

# Molecular Weight-Based Fractionation of Intact Proteins with Liquid Phase Recovery using the GELFREE™ 8100

Nghia Chiem, Christopher Dill, Jay Harkins, Peter Osucha, Chuck Witkowski, Jeremy Norris  
Protein Discovery, Knoxville, TN

## INTRODUCTION

Sample complexity necessitates extensive fractionation for in-depth proteomic analysis using mass spectrometry. Preparative 1D gel electrophoresis using SDS-PAGE provides broad molecular weight fractionation of intact proteins and unbiased sampling of protein classes (hydrophobic, acidic, basic, etc.), driving its widespread use in bottom-up and top-down proteomics. However, protein fractions separated on the gels must be extracted into liquid phase for subsequent analysis using mass spectrometry, currently requiring tedious gel slicing and yielding relatively poor recoveries.

This work describes the development and characterization of a commercial system using a novel technology, termed gel Elution Liquid Fraction Entrapment Electrophoresis (GELFREE), for molecular weight-based fractionation of intact proteins with liquid phase recovery. Eight samples are run in parallel using a programmable control module in ca. 90 minutes. The system is shown to provide robust fractionation over the mass range 3.5kDa – 150kDa with high loading capacity, reproducibility, and recovery.

## MATERIALS AND METHODS

**Preparation of the Sample:** Bovine liver was lysed in T-Per in the presence of protease inhibitors and homogenized in a Dounce homogenizer. Total protein was quantified using a Lowry assay and adjusted to a final concentration of 10µg/µL. Prior to use, the bovine liver homogenate (BLH) was centrifuged at 500 g for 10 minutes and a 12µL aliquot (1.12mg) was added to 30µL sample buffer, 5µL reducing agent (1M DTT) and heated at 95°C for 5 min.

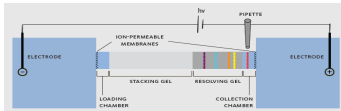
**Preparation of Cartridge:** The storage buffer was removed from all reservoirs and chambers. The anode buffer reservoirs were each refilled with 8mL of 1X tris-tricine running buffer and the collection chambers were each refilled with 100µL of 1X tris-tricine running buffer. The cathode reservoirs were each refilled with 5.5mL tris-tricine running buffer, which automatically fills the sample loading chamber with the correct volume.

**Performing the Run:** The Running Buffer was removed from the Sample Loading Chamber and immediately replaced with 150µL of buffered BLH sample using an 8-channel pipettor. The instrument was set at constant 100V, 3W max power, per channel. Pauses were programmed to occur at 5, 14, 15, 16, 17, 18, 20, 22, 24, 26, 28, 33, 38, 43, 58, and 73 minutes. At 5 minutes, the Cathode Reservoirs were filled with Running Buffer to a total volume of 8mL. The run was resumed and at each of the next 16 pauses, the solutions in the Sample Collection Chambers were harvested using an 8-channel pipettor and refilled with 100µL Running Buffer in each. At 28 minutes and 58 minutes, the Running Buffer was replaced with 8mL in the Cathode Buffer Reservoir and 8mL in the Anode Buffer Reservoir.

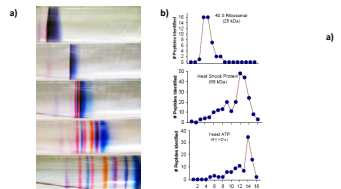
**1D Gel Analysis:** 5µL reducing sample buffer (5% 1M DTT) was added to 20µL of each GELFREE fraction, heated at 95°C for five minutes, and loaded on a 10–20% tris-glycine gel (Invitrogen, Carlsbad CA). 10µL of the Mark 12™ Unstained Standard was loaded for MW calibration. 3µL of BLH stock was added to 17µL of 180 H<sub>2</sub>O and 5µL reducing sample buffer, heated at 95°C for 5 min, and loaded as a control. Electrophoresis was performed at constant 125V for 2 hrs, after which gels were removed from the cassette and Silver stained using standard protocols.

**Reproducibility and Recovery Experiments:** For visual reproducibility assessment, 10µL ColorBurst™ (Sigma, St. Louis MO) was added to 90µL of 180 H<sub>2</sub>O and 28µL Sample Buffer and 7µL of 1M DTT and loaded as previously described. The cartridge was run at constant 100V, max 3W per channel for 15 minutes, the gel tubes removed and imaged. For BSA recovery and reproducibility, 12µL of 2µg/µL solution BSA (Thermo Fisher Scientific, Rockford, IL) was added to 100µL 180 H<sub>2</sub>O, 30µL Sample Buffer and 8µL 1M DTT, heated at 95°C for 5 min, and loaded as previously described. The instrument was run at constant 100V, max 3W per channel. A 10 minute fraction, totaling 170µL, was collected between 25 and 35 minutes. For 1D gel analysis, 15µL of each GELFREE fraction was added to 5µL reduced sample buffer, heated at 95°C for 5 min and loaded on a tris-glycine gel, along with 10µL of the Mark12™ Unstained Standard and 1µL stock BSA in 14µL H<sub>2</sub>O was loaded in lane 1D. Electrophoresis was performed at 125V for 2 hrs, and the gel was removed and stained using SimplyBlue™ SafeStain (Invitrogen, Carlsbad CA) according to the manufacturer's procedures. The gels were then scanned and relative intensities determined using the Volume Contour tool of Quantity One® gel analysis software (Bio-Rad Laboratories, Richmond WA).

