

ARGONNE NATIONAL LABORATORY

PROCEDURES FOR TWO-DIMENSIONAL ELECTROPHORESIS OF PROTEINS

Carol S. Giometti and Sandra L. Tollaksen

November 3, 2010

INTRODUCTION

1 SAMPLE PREPARATION

1.1 GENERAL SAMPLE PREPARATION

1.2 PROTEIN ANALYSIS

1.2.1 DENATURED PROTEINS

1.2.2 NATIVE PROTEINS

2 FIRST DIMENSION SEPARATION

2.1 CASTING GELS IN THE ISO APPARATUS

2.2 PREFOCUSING

2.3 ISO

2.4 IPG

2.5 BASO (NEPHGE)

2.6 ACIDO

2.7 GEL RECOVERY

2.8 CLEANING THE ISO SETUP

3 SECOND DIMENSION SEPARATION

3.1 CASTING DALT GELS

3.2 CASTING DALT GELS MANUALLY

3.3 CASTING DALT GELS WITH COMPUTER ASSISTANCE

3.4 ADDITION OF A STACKING GEL

3.5 LOADING ISO GELS ONTO DALT PLATES

3.6 RUNNING THE DALT SYSTEM

3.7 CLEANING THE DALT PLATES

3.6 DRAINING THE DALT TANK

- 4 [1-D PROTEIN SEPARATION](#)
- 5 [PROTEIN DETECTION](#)
 - 5.1 [COOMASSIE BLUE](#)
 - 5.2 [SILVER STAIN](#)
- 6 [DIGITIZING GELS](#)
 - 6.1 [IMAGE CAPTURE WITH THE MIGHTEX CAMERA](#)
 - 6.2 [IMAGE PROCESSING WITH THE PMGCAM APPLICATION](#)
- 7 [PROTEIN TRANSFERS](#)
- 8 [WESTERN BLOTS](#)
 - 7.1 [HRP COLOR DEVELOPMENT](#)
 - 7.2 [CHEMILUMINESCENCE](#)
- 9 [IN-GEL DIGESTION OF SPOTS IN GELS](#)
 - 8.1 [EQUIPMENT AND SOLUTIONS TO PREPARE FOR IN-GEL DIGEST](#)
 - 8.2 [IN-GEL ENZYME DIGESTION OF COOMASSIE-STAINED GELS](#)
 - 8.3 [IN-GEL ENZYME DIGESTION OF SILVER-STAINED GELS](#)
- 10 [CULTURING AND HARVESTING BACTERIA](#)
- 11 [REFERENCES](#)
- 12 [APPENDICES](#)
 - A. [WORK SAFETY](#)
 - B. [WASTE MANAGEMENT](#)
 - C. [RECIPES](#)

Director of the Proteomics Group: Carol S. Giometti (csgiometti@anl.gov)

For technical questions contact: Kristene Henne (khenne@anl.gov)

INTRODUCTION

The ISO-DALT system is a high-throughput approach to analysis of proteins by two-dimensional gel electrophoresis (2DE). Originally built at Argonne National Laboratory under the direction of Norman and Leigh Anderson ([1978a](#), [1978b](#)), the ISO-DALT system provides the capability to generate and run first-and-second-dimension gels in multiples of 20.

Modifications of the chemistry used for first-dimension separation in the ISO apparatus have been developed to allow the resolution of very [acidic](#) and very [basic](#) proteins, in addition to the classical use of [isoelectric focusing](#) for the separation of neutral proteins. Modifications of the polyacrylamide gel composition for the second-dimension separation in the DALT apparatus can be made to adjust the molecular weight range of proteins resolved. Thus, the spectrum of proteins that can be surveyed by 2DE has been broadened beyond that originally described by [Patrick O'Farrell](#) in 1975.

The Argonne ISO-DALT system has stood the test of time, still producing 2DE patterns of superior quality. The procedures described in this Web-based manual have evolved over more than three decades of use and include the most current recipes and electrophoresis procedures used by the Argonne Proteomics Group. The details of some procedures are specific to the Argonne ISO-DALT system and may require modification for use with commercially available electrophoresis systems. Work safety and waste management procedures are described in the appendices at the back of the manual, as are recipes, which are generally applicable independent of the equipment used.

