

Comparing Gel-Eluted Liquid Fraction Entrapment Electrophoresis to Gel-LC for the Analysis of Complex Proteomics Samples

James B. Harkins IV, Nghia Chiem, Christopher Dill, Cindy Brown, Charles E. Witkowski II, Jeremy L. Norris

Protein Discovery, Inc., Knoxville, TN

OVERVIEW

It has long been understood that sample fractionation is critically important to generating quality, comprehensive proteomics data. In spite of the continual improvements in speed and sensitivity of mass spectrometers, these instruments are still unable to adequately overcome the enormous challenge of most biological samples with multiple dimensions of separation prior to mass analysis. In response, there are numerous options for sample fractionation, ranging from simple one-dimensional gels to sophisticated chromatographic techniques. Each of these techniques has unique advantages and drawbacks; however, it is a proven principle that high quality fractionation improves one's ability to generate quality data.

Two recent studies^{1,2} evaluated many of the commonly used methods for sample fractionation on both the peptide level and the protein level. Each confirmed that even among the most sophisticated and popular techniques, protein-level fractionation using one-dimensional gels (GelC-MS/MS) was most effective for proteomics. These studies conclude that GelC-MS/MS outperforms other techniques when examining total protein identifications and unique peptide identifications. These studies find that "GelC gives the richest and most consistent protein identification data, with the added advantage of information on molecular weight"².

In spite of the superior performance of 1D gels relative to other techniques, there are some disadvantages of GelC-MS/MS. Protein recovery is difficult, requiring the protein to be digested for recovery. Conventional, commercially available 1D gels have limited loading capacity, making detection of low abundance proteins challenging. One-dimensional gels have poor reproducibility, making it difficult to reproducibly isolate proteins of interest.

In this study, we introduce an improved method of GelC-MS/MS that utilizes a precast, high capacity PAGE separation to isolate distinct molecular weight fractions in solution phase. Since proteins are recovered in solution, recovery is much higher (>90%). This feature, combined with high loading capacity (>500 µg), lead to an increase in proteome coverage and data quality.

References

- Fang, Y, et al. Quantitative Analysis of Proteome Coverage and Recovery Rates for Upstream Fractionation Methods in Proteomics. *J. Proteome Res.* 2010, ASAP.
- Piersma, S.R, et al. Workflow Comparison for Label-Free, Quantitative Secretome Proteomics for Cancer Biomarker Discovery: Method Evaluation, Differential Analysis, and Verification in Serum. *J. Proteome Res.* 2010, ASAP.

Gelfree™ 8100 Fractionation System

Molecular Weight-Based Fractionation with Liquid-Phase Recovery

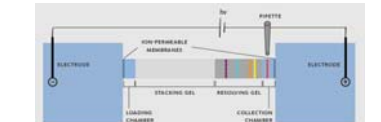
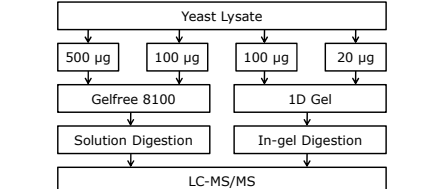


Figure 1: Schematic of the Gelfree™ 8100 Fractionation System. The technology uses SDS-PAGE to separate analytes based on molecular weight. As molecular weight fractions elute from the end of the gel, they are retained in a liquid layer defined by the end of the gel and a molecular weight cut-off membrane.

EXPERIMENTAL DESIGN

This study compares traditional GelC-MS/MS using a 1 mm precast 1D gel and in-gel digestion to the same fractions prepared using Gelfree 8100 and in-solution digestion. In short, a single yeast lysate was divided into aliquots for preparation using both techniques. Aliquots of 500 and 100 µg were fractionated using Gelfree 8100 while 100 and 20 µg aliquots were separated using 1D Gel. Gelfree fractions were subjected to tryptic digestion in solution. Gel bands that correspond to identical molecular weight fractions for four of the Gelfree fractions (5-8) were excised from the 1D gel lanes and digested using in-gel tryptic digestion. Each sample set was analyzed using nanoLC-MS/MS on an ion trap mass spectrometer.



METHODS

Sample: Whole cell lysate of *S. cerevisiae*.

Gelfree fractionation: Proteins of molecular weight between 3.5-150 kDa were separated using Gelfree 8100 Fractionation System using an 8% Tris-acetate/HEPES buffer system (Protein Discovery). Samples were prepared for separation by desalting using Zeba Spin Desalting Column (Pierce Biotechnology). Loading amounts of 500 µg and 100 µg were run in parallel. The fractions were separated into 12 fractions using manufacturer's recommended protocol and stored at -80°C.

