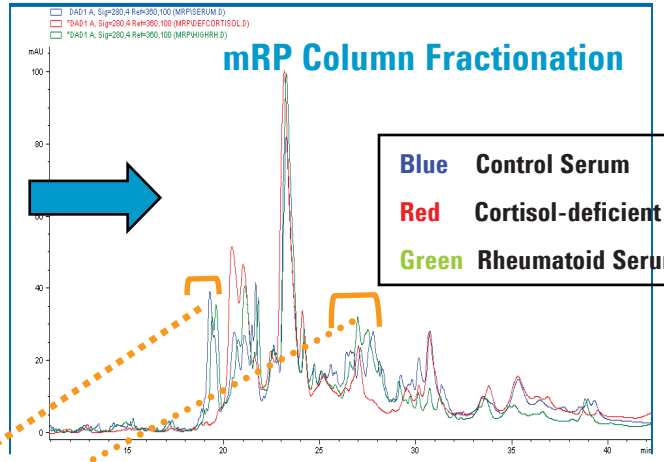
Low abundance proteins



Simultaneously:

- *Concentrate*
- *Desalt*
- *Fractionate*

mRP Column Fractionation

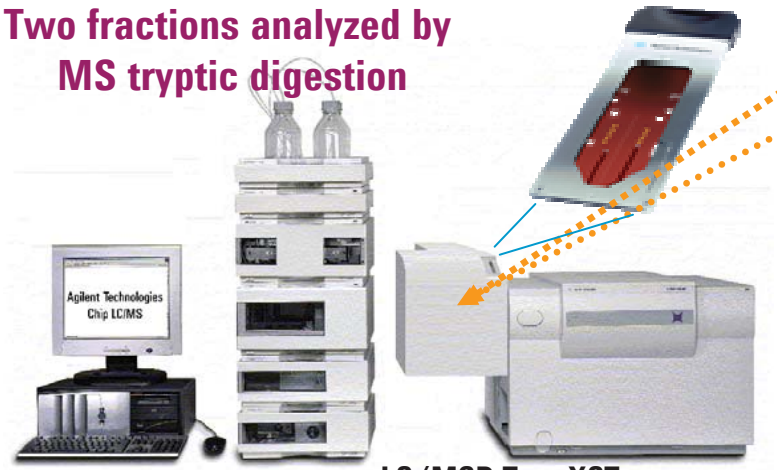


Blue Control Serum

Red Cortisol-deficient Serum

Green Rheumatoid Serum

Two fractions analyzed by MS tryptic digestion



LC/MSD Trap XCT

