Evaluation of Stain-Free Gels For GeLC-MS Applications

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Introduction

GeLC-MS is a widely used proteomics workflow that involves an initial electrophoretic step of resolving protein mixtures on a 1-D SDS-PAGE gel. Subsequently, the gel is stained for visualization of protein lanes and each lane sliced into discrete fractions along the length of the gel. Finally, each fraction is processed for in-gel protein digestion, peptide purification, and LC-MS/MS analysis.

Reports have demonstrated that when compared to in-solution protein digestion and fractionation, GeLC-MS enables higher total protein identifications and hydrophobic membrane protein identifications are particularly enhanced (Fang et al. 2010; Piersma et al. 2010). In spite of these benefits, improvements in the traditional GeLC-MS workflow in terms of speed and ease of processing would enable its wider adoption and deeper investigations of complex proteomes.

With these interests in mind, we tested the suitability of Criterion™ TGX Stain-Free™ gels, which have faster electrophoretic run times and make manual staining and destaining unnecessary, for use in a simplified GeLC-MS workflow.

Background

Stain-free technology is based on a UV-induced trihalo compound modification of tryptophan residues contained in proteins after separation by electrophoresis. The system uses standard reagents, sample preparation methods, and electrophoresis protocols. After electrophoresis, the gel is removed from the cassette, activated by UV irradiation, and produces a fluorescent signal that is detected and captured by a CCD camera. In 2.5–5 minutes, the system provides an image of the protein that is consistent with the image achieved using standard SDS-PAGE/Coomassie staining methods.
U.S. patents 7569130 and 8007646.

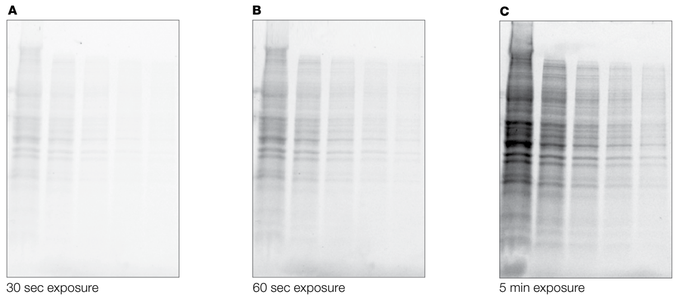

Fig. 1. Representative gel images of HeLa lysate separated on Criterion Stain-Free TGX gel, imaged at 30 sec (A), 60 sec (B), and 5 min (C).

Methods

Protein Electrophoresis and Collection of Individual Gel Slices

50 µg protein from 3T3 whole cell lysates was electrophoresed on 4–15% Criterion™ Tris-HCl or Criterion TGX Stain-Free gels in triplicate. After electrophoresis, Tris-HCl gels were visualized by Coomassie staining and destaining (~3 hr). After destaining was completed, eight slices covering one single entire lane were cut and processed for in-gel trypsin digestion and post-digestion sample processing. In the case of samples run on TGX Stain-Free gels, gels were visualized using a Gel Doc™ EZ imager with 5 min UV activation and eight slices covering an entire single lane cut out identically as above for in-gel digestion and post-digestion peptide purification. Each gel slice was assumed to contain ~6 µg protein.

In-Gel Digestion (adapted from Shevchenko et al. 1996)

Gel slices were initially washed with water, twice with 1:1 100 mM NH_4HCO_3/acetonitrile for 15 min each, and then washed once with 2x volume of acetonitrile. Gel slices were rehydrated in 100 mM DTT/100 mM NH_4HCO_3, incubated at 56°C for 1 hr and removed for cooling to room temperature. The liquid was removed and proteins alkylated by 50 mM iodoacetamide/100 mM NH_4HCO_3 for 30 min at room temperature in the dark. After removal of alkylating buffer, gel slices were washed with acetonitrile and NH_4HCO_3 as above, until all the Coomassie stain was removed. The last wash was performed with 100% acetonitrile and samples processed for in-gel trypsin digestion. Gel slices were rehydrated in 100 mM NH_4HCO_3, 5 mM $CaCl_2$, 1.5 ng/ml trypsin for ~20 min on ice. The supernatant was removed and replaced with 40–70 µl of the same buffer without trypsin and digestion continued overnight at 37°C.