SDS removal: SDS was removed from the Gelfree fractions using Pierce Detergent Removal Spin Columns.

Digestion of Gelfree fractions: Each solution was recovered in approximately 150 µl of buffer.

- An aliquot of 15 µl of 1% PPS Silent Surfactant in 50 mM NH₄HCO₃ was added.
- A volume of 1.5 µl of 0.5 M DTT was added, incubate for 30°C for 30 minutes.
- A volume of 4.5 µl of 0.5 M IAA was added, incubate in the dark at room temperature for 30 minutes.
- One micromolar of trypsin was added. (Assume that protein load is divided equally into 12 fractions for a total protein content of 42 µg and 8 µg for the 500 and 100 µg load, respectively. Target of 1:50 trypsin:protein, adding no less than 1 µg for low total protein.)
- Sample incubated overnight (16 hrs) at 37°C.
- A volume of 45 µl of 1M HCl was added to quench digestion and cleave PPS.
- Sample centrifuges at 14000 x g for 10 minutes.
- The sample volume was reduced to 50 µl by vacuum centrifugation.

1D gel analysis: Gel analysis was performed using 10-20% Tris-Glycine gels (Invitrogen). Two different protein loads of 100 µg and 20 µg were analyzed. For comparison, 4 Gelfree fractions were run on the same gel in order to select the identical molecular weight fractions. The analysis was performed using Spectrum Mill and Excel. Only the most valid protein bands staining. Gel bands equivalent in molecular weight to each of the fractions (5-8) were excised from the two right lanes and were divided into pieces.

Mass Spectrometry: Samples were analyzed in duplicate by nanoLC-MS/MS using an Agilent 6340 ion trap mass spectrometer equipped with Chip-LC. Samples were separated using a 60 minute gradient on a 0.075 x 150 mm column. One microliter fractions were desalted and concentrated using an inline trap column. Data were analyzed using Spectrum Mill and Excel. Only the most valid protein identifications are compared, requiring a score of 9 for protein identifications and a score of 20 for peptides with >70% of spectral features matched.

In-gel digestion: Adapted procedure from Shevchenko, et al. *Nature Protocols*, 1(5), 2856.

- NH₄HCO₃ was removed from the gel plugs.
- A volume of 0.5 ml of acetonitrile was added, vortex and incubate for 10 minutes until the gel plugs become white.
- Acetonitrile removed.
- An aliquot of 25 µl of 10 mM DTT was added, sample incubated at 50°C for 30 minutes.
- Cooled to room temperature.
- Added 500 µl of acetonitrile, dehydrate, remove liquid.
- An aliquot of 25 µl of 50 mM IAA was added, sample incubated at room temperature for 30 minutes.
- Dehydrated gels.
- Added 50 µl of trypsin (13 ng µl⁻¹ in 10% acetonitrile/10 mM NH₄HCO₃).
- Refrigerated for 30 minutes.
- Added additional trypsin if necessary to cover the gel plugs.
- Refrigerated an additional 90 minutes.
- Incubated at 37°C (16 hrs) overnight.
- Added an aliquot of 100 µl of extraction buffer (1:2 (v/v) 5% formic acid/acetonitrile).
- Incubated for 15 minutes at 37°C.
- Withdrew supernatant using a gel loading tip.
- Vacuum centrifuged supernatant to dryness.
- Reconstituted in 50 µl of 0.1% formic acid.

Rat Brain: Rat brain sample (Figure 7) was prepared from fresh frozen tissue as described in Wisniewski, et al., *J. Proteome Res.*, ASAP.

RESULTS

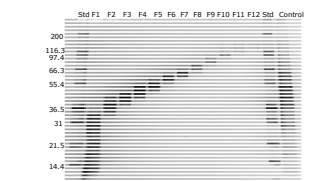


Figure 2: Fractionation of *S. cerevisiae* by Gelfree 8100. The lysate was fractionated into 12 fractions ranging in molecular weight from 3.5 to 150 kDa. The fractions were visualized using 1D gel electrophoresis, followed by silver staining. Fractions 5-8 were selected compared against Gelfree. This figure shows 500 µg loading of Gelfree 8100.