Spectrum Mill

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

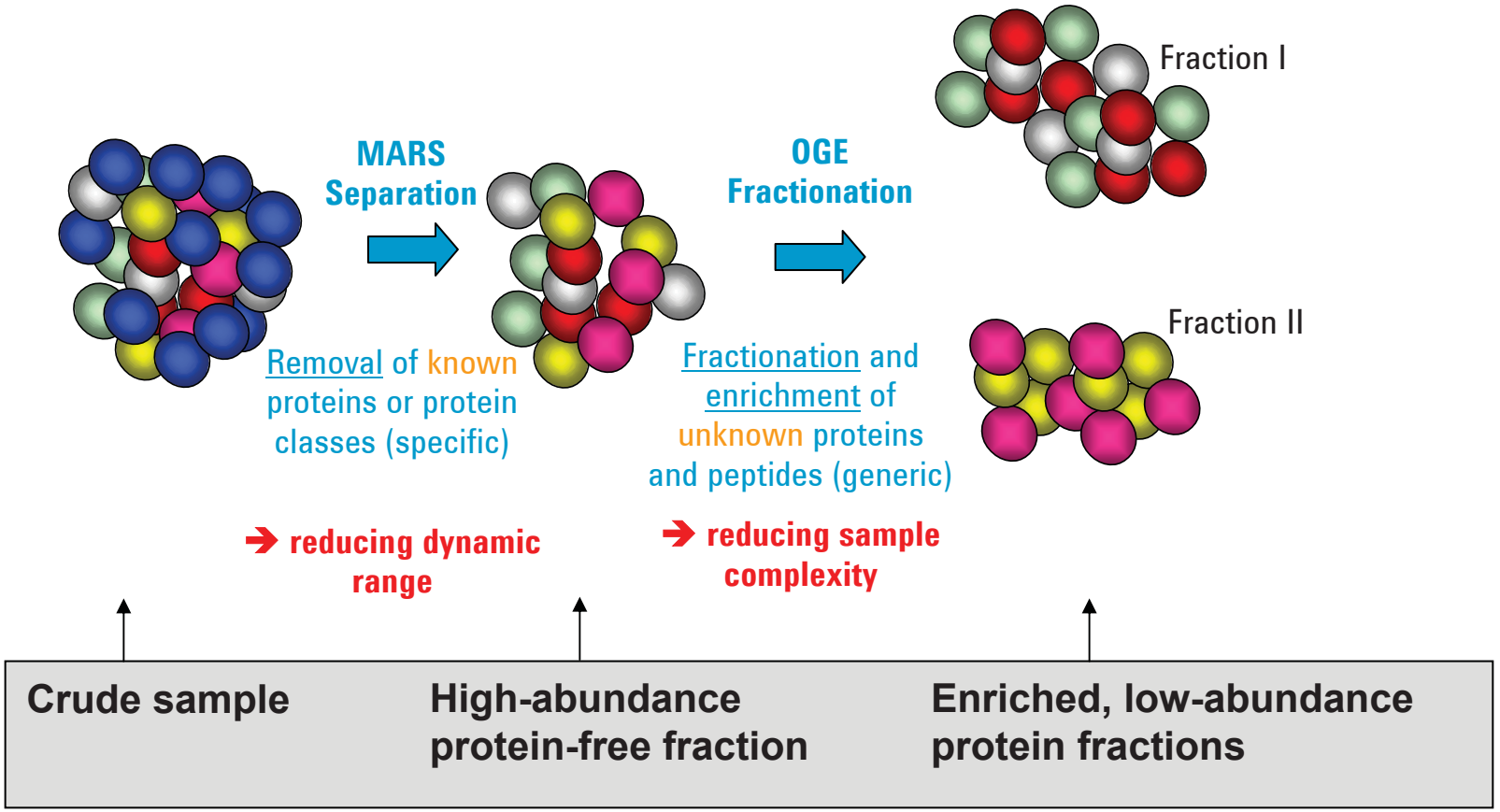
Agilent Technologies

Off-Gel Electrophoresis

Technology and Applications

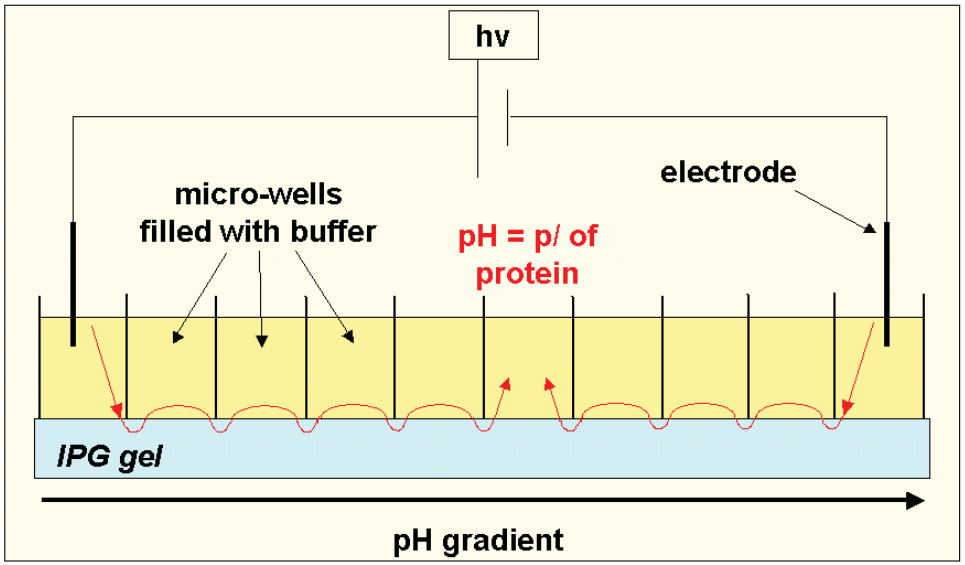
Strategy

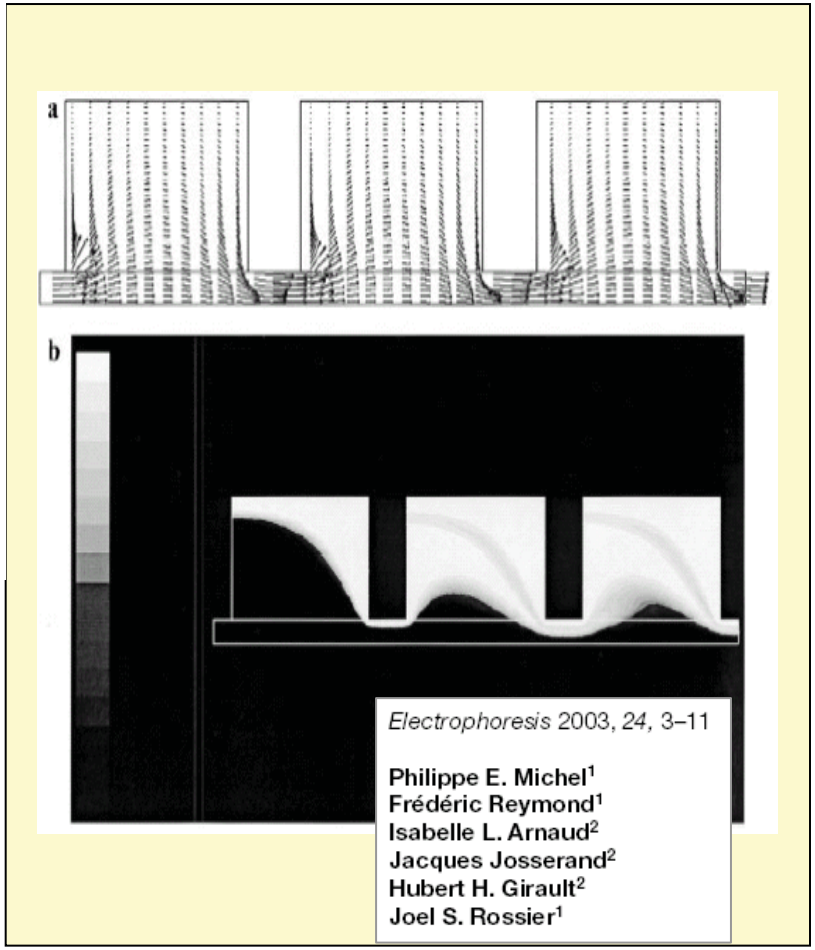
Towards Low Abundance Proteins with Immunodepletion & OGE