Post-Digestion Peptide purification (adapted from Rappsilber et al. 2003)

After overnight tryptic digestion in microcentrifuge tubes, samples were vacuum dried, reconstituted in ~0.2–0.4% TFA, and processed for C18 purification of peptides. Each gel slice was assumed to contain ~6 µg tryptic peptides and processed for C18 purification using 3 punches (made using an 18 gauge blunt tip syringe needle) of Empore reversed-phase extraction disks in a stage-tip. ~1.5 µg peptide was analyzed on a Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) as described herein.

Peptides were dissolved in 16.5 µL of load solvent (98:2:0.01 water/acetonitrile/formic acid) and 5 µl loaded directly onto a 12 cm × 75 µm fused silica pulled-tip capillary column packed in-house with Magic C18AQ, 5 µm, 200 Å pore size resin (Michrom BioResources, Inc.) with load solvent at a flow rate of 800 nl/min using a Nano LC-1D Plus system and a MicroAS autosampler.

Peptides were eluted using a gradient of 10–40% acetonitrile in 0.1% formic acid over 55 min with a constant flow of 320 nl/min. The column was mounted in a nanospray source directly in line with a Velos Orbitrap mass spectrometer. Spray voltage was at 2.2 kV and the heated capillary maintained at 260°C. The orbital trap was set to acquire survey mass spectra (m/z 300–2000) with a resolution of 30,000 at m/z 400 with a target value set to 1E6 ions or 500 ms. The six most intense ions from the full scan were selected for fragmentation by higher-energy collision induced dissociation activation (normalized collision energy, 40% activation time 0.1 msec, fixed first m/z value at 111) in the HCD multipole with automatic gain control settings of 100,000 ion or 500 ms concurrent with full-scan acquisition in the orbital trap. For enhanced mass accuracy, the lock mass option was enabled for real-time calibration with polysiloxane peak at 445.1200 m/z. Precursor ion charge state screening was enabled; unassigned and singly charged species were rejected. Dynamic exclusion set to a maximum of 50 entries with a maximum retention period of 15 sec and mass window of –0.7 to 1.25 amu. Data were acquired using Xcalibur software v 2.1.0 SP1.

Database Searches

Collected spectra were searched against the 2011 version of the uniprot mouse database (47932) to which the reverse complement of each protein was appended along with common contaminants using SEQUEST V27Rev12. Fixed modifications used was +57 Da on Cys (alkylation by iodoacetamide) and variable modifications used were +16 Da Met (oxidation), +32 on Trp (formylkynurein), and +58 on Trp (hydroxylethanone). All protein identifications were filtered at 1% global false discovery rate (1% false discovery rate at protein level) using Scaffold Version 3.0.

Table 1. LC-MS gradient and solvents used for elution of peptides in line with mass spectrometer.

Time, min	% B
0	2
5	10
60	40
60.1	80
70	80
70.1	2
80	2

Solvent A: 98:2, H_20:acetontrile, 0.1% formic acid
Solvent B: 98:2, acetontrile: H_20, 0.1% formic acid