## GEL ELUTION LIQUID FRACTION ENTRAPMENT ELECTROPHORESIS - BACKGROUND



**Figure 1: Schematic of the GELFREE device.** 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.



**Figure 3: GELFREE separates proteins into distinct fractions based on molecular weight.** a) Protein separation inside the tube gel shows the resolution of labeled proteins ranging from 6–220 kDa. b) Using bottom-up LC/MS/MS approaches, protein separation is confirmed.

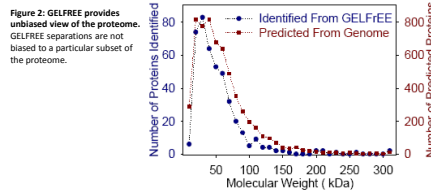
Data provided by John C. Tran, *Development of Effective Proteomic Separations for Biological Analysis Using Mass Spectrometry*, (2008) Dissertation, Dalhousie University, Halifax, Nova Scotia.

## GELFREE™ 8100 FRACTIONATION SYSTEM

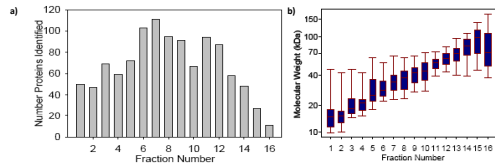
The GELFREE™ 8100 system is comprised of two components, the GELFREE Instrument and the GELFREE Cartridge.



**Figure 4:** The GELFREE instrument (left) is an eight channel electrophoretic controller capable of simultaneously supplying constant current or voltage to each of the eight channels in the cartridge (right). A touch screen display located on the front of the instrument allows the user to program the voltage/current to apply to each cell, as well as the fraction entrapment intervals. To use the system, the user programs the sequence (or chooses from a pre-programmed sequence) for each of the eight electrophoretic cells and starts the experiment via the touch screen interface. The device automatically pauses the experiment whenever the time interval has expired, allowing the user to extract the molecular weight fraction of interest. Measurement information for the eight channels is displayed to the user in tabular and graphical format during the course of an experiment. Additionally, this run information is exportable in text delimited format using a USB port located on the back of the instrument.

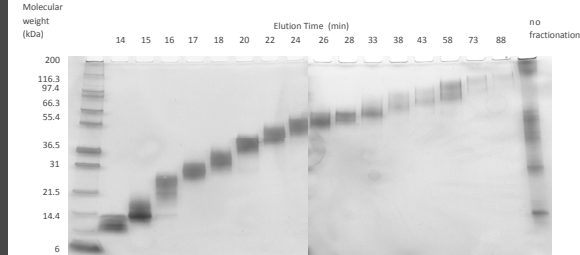


**Figure 2: GELFREE provides unbiased view of the proteome.** GELFREE separations are not biased to a particular subset of the proteome.

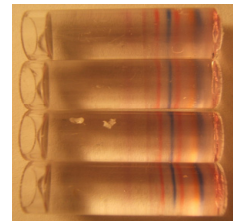


**Figure 4: GELFREE is compatible with proteomics techniques such as LC/MS/MS.** Protein fractions were analyzed using bottom-up LC/MS/MS techniques. a) Identified proteins from each fraction of a complex biological sample and b) calculated molecular weight of each identified protein. Larger proteins are identified in later fractions.

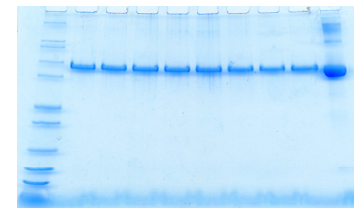
## RESULTS



**Figure 5: GELFREE provides intact protein molecular weight-based fractionation with liquid phase recovery.** Each of the 16 fractions collected from the GELFREE cartridge was run on a 1D tris-glycine gel and silver stained. The gel shows the molecular weight based fractions from bovine liver homogenate.



**Figure 6: GELFREE provides excellent gel-to-gel reproducibility.** ColorBurst™ replicates separated simultaneously using the GELFREE system.



**Figure 7: Recovery from GELFREE is >60% and reproducibility of recovery is excellent (CV<10%).**

Column	% Adj. Vol.	% Recovery
1.0	10.3	62.6
2.0	10.4	63.5
3.0	10.8	66.0
4.0	11.8	71.9
5.0	11.1	67.8
6.0	9.0	54.7
7.0	9.6	58.6
8.0	10.5	64.0
Control	16.4	

Average	Standard Deviation	% CV
63.6	5.3	8.4

**Figure 8: Recoveries as calculated using QuantityOne™ from GELFREE.**

## CONCLUSIONS

- The GELFREE technology provides broad mass range fractionation and molecular weight-based fraction targeting
- Analytes are recovered intact for characterization of post-translational modifications, truncations, and other protein alterations
- SDS-PAGE provides non-biased sampling of protein classes (hydrophobic, acidic, basic, low molecular weight, etc.)
- The GELFREE™ 8100 provides high protein recoveries across the entire mass range 3.5kDa – 150kDa
- Liquid fractions are easy to harvest with a pipette (no slicing of gel bands)
- Permits in-solution digestion rather than in-gel digestion
- High reproducibility (RSD < 5%, n=6)
- Loading capacity is ~1mg total protein in bovine liver homogenate per channel
- Fractions are suitable for downstream preparation and analysis using LC-MS, MADI-TOF, or western blot
- Molecular weight-based fractionation provides information that is essential for distinguishing peptides from multiple gene products
- 8 channels are run simultaneously in less than 90 minutes