MARS: Multiple Affinity Removal System
OGE: Off-Gel Electrophoresis

pI-based Fractionation:
Off-Gel-Electrophoresis



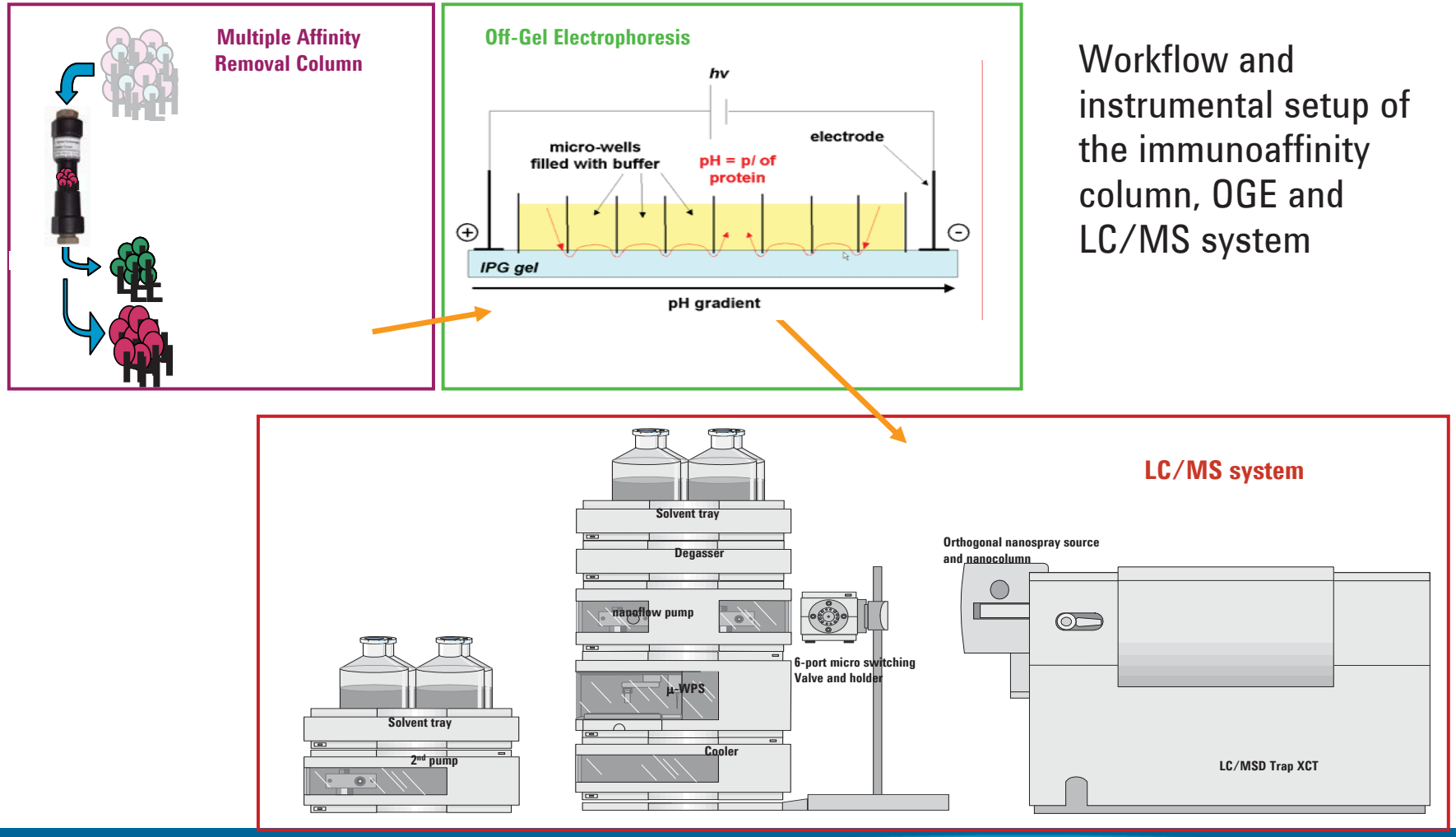


pH gradient	immobilized (IPG gels)
Number of fractions	≤ 30
Fraction volume	0.05 - 0.1 ml
Resolution	0.05-0.5 pH
Separation time	5 - 20 h

Agilent Technologies

Plasma Proteome Workflow

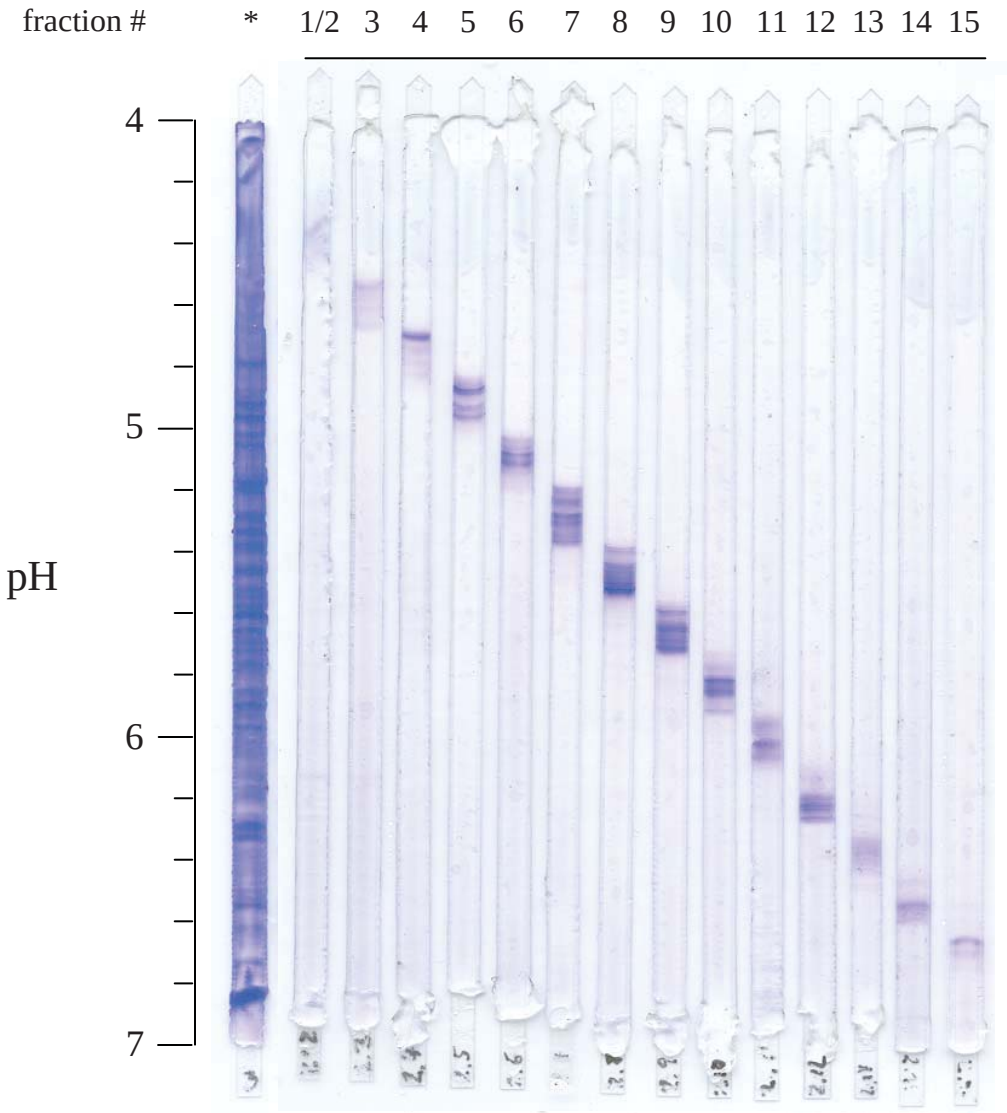
Immunodepletion, OGE, LC/MS



Workflow and instrumental setup of the immunoaffinity column, OGE and LC/MS system

