Springer Protocols

Methods in Molecular Biology 527

Phospho-Proteomics

Methods and Protocols

Edited by
Marjo de Graauw

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METHODS IN MOLECULAR BIOLOGY™

Phospho-Proteomics

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Edited by

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Division of Toxicology, Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, Leiden, Netherlands

Humana Press

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ISBN: 978-1-60327-833-1 e-ISBN: 978-1-60327-834-8
ISSN: 1064-3745 e-ISSN: 1940-6029
DOI: 10.1007/978-1-60327-834-8

Library of Congress Control Number: 2008944207

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Preface

Phosphorylation of proteins on specific amino acid residues is a key regulatory mechanism in cells. Protein phosphorylation controls many basic cellular processes, such as cell growth, differentiation, migration, metabolism, and cell death, and is in itself regulated by the activity of kinases and phosphatases. Identification of differentially phosphorylated proteins by means of phospho-proteomics therefore increases our insight into the signal transduction pathways that are activated in cells in response to different stimuli, such as growth factor stimulation or exposure to toxicants.

Phospho-proteomics presents both well-established protocols and some of the newest strategies for the identification and evaluation of protein phosphorylation on Tyr, Ser, and Thr residues. Detailed protocols and methodologies are included that focus on twodimensional gel electrophoresis and protein phosphorylation, enrichment of phosphoproteins and peptides, quantitative analysis of phosphorylation by labeling and MS analysis, and antibody and kinase arrays. In addition, this volume contains three chapters on bioinformatics and phosphosite prediction and two reviews providing an overview of recent advances in the identification of phosphoproteins by mass spectrometry and an outline of different chemical tagging strategies.

Phospho-proteomics is aimed at those who wish to gain insight into signal transduction pathways by studying and identifying protein phosphorylation. It is written for a broad audience ranging from researchers who are new to phosphoproteomic techniques and for those with more experience in the field. This book covers those proteomic methods and technologies that are widely used and have been well-established in the prominent proteomic labs. In addition, several protocols have been included that point to a new direction in the phospho-proteomics field.

Finally, I thank my colleagues at the Division of Toxicology, LACDR, Leiden, for their support and all the authors who kindly contributed their time, expertise, suggestions, and enthusiasm.

Leiden, The Netherlands ***Marjo de Graauw, PhD***

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Chapter 1

A High-Resolution Two Dimensional Gel- and Pro-Q DPS-Based Proteomics Workflow for Phosphoprotein Identification and Quantitative Profiling

Ganesh K. Agrawal and Jay J. Thelen

Summary

The two-dimensional (2-D) gel-based proteomics platform remains the workhorse for proteomics and is fueled by a number of key improvements, including fluorescence-based stains for detection and quantification of proteins and phosphoproteins with high sensitivity and linear dynamic ranges. One such stain is Pro-Q diamond phosphoprotein stain (Pro-Q DPS), which binds to the phosphate moiety of phosphoproteins irrespective of the phosphoamino acid. We recently introduced a modified Pro-Q DPS protocol to detect phosphoprotein spots on 2-D gels with very low background addressing some prime concerns, including high cost and reproducibility of Pro-Q DPS. The major modifications were a threefold dilution of Pro-Q DPS and the use of threefold less volume of the diluted staining solution. In this chapter, use of the modified Pro-Q DPS protocol along with the 2-D gel-based proteomics for phosphoprotein detection and quantification is described in detail. This 2-D gel- and Pro-Q DPS-based proteomics workflow has seven major steps: preparation of total protein, separation of proteins by 2-D gel electrophoresis, detection of phosphoprotein and total protein, image analysis and quantitative expression profiling, excision of 2-D spots, mass spectrometry analysis, and data processing and organization. Involvement of the modified Pro-Q DPS protocol in this proteomics workflow alone reduces the overall cost by at least ninefold for conducting phospho-proteomics analysis on a global scale, thereby making this entire process economically attractive to the scientific community.

Key words: Two-dimensional polyacrylamide gel electrophoresis, Reversible protein phosphorylation, Proteomics, Phospho-proteomics, Phosphoproteome, Phosphoproteins, Quantification, Method, Protocol.