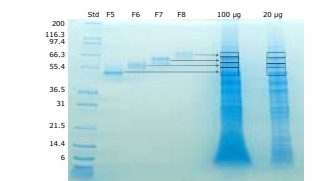


Figure 3: Preparation of *S. cerevisiae* for GelC-MS/MS analysis. The lysate was separated using 1D gel electrophoresis. Aliquots from the Gelfree fractions were run in parallel to align with the unfractionated sample. The molecular weight ranges corresponding to each Gelfree fraction were excised and digested using standard protocols. The range of the four fractions chosen is approximately 15 kDa (53-68 kDa).

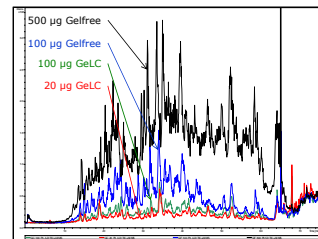


Figure 4: Total ion chromatograms of fraction five from each of the four conditions tested. Each of the four samples was diluted to identical volumes and equal injection volumes were compared. The total ion current represents the relative recovery of digested peptides from each condition. Even considering equivalent loading amounts of 100 µg, substantially greater numbers of peptides were detected using Gelfree 8100.

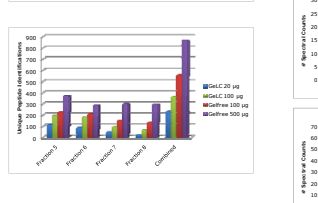
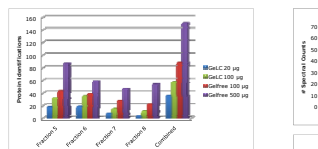


Figure 5: The combination of high recovery and high loading capacity significantly increases the number of proteins and unique peptides identified. Comparison of the total number of valid proteins identified using Gelfree with those identified using conventional GelC reveals significantly more proteins and peptides are identified using Gelfree 8100. This increase of approximately 50% can be directly correlated with both the higher loading capacity and the increase in the percentage of total protein recovered from the Gelfree 8100.

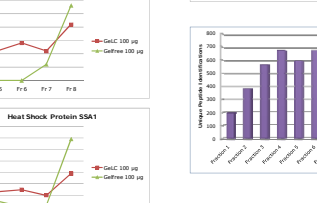
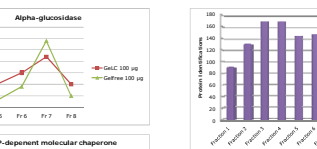


Figure 6: High loading capacity of Gelfree provides increased resolution relative to conventional 1D gels. Examination of spectral counts of identified proteins across all fractions reveals that proteins are spread across fewer fractions, reducing redundancy of protein identifications across fractions at equivalent protein loading.

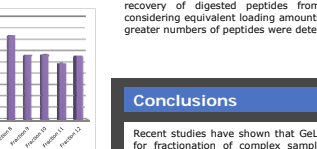


Figure 7: Gelfree 8100 performance for the 12 fractions of rat brain extract. The total number of unique proteins and peptides identified across twelve Gelfree 8100 fractions was evaluated. A total of 1245 unique proteins and 5270 unique peptides were identified across the mass range 3.5-150 kDa. Samples were fractionated using the Mid-Mass Gelfree Cartridge kit. Samples were prepared for analysis using Emitter-Assisted Spray Preparation (EAS). Samples were analyzed using a 3D ion trap.

Conclusions

Recent studies have shown that GelC-MS/MS is the method of choice for fractionation of complex samples in preparation for bottom-up proteomics. Fractionation of intact proteins using the Gelfree 8100 Fractionation System yields superior results when compared directly to GelC-MS/MS for the same range of molecular weights.

- The advantages observed include:
- ✓ Programmable, reproducible fractionation according to protein molecular weight.
 - ✓ Unbiased protein fractionation compatible with all protein classes (hydrophobic, acidic, basic, LMW, etc.).
 - ✓ High loading capacity: >500 µg of a complex cell lysate.
 - ✓ High recovery: >90% of protein fractions in liquid form permits in-solution digestion of proteins or direct analysis of intact proteins.
 - ✓ An increase in the number of statistically valid protein identifications and unique peptide identifications.
 - ✓ Higher resolution fractionation at a given protein load relative to conventional 1D gels, a consequence of higher loading capacity.
 - ✓ Minimal to no loss of proteins identified using GelC-MS/MS.

Other applications include sample fractionation for top-down proteomics, bottom-up proteomics and characterization of protein therapeutics. Gelfree 8100 is compatible with depletion, IEF, protein digestion, and western blotting.