Agilent Technologies

Analysis of OGE Fractions by 1D Electrophoresis



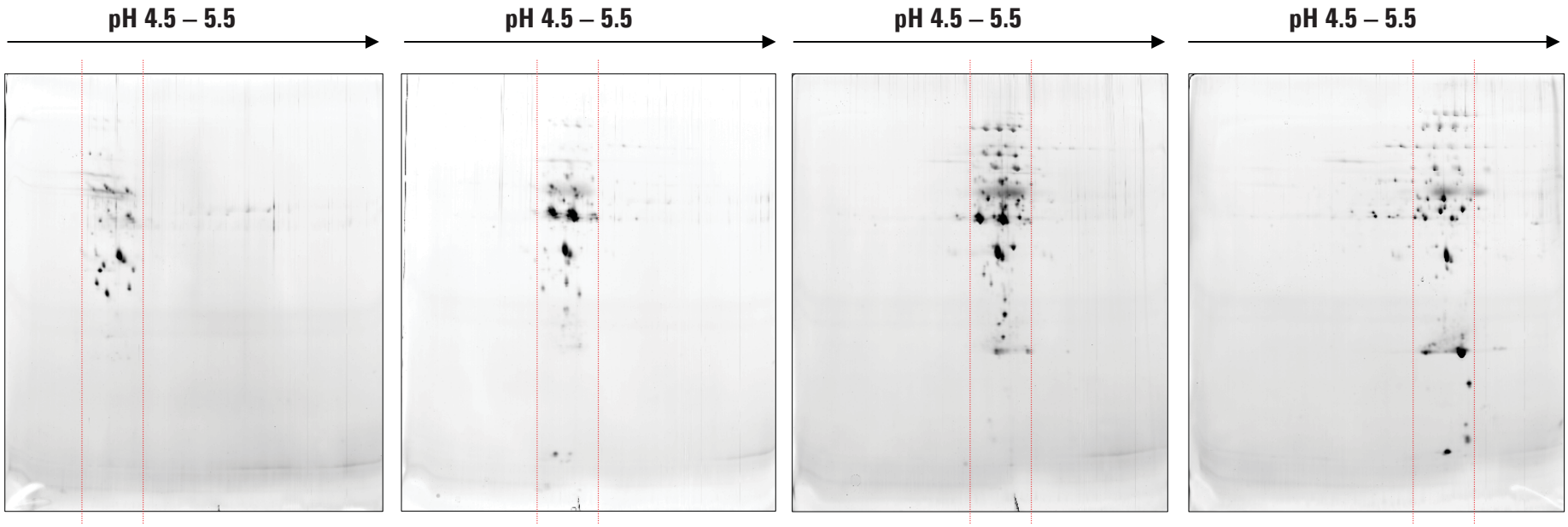
E. coli cell extract

Coomassie Brillant Blue stain

* unfractionated sample

Analysis of OGE Fractions by High Resolution 2DE Albumin-Depleted Human Plasma, Silver Stain; Experiment done by Lab of Prof. Tissot/CHUV

target: Δ pH 0.16



fraction #4
pH 4.77-4.93

fraction #5
pH 4.93-5.10

fraction #6
pH 5.10-5.26

fraction #7
pH 5.26-5.42

→ almost no overlap between fractions

Agilent Technologies

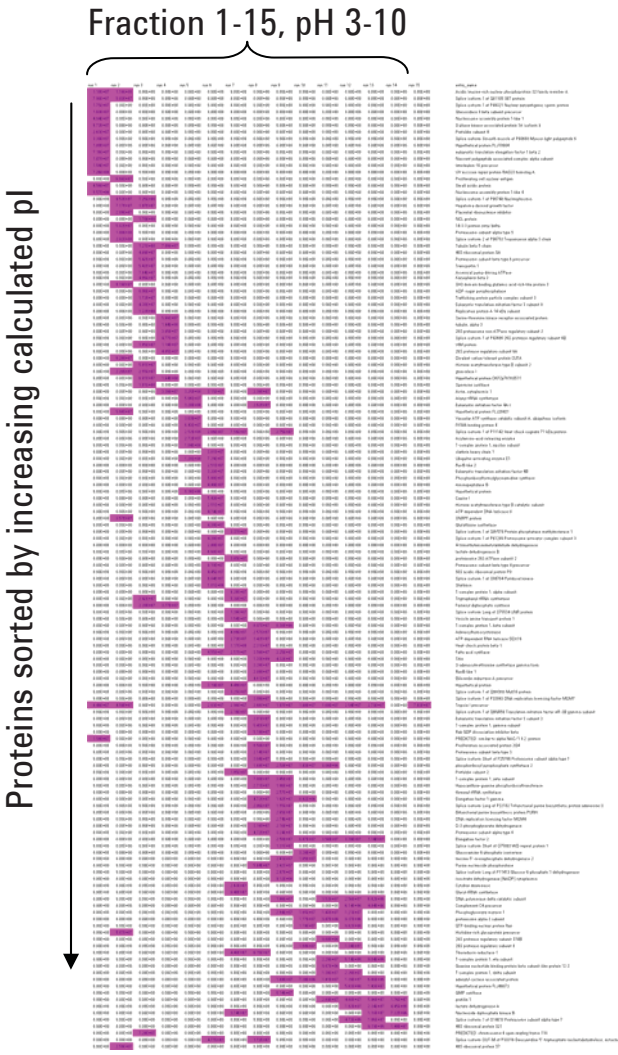
Applications

Protein Samples:

- Immunodepleted serum/plasma
- Cerebrospinal fluid (CSF)
- HeLa proteasome cell extract
- Mammalian macrophage cell extract
- preB 697 cell extract
- Bacterial lysates
    - *E. coli*
    - *H. influenzae*

Peptide samples

OGE Fractionation of a Protein Fraction from HeLa S3 Cell Extract* analyzed by Chip-LC/MS



Prefractionation of this cell extract leads to significantly greater number of proteins identified:

144 proteins total for 15 OGE fractions

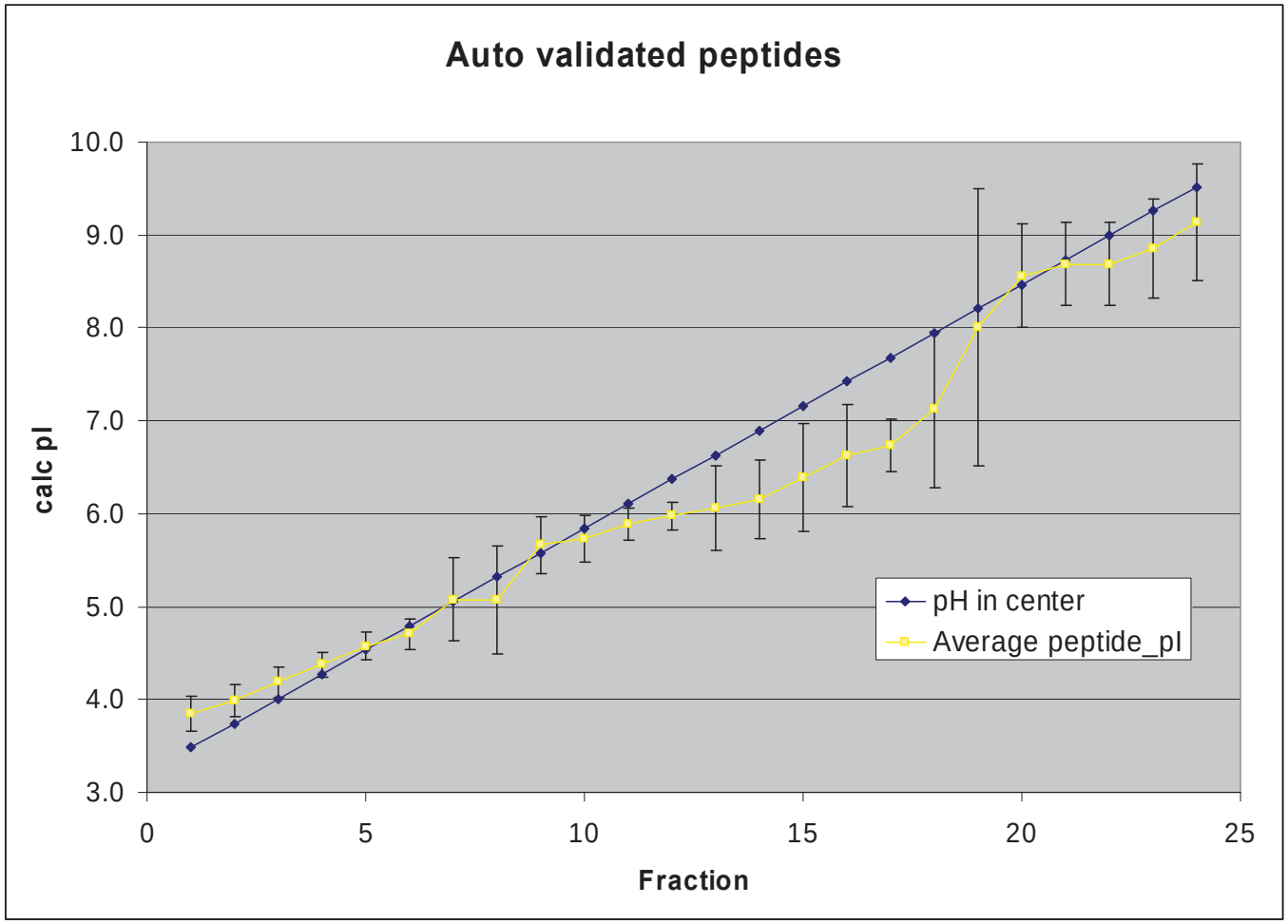
20 proteins total for 15 injections of unfractionated sample

