Protein Fractionation Techniques, HPLC and OFFGEL Fractionation

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April 2, 2008
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)

\[ \text{positively charged} \quad \text{balanced} \quad \text{negatively charged} \]

\[ \text{pH < pI} \quad \text{pH = pI} \quad \text{pH > pI} \]

(anode) (cathode)

pH gradient

Low pH \quad \text{High pH}
**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
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”.

OFFGEL Workflow overview
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 for each individual sample 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 Brilliant Blue stain

* unfractionated sample

E. coli lysate

Standard in-gel IEF and staining
OFFGEL Protein Fractionation
4 times more proteins detected with OFFGEL
OFFGEL Increases MS Sensitivity

example workflow

4-fold increase of detected proteins due to OFFGEL fractionation
OFFGEL Peptide Fractionation

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

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

OFFGEL load
1% bLG
0.1% bLG
0.01% bLG
no bLG

OFFGEL Fraction pH 4.8
1% bLG
0.1% bLG
0.01% bLG
no bLG

Agilent Technologies
BioColumn Separations
April 2, 2008
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

**OFFGEL**
pl-based Protein Fractionation

**mRP**
Protein Fractionation

**Multiple Affinity Removal System**

**OFFGEL Electrophoresis System**

**Macroporous Reverse Phase Column**
mRP (Macroporous Reverse Phase) Column

High Recovery Protein Fractionation

mRP Column

![Graph Image]
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 prefractonation, desalting and concentrating applications, including:
  – Whole cell lysates
  – High recovery membrane protein fractionation
mRP-C18 Recovery using Immunodepleted Serum

<table>
<thead>
<tr>
<th>Protein Conc. Pre-mRP*</th>
<th>Protein Conc. No column</th>
<th>Protein Conc. mRP recovery</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>47 µg</td>
<td>49.8 µg</td>
<td>49.3 µg</td>
<td>99%</td>
</tr>
</tbody>
</table>
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

(1) Hemopexin

(2) Apolipoprotein

Complement component C4 and α-1-acid-glycoprotein not fully resolved.

**Macroporous Reverse Phase C18**

270 ug depleted Human serum

(1) Hemopexin

(2) Apolipoprotein

α-1-acid-glycoprotein

Complement component C4
Applications for mRP-C18: Protein Fractionation

(4.6 x 50mm mRP-C18)
mRP Fractionation of Depleted Human Serum

A. Removal of 6 most abundant Proteins

1. Immunodepletion
2. Low Abundance proteins

B. Fractionation of the low abundant proteins

mRP Column

Blue – Control Serum
Red - Cortisol deficient Serum
Green – Rheumatoid Serum

LC/MS system

2 Fractions Analyzed by MS

Tryptic digestion

Data Analysis

<table>
<thead>
<tr>
<th>Serum #</th>
<th>Control #</th>
<th>Low Abundance</th>
<th>High Abundance</th>
<th># Unique Peptides</th>
<th>Score</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.0E+01</td>
<td>0.0E+00</td>
<td>8.1E+00</td>
<td>12</td>
<td>1.15</td>
<td>apolipoprotein H (beta-2-glycoprotein I)</td>
</tr>
<tr>
<td>8</td>
<td>1.94E+01</td>
<td>0.0E+00</td>
<td>2.2E+01</td>
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<td>apolipoprotein H (beta-2-glycoprotein I)</td>
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<tr>
<td>0</td>
<td>0.0E+00</td>
<td>1.60E+00</td>
<td>28.67</td>
<td>2</td>
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<td>complement component 1 inhibitor precursor</td>
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<td>0.0E+00</td>
<td>30.34</td>
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<tr>
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<td>0.0E+00</td>
<td>0.00E+00</td>
<td>2</td>
<td></td>
<td>complement factor B precursor</td>
</tr>
<tr>
<td>0</td>
<td>0.0E+00</td>
<td>0.0E+00</td>
<td>3.13E+00</td>
<td>2</td>
<td></td>
<td>complement factor B precursor</td>
</tr>
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<td>0.0E+00</td>
<td>3.40E+00</td>
<td>2</td>
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<td>hemopexin</td>
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<tr>
<td>0</td>
<td>0.0E+00</td>
<td>0.0E+00</td>
<td>4.13E+00</td>
<td>2</td>
<td></td>
<td>alpha-1-acid glycoprotein 2 precursor</td>
</tr>
</tbody>
</table>
Serum Proteomics Workflow