Results and Conclusions

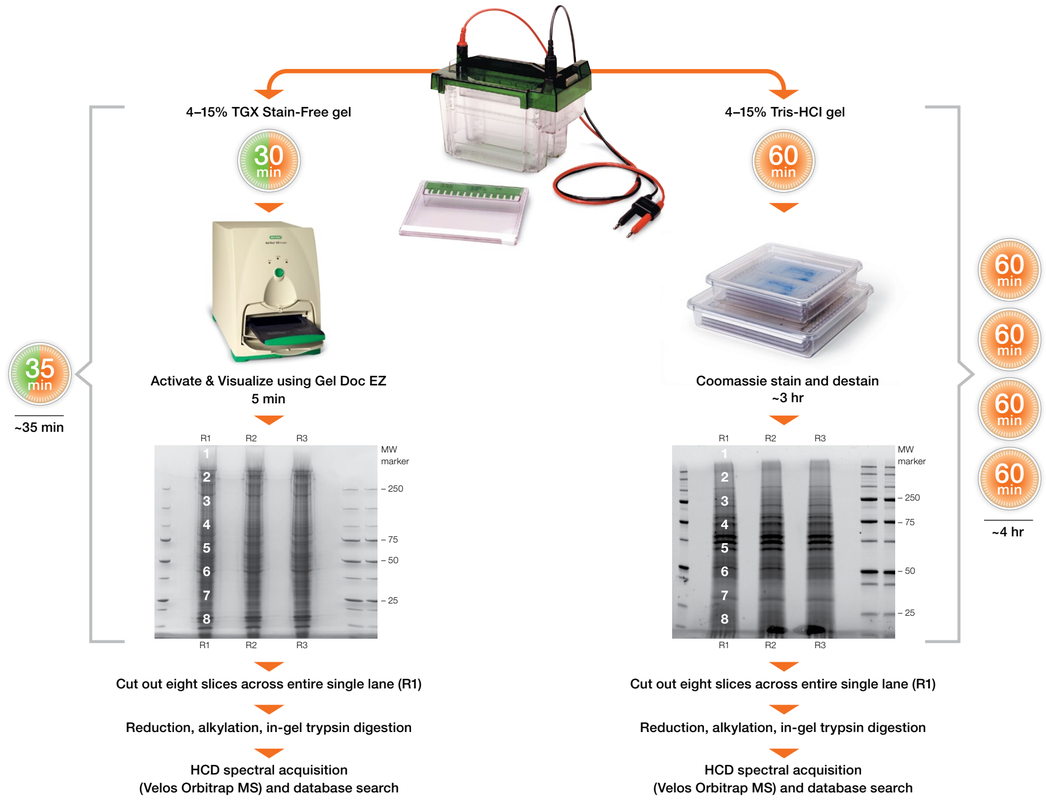

Fig.2. Comparison of the 3T3 cell lysate separated on Criterion gels. 50 µg of mouse 3T3 lysate electrophoresed on Criterion 4–15% TGX Stain-Free gel (left) or 4–15% Tris-HCl gel (right) in triplicate (R1, R2, R3). Lane/Sample R1 was processed for GeLC-MS after visualization by Gel Doc EZ (left) or Coomassie staining and destaining (right).

Table 2. Results from GeLC-MS workflow using 4-15% TGX Stain-Free and Tris-HCl gels.

Results	TGX Stain-Free	Tris-HCl
Proteins (1% global FDR)	1,225	1,129
Peptides (0.2% global FDR)	15,802	15,541
Spectra	38,999	37,528
% Identified spectra	41%	41%

1420 total proteins (1% global FDR).

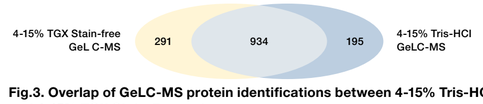

Fig.3. Overlap of GeLC-MS protein identifications between 4-15% Tris-HCl and 4-15% TGX Stain-Free gels.

Conclusions

1. Near identical protein, peptide, and spectral identifications (+/- 10% for each category) using Tris-HCl and TGX Stain-Free gels for GeLC-MS.
2. TGX Stain-Free gels are suitable with GeLC-MS workflows.
3. Benefits of using TGX Stain-Free gels:
   – Faster gel run times compare to conventional Tris-HCl gels
   – UV activation eliminates the need for staining and destaining of lanes (savings of at least 3 hr, depending on staining method used) and associated variability/handling

References

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