* binding to anion exchange resin

Agilent Technologies

OGE Fractionation of Peptides - E.Coli Tryptic Digest

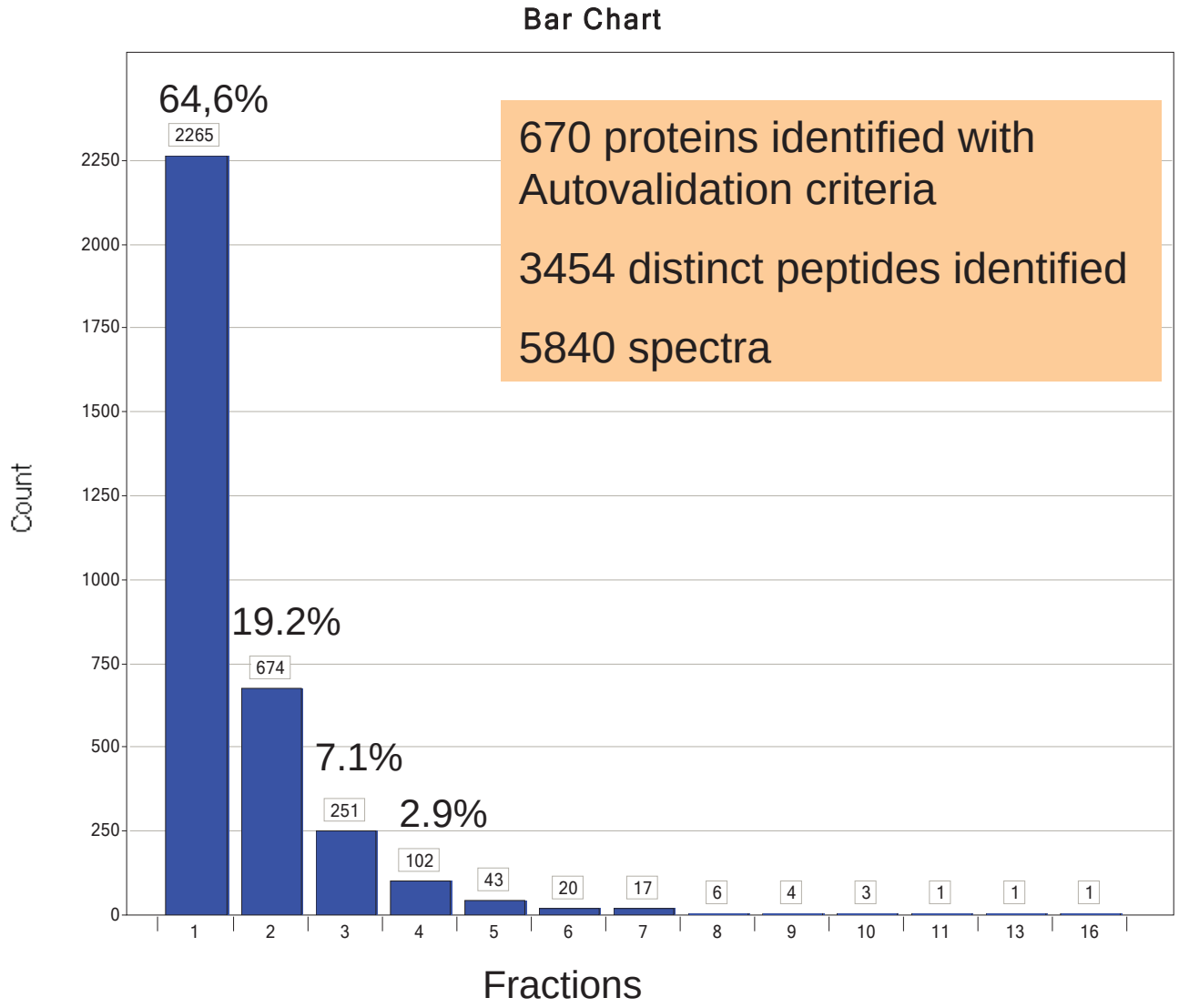
Average Peptide pI with Standard Deviation for Autovalidated Peptides



Agilent Technologies

OGE Fractionation of Peptides - E.Coli Tryptic Digest

Number of Peptide Identifications in Number of Fractions



670 proteins identified with Autovalidation criteria

3454 distinct peptides identified

5840 spectra

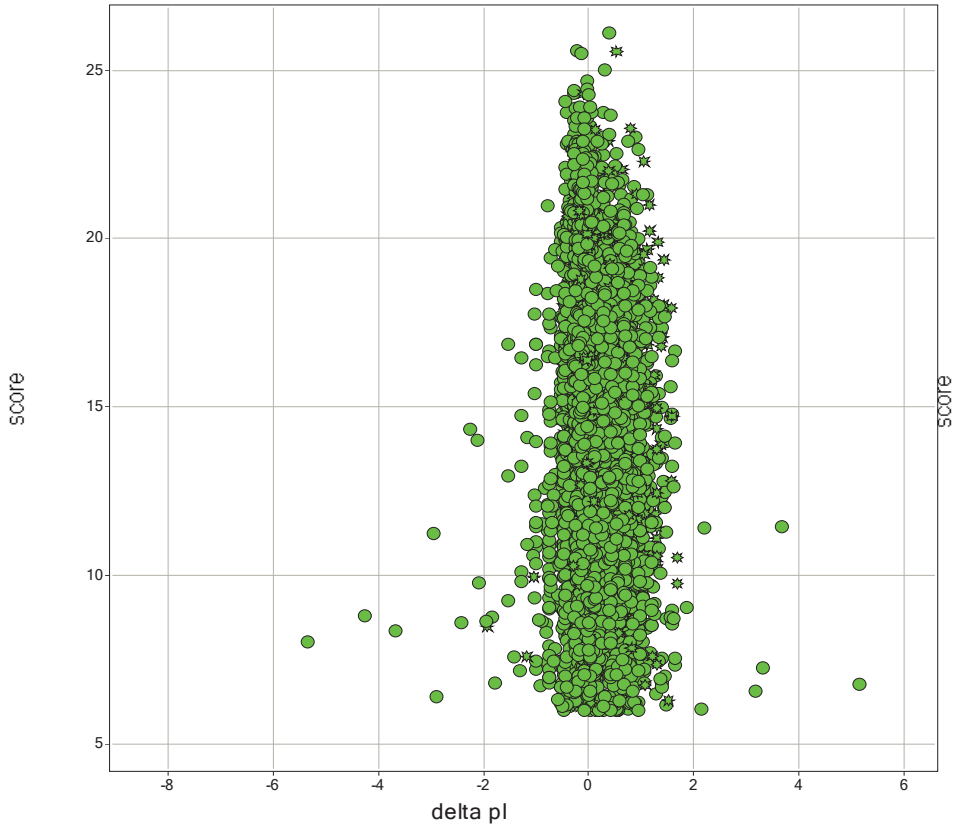
=> The majority of peptides are found in only 1 or 2 fractions!