Marjo de Graauw (ed.), *Phospho-Proteomics, Methods and Protocols, vol. 527*
© 2009 Humana Press, a part of Springer Science+Business Media, New York, NY
Book DOI: 10.1007/978-1-60327-834-8_1

1. Introduction

Two-dimensional gel electrophoresis (2-DGE) coupled with mass spectrometry (MS) is generally known as a 2-D gel-based proteomics platform. 2-DGE separates proteins by their isoelectric point (p*I*) through an immobilized pH-gradient (IPG) gel matrix in the first dimension and then by molecular weight in the second dimension *(1)*. 2-D gel-based proteomics is now considered a mature and well-established technique and remains the most widely and routinely used technique in the proteomics field. This is mainly due to continuous improvements in separation and detection technologies, such as narrow-range IPG strips and fluorescence-based protein labeling and stains *(1, 2)*.

Fluorescence-based stains were recently developed to detect proteins, including post-translationally modified proteins with high sensitivity and linearity. One such stain is the Pro-Q diamond phosphoprotein stain (Pro-Q DPS). Pro-Q DPS binds directly and specifically to the phosphate moiety of phosphoproteins at levels as low as 1 ng and is fully compatible with other stains and MS *(3, 4)*. Therefore, a combination of 2-DGE and Pro-Q DPS allows quantitative analysis of phosphoproteins on a global scale, a major advancement in the phospho-proteomics field. Phosphoproteomics is an emerging field *(5, 6)*, the goal of which is to map phosphorylation networks and understand protein phosphorylation events controlling numerous cellular processes. Quantitative and sensitive detection of phosphoproteins is integral to these objectives.

Recently we introduced a modified Pro-Q DPS protocol that addressed the prime concerns of high cost and reproducibility, which were limiting the utilization of this novel technology *(7)*. The major changes to the manufacturer's protocol include a threefold dilution of Pro-Q DPS by diluting the stock solution, of which a third less volume, compared with that recommended, is used. This practical change in staining solution reduces the overall cost by ninefold for performing a large-scale phosphoproteomics analysis, making the 2-D gel- and Pro-Q DPS-based proteomics workflow more attractive for routine use. Moreover, the required volume of other solutions, such as fixation and destaining, was determined to be half of the recommended volume. The optimized conditions of this modified protocol are summarized for a large gel (size, 26 cm × 20 cm × 1 mm) in **Table 1** and have been utilized in both plant and animal phospho-proteomics analyses *(8–10)*.

In this chapter, we describe the 2-D gel- and Pro-Q DPS-based proteomics workflow for identification and quantitative profiling of phosphoproteins, which is mainly based on two publications – the modified Pro-Q DPS protocol *(7)* and the

Table 1
A modified Pro-Q DPS protocol for phosphoprotein detection in large-format 2-D polyacrylamide gels

Step	Solution	Amount (mL)	Time (min)	Dark incubation	Reuse of solution
1	Fixation	250	2 × 30	Not required	Yes
2	Washing	250	2 × 15	Not required	No
3	Staining	150	120	Required	Yes
4	Destaining	250	4 × 30	Required	Yes
5	Washing	250	2 × 5	Required	No

application of this simple, economical protocol in large-scale identification and quantification of phosphoproteins expressed in developing rapeseed *(9)* – along with the ongoing work in our laboratory. This proteomics process involves seven major steps: (1) preparation of total protein, (2) separation of proteins by 2-D gel electrophoresis, (3) detection of phosphoproteins and total proteins, (4) image analysis and quantitative expression profiling, (5) excision of 2-D spots, (6) MS analysis, and (7) data processing and organization. The published reports and our unpublished data demonstrate that this system is highly suitable and reproducible for performing phosphoproteomic analyses on a large scale.

2. Materials

2.1. General

1. Fresh TEMED (*N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine) is important for polymerization of gel (*see* **Note 1**).
2. Unless otherwise stated, all solutions were prepared in deionized water (18.2 MΩ conductivity).
3. Filter sterilization of solutions using either 25-mm syringe filter (0.2 μm, nylon; Fisher Scientific, Houston, TX) or steritop (0.22 μm; Millipore Corporation, Billerica, MA) depending on the solution volume.