1. Blood Sample from Patient
2. Serum/Plasma sent to lab
3. Remove High Abundant Protein
4. Fractionation of Proteins
5. Mass Spectrometer + Software ID peptides and verify presence of protein (quantitation)
6. 1-D (Reverse Phase) or 2-D (Ion Exchange + Reverse Phase) Chromatography
7. Trypsin enzyme digestion of proteins into peptides
8. Fractionation of Proteins and/or Peptides
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

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Total Acquisition Time (hours)</th>
<th># MS/MS Collected</th>
<th># Proteins Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Serum**</td>
<td>36</td>
<td>67,997</td>
<td>40</td>
</tr>
<tr>
<td>Immunodepleted Human Serum</td>
<td>24</td>
<td>46,808</td>
<td>170</td>
</tr>
<tr>
<td>Fraction #16</td>
<td>22</td>
<td>59,767</td>
<td>144</td>
</tr>
<tr>
<td>Fraction #22</td>
<td>22</td>
<td>59,598</td>
<td>114</td>
</tr>
<tr>
<td>Fraction #28</td>
<td>22</td>
<td>58,473</td>
<td>96</td>
</tr>
<tr>
<td>Fraction #32</td>
<td>22</td>
<td>54,758</td>
<td>107</td>
</tr>
<tr>
<td>Combined Fractions</td>
<td>88</td>
<td>232,596</td>
<td>461</td>
</tr>
</tbody>
</table>
Human Brain Membrane Lipid Raft Prep (500µg)

PC = phosphatidylcholine
SM = sphingomyelin
Human Brain Membrane Lipid Raft Prep

Selected Excised Bands Which are Integral 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

Blue – postrun column injection after 5 separations

Red – prerun column injection
Hela Cell Lysate mRP Fractionation

4.6 x 50mm mRP C18 column
Hela Cell Membrane Protein Fractionation and ID

HeLa Cell Membrane Proteins

- Solubilization
- mRP

Strategy 1
In-Gel Digestion

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

Strategy 2
In-Solution Digestion

- 17 mRP fractions
- Total Acquisition Time (hrs): 102
- # MS/MS Spectra Collected: 412,741
- # Distinct Peptides Matched: 5383
- # Total Proteins Identified: 954
- # Membrane Proteins Identified: 470
- # Integral Membrane Proteins Identified: 337

Total man hours (labor): 3-4 days

1D nano-chip LC/MS/MS

2D nano-chip LC/MS/MS

Total man hours (labor): 4 hours
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

<table>
<thead>
<tr>
<th>Brief Description</th>
<th>Macroporous Reverse Phase (mRP)</th>
<th>OFFGEL Electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC Column for protein fractionation</td>
<td>Instrumentation and consumable kits for protein and peptide fractionation</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target Applications</th>
<th>Protein Discovery &amp; Characterization, Biomarker Validation</th>
<th>Protein Discovery, Protein Characterization</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Sample Types</th>
<th>Proteins</th>
<th>Proteins or peptides</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Operating Conditions</th>
<th>70-90°C, reverse phase</th>
<th>Cooled samples, aqueous buffer</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Downstream Compatibility</th>
<th>LC-MS*, LC-UV, MALDI*, 2-D gels</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Loadable Sample Amount</th>
<th>2µg-300 µg</th>
<th>50µg-5mg</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Fractionation Principle</th>
<th>Reverse phase (hydrophobicity)</th>
<th>Isoelectric point (pI)-based</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Run-time</th>
<th>60 min/gradient</th>
<th>8 hours-36 hours, up to 16 samples in parallel</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Recovery</th>
<th>95-99%</th>
<th>Protein: 70%, peptides: &gt;80%</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Knowledge Transfer</th>
<th>No need for additional HPLC training, similar to any other reverse phase separation</th>
<th>2-D gel users can easily transfer IEF parameters to OFFGEL fractionations or validate OFFGEL vs. in-gel IEF</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>How to use mRP and OGE together</th>
<th>OFFGEL protein fractionation followed by mRP for second dimension fractionation and desalting</th>
</tr>
</thead>
</table>

* 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

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