Agilent Technologies

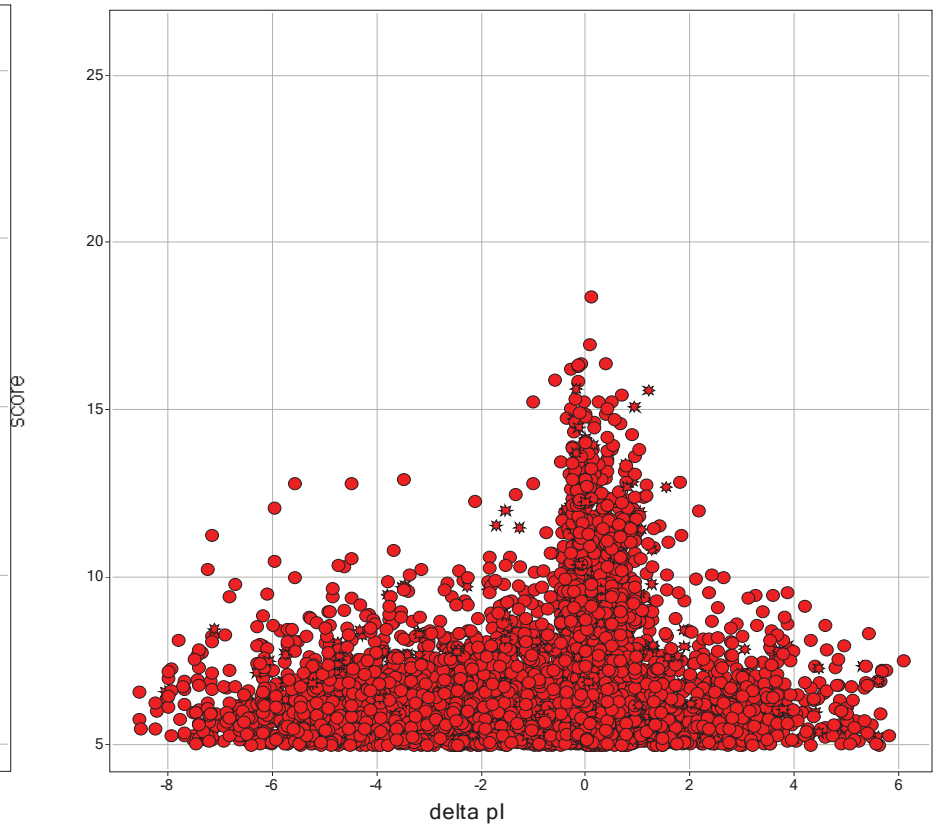
OGE Fractionation of Peptides

Trypsin Digested *E-Coli* Lysate

Peptide scores plotted against delta pI (3454 peptides, 5840 spectra, valid after autovalidation)



Peptide scores plotted against delta pI (5251 spectra, not valid after autovalidation)

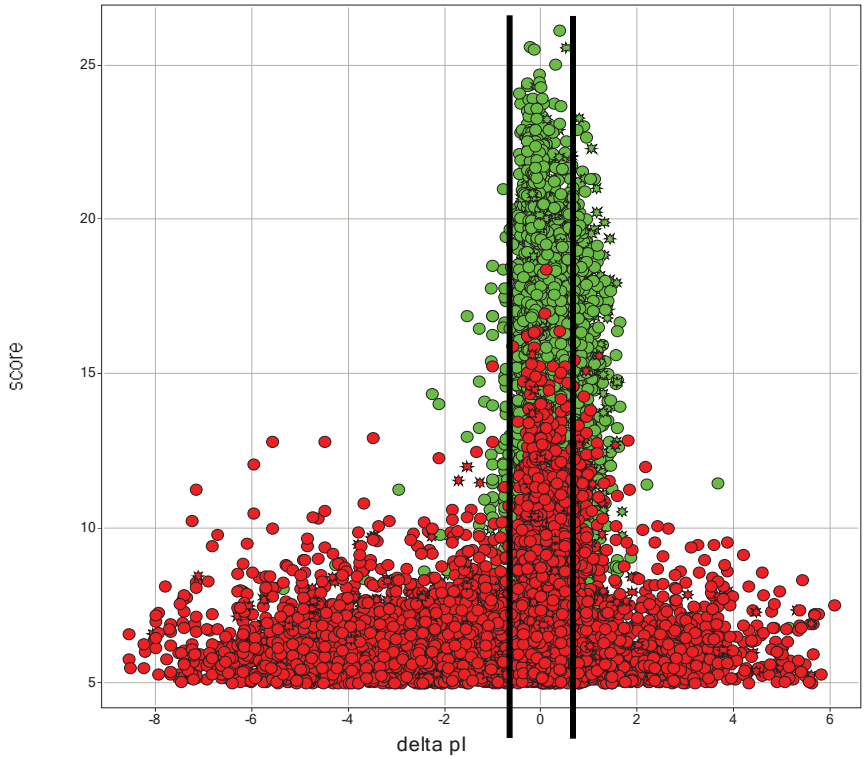


Can be used to search for charged PTM's, peptides with high delta pI and high score are possible candidates.

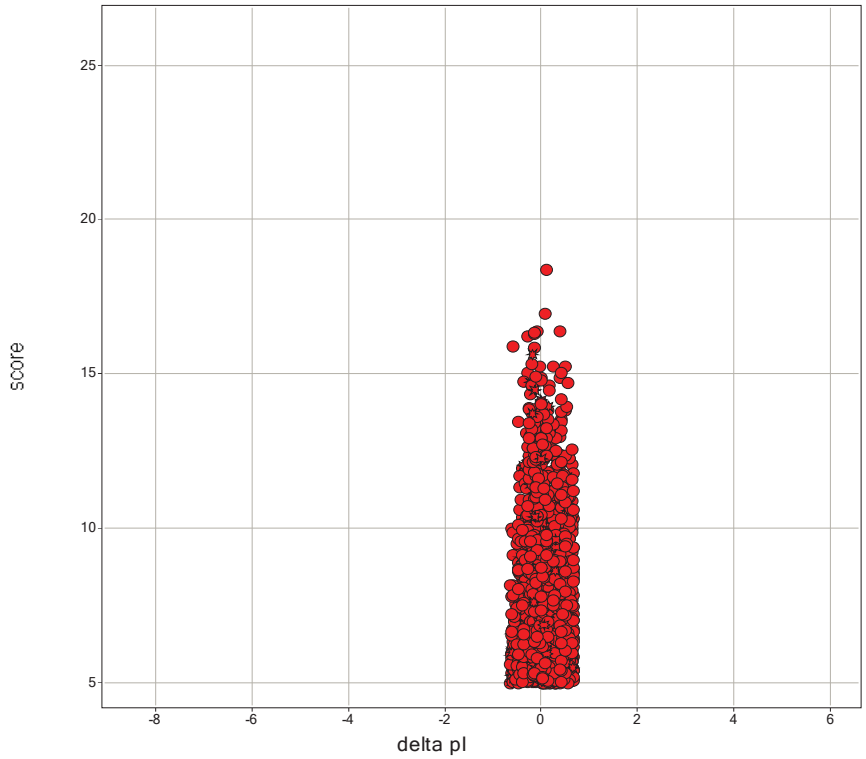
OGE Fractionation of Peptides

Trypsin Digested *E-Coli* Lysate

Peptide scores plotted against delta pI (11091 spectra, valid after autovalidation = green, not validated = red)