2.2. Materials and Storage

1. Collect biological materials, immediately freeze with liquid nitrogen, and store at –80°C.

2.3. Total Protein Extraction: Phenol–Ammonium Acetate/ Methanol Method

1. Mortar and pestle.
2. Extraction buffer: 100 mM Tris–HCl, pH 8.8, 10 mM (w/v) ethylenediamine tetra-acetic acid (EDTA), 900 mM (w/v) sucrose, 0.4% (v/v) 2-mercaptoethanol. Store at 4°C (*see* **Note 2**).
3. Phenol buffered with Tris–HCl, pH 8.8. Store at 4°C (*see* **Note 3**).
4. Extraction buffer plus phosphatase and protease inhibitors: Add 5 mM (w/v) sodium vanadate (or sodium meta vanadate), 5 mM (w/v) sodium fluoride, 25 mM (w/v) glycerophosphate disodium salt pentahydrate, and protease inhibitor cocktail complete EDTA-free tablet (1 tablet/10-mL solution) to the extraction buffer (*see* **step 2** of **Subheading 2.3**) and mix until dissolved (*see* **Note 4**).
5. Phenol extraction buffer: Add equal volume of extraction buffer (plus phosphatase and protease inhibitors solution from **step 4**) and buffered phenol (*from* **step 3**) in a 15-mL Falcon tube and mix before use.
6. Ammonium acetate/methanol solution: 100 mM (w/v) ammonium acetate in 100% methanol. Store at 4°C.
7. 80% (v/v) acetone in deionized water. Store at 4°C.
8. 70% (v/v) ethanol in deionized water. Store at 4°C.
9. Isoelectric focusing (IEF) extraction solution: 8 M (w/v) urea, 2 M (w/v) thiourea, 2% (w/v) CHAPS, 2% (v/v) Triton X-100, and 50 mM (w/v) DTT. Store in single-use aliquots of 1 mL at −20°C.
10. Balance and load shaking tray (Nutator; Becton Dickinson, Franklin Lakes, NJ).
11. Dounce tissue grinder (15 mL, Wheaton, Millville, NJ).

2.4. Protein Quantification: A Modified Bradford Method

1. SDS (sodium dodecyl sulfate) running buffer: 25 mM Tris-base (w/v), 192 mM (w/v) glycine, and 0.1% (w/v) SDS. Prepare 10× SDS running buffer and keep at room temperature (RT).
2. 0.5× SDS running buffer. Store at RT.
3. 1 mg/mL bovine serum albumin (BSA) in 0.5× SDS running buffer. Store at −20°C in aliquots (*see* **Note 5**).
4. Bradford dye: Dilute the Bradford protein assay dye (Bio-Rad, Hercules, CA) fivefold in deionized water before use.
5. Microtiter plate spectrophotometer (a 96-well plate reader; Thermo Electron Corporation, Multiskan MCC, Type 355).

2.5. Isoelectric Focusing of Total Proteins

1. Protean IEF Cell, Protean IEF system 24 cm disposable trays, and isoelectric point gel (IPG) focusing tray are from Bio-Rad.

2. Linear IPG strips (pH 4–7, 24 cm; GE Healthcare, Piscataway, NJ) (*see* **Note 6**).
3. IPG buffer (pH 4–7, 24 cm; GE Healthcare, Piscataway, NJ).

2.6. SDS-PAGE

1. Ettan DALTtwelve electrophoresis unit (GE Healthcare, Piscataway, NJ).
2. Acrylamide stock (30.8% T): 30% (w/v) acrylamide and 0.8% *N*,*N*′-methylene bis-acrylamide. Filter sterilize and store at 4–8°C (*see* **Note 7**).
3. 1.5 M Tris–HCl, pH 8.8. Filter sterilize and store at 4°C.
4. 0.5 M Tris–HCl, pH 6.8. Filter sterilize and store at 4°C.
5. 10% (w/v) SDS in deionized water. Filter sterilize and store at RT.
6. 10% (w/v) ammonium persulfate in deionized water. Use freshly prepared solution.
7. Displacement solution: 375 mM Tris–HCl, pH 8.8, 50% (v/v) glycerol, and trace of bromophenol blue in deionized water. Store at 4°C.
8. PeppermintStick phosphoprotein molecular weight standards (Invitrogen, Carlsbad, CA). Store in aliquots at −20°C (*see* **Note 8**).
9. SDS equilibration buffer: 50 mM Tris–HCl, pH 8.8, 6 M (w/v) urea, 30% (v/v) glycerol, and 4% (w/v) SDS. Filter sterilize and store in single-use aliquots at −20°C in 15-mL Falcon tubes.
10. Reduction solution: 2% (w/v) DTT in SDS equilibration buffer. Prepare right before use.
11. Alkylation solution: 2.5% (w/v) iodoacetamide in SDS equilibration buffer. Prepare fresh and protect from light.
12. Electrode wicks (Protean IEF system; Bio-Rad Laboratories, Hercules, CA).
13. Agarose sealing solution: Add 125 mg agarose to 1× SDS running buffer plus a few grains of bromophenol blue.
14. Kimwipes.
15. Rocking platform.

2.7. Phosphoprotein Detection with Pro-Q DPS

1. Large gel-staining tray, size 26 cm × 20 cm × 10 cm (Daigger, Vernon Hills, IL).
2. Orbital shaker.
3. Pro-Q DPS (Invitrogen, Carlsbad, CA). Store at 4°C (*see* **Note 9**).
4. Fixation solution: 50% (v/v) methanol, 10% (v/v) acetic acid in deionized water.
5. Washing solution: Deionized water.

6. Staining solution: Threefold diluted Pro-Q DPS (v/v) in deionized water.
7. Destaining solution: 50 mM sodium acetate–acetic acid, pH 4.0, 20% (v/v) acetonitrile in deionized water. To prepare 1 L of destaining solution, combine 50 mL of 1.0M sodium acetate, pH 4.0, 750 mL of deionized water, and 200 mL of acetonitrile (*see* **Note 10**).

2.8. Total Protein Detection with Colloidal CBB

1. Colloidal Coomassie Brilliant Blue (CBB) solution: 800 mL of ethanol, 3.2 g of brilliant blue G-250, 64 mL of phosphoric acid, 320 g of ammonium sulfate to prepare 4 L in deionized water (*see* **Note 11**).
2. Gel storage solution: To prepare 250 mL, use 25 mL of CBB solution, 2.5 mL of 100× sodium azide (2% (w/v) stock), and 222.5 mL of deionized water. Use 250 mL per 24 cm of large-format gel, shake for 5 min, and store at 4°C (up to 3–4 months).

2.9. Image Acquisition of 2-D Gels

1. Fluorescence image capture device: FLA 5000 laser scanner (Fuji Medical Systems, Stamford, CT) for scanning phosphoprotein gels.
2. Protein image capture device: ScanMaker 9800XL (Microtek, Carson, CA).

2.10. Image Analysis

1. Image quantification software: Image Gauge Analysis software (Fuji, Stamford, CT).
2. ImageMaster 2D Platinum software version 5 or version 6 (GE Healthcare, Piscataway, NJ)

2.11. 2-D Spots Excision and In-Gel Digestion

1. Spot picker (1.5 mm; The Gel Company, San Francisco, CA).
2. MultiScreen Solvinert – a 96-well filtration system (0.45 µm low-binding hydrophilic PTFE; Millipore, Bedford, MA).
3. Vacuum manifold system (Millipore, Bedford, MA).
4. Microplate shaker.
5. Polypropylene 96-well V-bottom sample collection plate.
6. 100 mM ammonium bicarbonate, pH 8.0 in MilliQ water. Store at RT.
7. In-gel wash solution: 50% acetonitrile and 50 mM ammonium bicarbonate, pH 8.0. 100 mL of in-gel wash solution: 50 mL of 100 mM ammonium bicarbonate, pH 8.0 and 50 mL of acetonitrile. Use freshly prepared solution.
8. In-gel trypsin solution: Use sequencing grade trypsin (Promega, Madison, WI). One vial contains 20 µg. Dissolve 20 µg in 5.1 mL of 50 mM ammonium bicarbonate, pH 8.0. Use freshly prepared solution. Store on ice until use.

9. In-gel extraction solution: 60% (v/v) acetonitrile and 1% (v/v) formic acid in MilliQ water.
10. CentriVap Console.

2.12. Mass Spectrometry and Data Analysis

1. 0.1% (v/v) formic acid in MilliQ water.
2. LTQ ProteomeX XL linear ion trap LC-MS/MS instrument and associated software for data processing such as SEQUEST algorithm as part of the BioWorks software suite (Thermo Fisher Scientific, Waltham, MA).