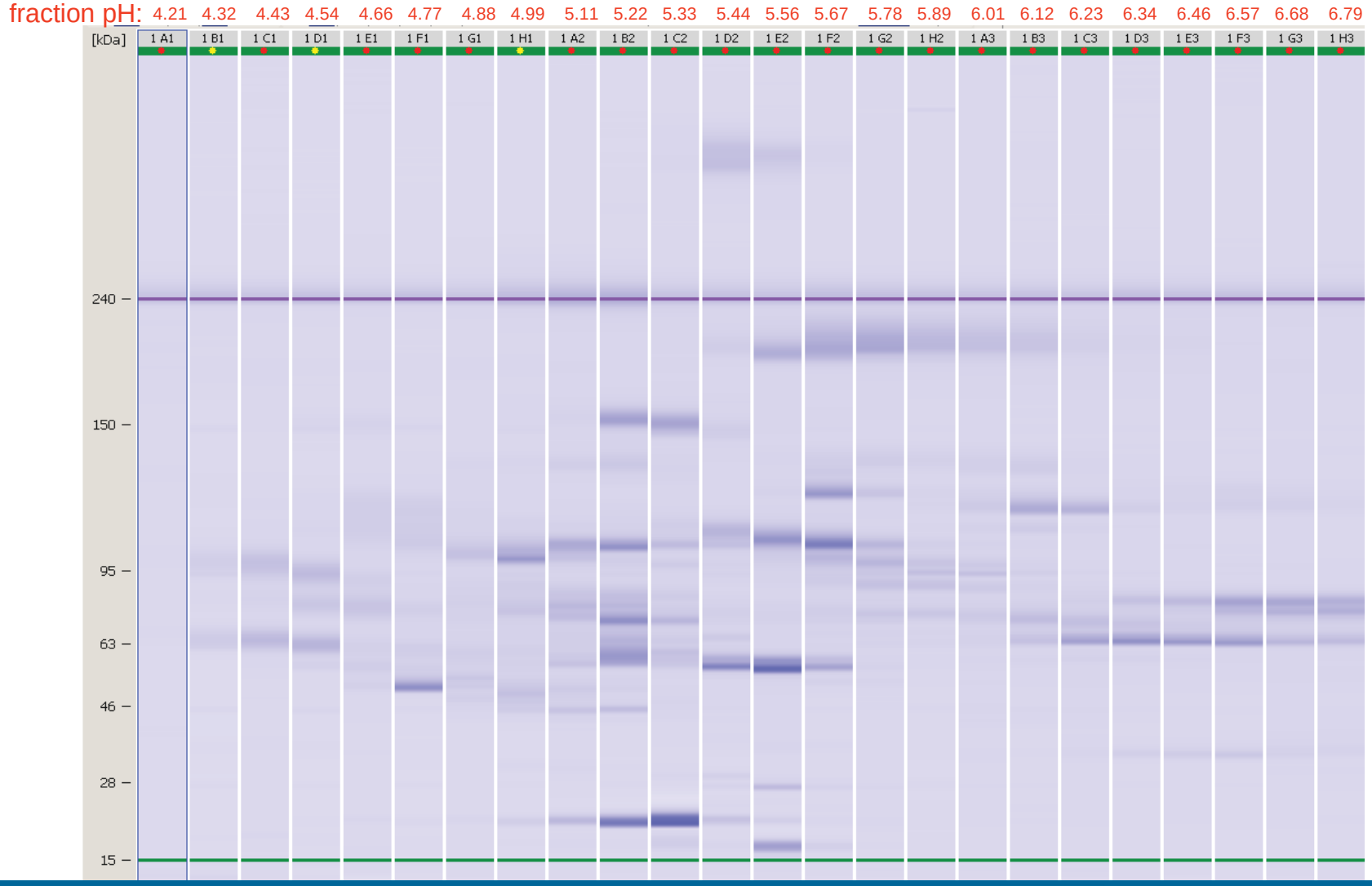


Peptide scores plotted against delta pI (1964 not validated spectra within ± 0.63 pH of center pH of well)



~35% of spectra not autovalidated are within ± 0.5pH.
Using this information, up to 20% more peptides can be identified!

Pseudo 2D Gel: OGE Fractions of Human Serum analyzed with the Protein 200-HT2 Assay on Agilent's 5100 ALP



Agilent Technologies

Outlook

• Protein prefractionation by immunodepletion, mRP and OGE enables a deeper dive into the plasma proteome and provides methods compatible with LC-MS based analysis.

• All prefractionation methods and tools integrate well together minimizing sample loss due to excessive sample manipulations

• OGE provides PTM-grade resolution of proteins and peptides and delivers fractions in solution (LC/MS compatibility)

• The mRP column provides highest recovery of protein samples (95+%) even on challenging protein samples such as integral membrane proteins

• The Multiple Affinity Removal System provides the highest sample capacity per ml resin and longest column lifetime when compared to other products. This translates to the lowest cost per ml of depleted sample.

• The Multiple Affinity Removal System also provides the greatest ease of use, highest selectivity and reproducibility (run to run, lot to lot) compared to any equivalent product.

Further Information

Bioreagents catalog – 5989-3431EN "The 2005-2006 Bioreagents Product and Resource Guide"

CD Compendium – Bioseparations 5989-4047EN

Weblink: http://www.agilent.com/chem