3. Methods

To identify and quantify phosphoproteins in large-format 2-D gels, it is important to prepare a high-quality protein sample that is free from interfering compounds such as carbohydrates and nucleic acids. Phenol–ammonium acetate/methanol method has been successfully used to extract proteins from developing seeds (rich in oil and carbohydrate) of soybean and rapeseed suitable for generating high-resolution 2-D gel reference maps *(9, 11, 12)*. The phenol-based extraction method has also been used by many other labs including Wolfram Weckwerth's group (Germany) for large-scale phosphoprotein analysis *(13)*. Based on these and other gel-based proteomics studies, it can be stated that the phenol–ammonium acetate/methanol method provides high-quality protein suitable for performing high-resolution 2-DGE while minimizing proteolysis and blocking endogenous phosphatase activity during protein isolation. Nevertheless, we cannot rule out the possibility of very low levels of endogenous phosphatase or protease activity. Taking this into account, it is always good to add phosphatase and protease inhibitors (*see* **step 4** of **Subheading 2.3**).

Isolated proteins are then separated by 2-DGE. Both biological and experimental replications are required for downstream 2-D gel image analysis and quantification. The 2-DGE technique has been combined with the modified method of Pro-Q DPS to detect and quantify phosphoproteins on a large scale. **Table 1** summarizes all steps including the required volumes of solutions for large-size gels, total incubation times and conditions, and whether solutions can be reused. Poststaining of the gel with total protein stains such as colloidal CBB (mentioned in this chapter), silver nitrate, or SYPRO Ruby provides insight into dynamics of proteins and phosphoproteins and also a landmark for excision of phosphoprotein spots.

Upon image analysis of 2-D gels and expression profiling, excised 2-D spots are in-gel digested with trypsin. Extracted

peptides are then subjected to MS and database analysis for identification of phosphoproteins. We have attempted to provide step-by-step information in this chapter. However, it is difficult to provide details on all the steps involved in this analysis. For example, it is almost impossible to describe the steps involved in the image analysis of 2-D gels using ImageMaster 2D Platinum software. In such cases, readers are encouraged to utilize the user manual for the ImageMaster software. We should emphasize that the detection of all distinct spots and manual editing of those spots are important steps in the 2-D gel image analysis. Recently, ImageMaster software has been updated and version 6 of this software is now being used.

3.1. Total Protein Extraction: Phenol–Ammonium Acetate/Methanol Method

1. Grind 250 mg of plant materials with liquid nitrogen in a mortar and pestle to obtain a powder (*see* **Note 12**).
2. Add 10 mL of phenol extraction buffer and continue grinding for an additional 30 s.
3. Transfer to a 15-mL Falcon tube and agitate on a nutator for 30 min at 4°C.
4. Centrifuge for 30 min at 2,800 g (4°C).
5. Transfer the upper-phase solution to a fresh 50-mL Falcon tube (*see* **Note 13**).
6. Add 5 volumes of ammonium acetate/methanol solution (ice cold), vortex, and incubate at –20°C overnight to precipitate the phenol-extracted proteins.
7. Centrifuge for 30 min at 2,800 g (4°C) to collect the precipitate.
8. Wash the pellet twice with ice-cold ammonium acetate/methanol solution, then twice with ice-cold 80% acetone solution, and finally with cold 70% ethanol solution (*see* **Note 14**).
9. Dry the final pellet (after removing the wash solution) for 20 min at 37°C (*see* **Note 15**).
10. Resuspend the final pellet in IEF extraction solution (*see* **Note 16**).
11. Centrifuge for 15 min at 28,000 g at RT.
12. Carefully transfer clear supernatant to a new 1.5-mL microfuge tube. Store in single-use aliquots at –80°C.

3.2. Protein Quantification: A Modified Bradford Method

1. Dilute the protein sample with 0.5× SDS running buffer in a 1.5-mL microfuge tube, and mix (*see* **Note 17**).
2. Use 0.5× SDS running buffer as blank and add 6 µL to the first column of the microtiter plate.

3. Add 1, 2, 4, and 6 µL of 1 mg/mL BSA standard in triplicate to wells and bring the final volume to 6 µL with 0.5× SDS running buffer.
4. Add 1 µL of tenfold diluted protein sample in triplicate to wells plus 5 µL of 0.5× SDS running buffer to make a total volume of 6 µL (*see* **Note 18**).
5. Add 200 µL of diluted Bradford dye to wells with protein.
6. Mix and incubate at RT for 5 min.
7. Vortex the microtiter plate on the spectrophotometer and measure absorbance at 595 nm (*see* **Note 19**).
8. Calculate the protein concentration.

3.3. Isoelectric Focusing of Total Proteins

1. Add 1 mg protein to a 1.5-mL microfuge tube and bring the final volume to 450 µL with IEF extraction buffer.
2. Add 2.25 µL (final concentration of 0.5%) of the correct IPG buffer to the same tube.
3. Mix the sample by vortexing and centrifuging at 28,000 g for 5 min to remove insoluble materials.
4. Pipette the supernatant into the IPG focusing tray starting from one end to the other (*see* **Note 20**).
5. Peel apart a dehydrated IPG strip (pH 4–7; 24 cm) using forceps and place the dried acrylamide side face down into the sample well in the IPG focusing tray (*see* **Note 21**).
6. Cover the focusing tray with the lid and keep inside a plastic bag to minimize evaporation.
7. Allow the IPG strip to rehydrate for 90 min at RT.
8. Overlay the IPG strip with mineral oil (~2.5 mL) to prevent dehydration (*see* **Note 22**).
9. Place the IPG focusing tray into Protean IEF cell unit.
10. Perform active rehydration (10 h at 50 V), followed by three-step focusing protocol: 100 V for 100 V h, 500 V for 500 V h, and 8,000 V for 99 kV h.

3.4. Assembling the Ettan DALTtwelve Gel Caster Unit

1. Tilt the DALTtwelve unit back so that it rests on its support legs (*see* **Note 23**).
2. Place a thicker separator sheet against the back wall to easily remove the last cassette from the gel caster unit after polymerization.
3. Clean each glass plate carefully with deionized water and ethanol using Kimwipes.
4. Fill the caster by alternating cassettes with separator sheets. End with a separator sheet, and then use the thicker separator

sheets to bring the level of the stack even with the edge of the caster.

5. Lubricate the foam gasket with a small amount of GelSeal and place it in the groove on the faceplate.
6. Turn four black-knobbed screws into the four threaded holes across the bottom until they are well engaged. Usually two to three full turns are enough.
7. Place the faceplate carefully onto the caster with the bottom slots resting on their respective screws. Screw the four remaining black-knobbed screws into the holes at the sides of the faceplate and tighten all eight evenly. The assembled unit is now ready to cast gels (*see* **Note 24**).

3.5. Casting 12% SDS-PAGE into the Ettan DALTtwelve Gel Caster

1. To cast twelve gels, add 300 mL of acrylamide stock, 188 mL of 1.5 M Tris–HCl, pH 8.8, 7.5 mL of 10% SDS, and 252 mL of deionized water in 1-L sidearm flask. This is the separating gel solution.
2. Place the flask on stir plate, add a medium size stir bar, and stir the solution.
3. Connect sidearm to vacuum, cover top opening with solid rubber stopper, and apply vacuum for 30 min.
4. Turn off vacuum, remove rubber stopper, and disconnect hose.
5. Add 3.6 mL of 10% ammonium persulfate while stirring the solution.
6. Add 120 μL of TEMED and continue to stir for 30 s. Move quickly to cast the gels.
7. Slowly pour the gel solution into the caster through the hydrostatic balance chamber until it is about 2 cm below the desired gel height.
8. Pour the displacement solution into the chamber until it is 0.25 cm below the surface of glass plates, and then immediately place the feed tube into the grommet to stop the flow.
9. Very slowly overlay each gel with 1.5 mL of deionized water.
10. Cover the upper portion of the unit with plastic wrap to prevent dehydration.
11. Allow polymerization for 16 h.
12. Bring the unit near a sink, carefully disassemble, and scrape off access acrylamide (*see* **Note 25**).
13. Remove the gel cassette from the caster by pulling forward on the separator sheets, rinse the outer surface of each gel cassette with deionized water to remove any polyacrylamide particles, and place gel cassettes in a cassette rack.