Although the vendors and products described pertain to the work done by the Argonne Proteomics Group, they are not specifically endorsed or recommended by Argonne National Laboratory. The authors of this manual have 65 combined years of work with 2DE and have run over 80,000 gels. Thus, the procedures outlined in this document have been well tested, fine-tuned, and now updated. We acknowledge the work of Joelle Krieger Salazar, who has tested some of our newer methods.

1 SAMPLE PREPARATION

1.1 GENERAL SAMPLE PREPARATION

The solutions for sample preparation can be made in fairly large quantities (up to 1 L) and stored frozen at $-70\text{ }^{\circ}\text{C}$ in small aliquots. The following four solutions have been optimized for the samples specified. Experimentation with these methods and chemicals is advised to find the best conditions for other types of samples.

For all of the solubilization conditions described below, samples should be centrifuged at $22\text{ }^{\circ}\text{C}$ for 1 h at approximately $100,000 \times g$ in an ultracentrifuge or for 10 min at 100,000 rpm ($435,000 \times g$) in a Beckman TL-100 centrifuge. For mixes containing 9 M urea, use 1.5 mL polyallomer centrifuge tubes, as polycarbonate tubes may crack. The supernatant is recovered for analysis by 2DE. Samples may be analyzed immediately or stored frozen ($-70\text{ }^{\circ}\text{C}$) for future analysis. The small translucent pellet that contains DNA and cell debris is discarded.

1. [SDS mix](#) (body fluids such as serum, plasma, or amniotic fluid). To a $10\text{ }\mu\text{l}$ sample, add $20\text{--}30\text{ }\mu\text{l}$ of SDS mix. Heat the sample on a $95\text{ }^{\circ}\text{C}$ heating block for 5 min to achieve maximum solubilization and to inactivate any proteolytic enzymes.
2. [NP-40/urea mix](#) (solid tissue samples, isolated cells, and pure proteins). For a wet tissue sample, use a volume of mix that is eight times the blotted wet weight of the sample (e.g., for a 10 mg sample, use $80\text{ }\mu\text{l}$ of mix). For a frozen tissue sample, pulverize the sample on a platform chilled on dry ice and use a volume of mix that is four times the weight of the powdered sample. Homogenize (or sonicate) before centrifuging the sample. For solubilizing isolated cells, use 5×10^6 cells per $50\text{ }\mu\text{l}$ of mix when Coomassie blue detection will be used. When silver-stain detection will be used, use 5×10^5 cells per $50\text{ }\mu\text{l}$ of mix. Disperse the cell pellet by tapping the bottom of the tube gently prior to addition of the solubilization mix. After the mix is added, mix the cell lysates well by drawing the solution up and down in a pipette tip. For pure protein samples, mix with solubilization mix to obtain a final protein concentration of 1 mg/mL. Keep the frothing to a minimum, and **do not heat** any protein sample in the presence of urea, because carbamylation of the proteins will occur.
3. [Urea mix without NP-40](#) (urine). Mix 10 mg of lyophilized urinary proteins with $100\text{ }\mu\text{l}$ of the mix. Load $10\text{ }\mu\text{l}$ (or less) of the solution onto the ISO gel.

4. [NP-40/urea/DTE mix](#) (muscle). For a wet tissue sample, use a volume of mix that is eight times the blotted wet weight of the sample. For a frozen tissue sample, pulverize the sample on a platform chilled on dry ice and use a volume of mix that is four times the weight of the powdered sample. Homogenize (or sonicate) before centrifuging the sample.

1.2 PROTEIN ANALYSIS

1.2.1 DENATURED PROTEINS

The protein concentrations of samples in solubilization mixtures containing NP-40/urea and mercaptoethanol can be determined using a modified Bradford protein assay ([Ramaqli and Rodriguez 1985](#)). The optimal protein load for Coomassie blue staining of whole cell lysates is 150-300 μg protein, while 20-60 μg of protein is recommended for silver-stained gels.

1. Prepare an ovalbumin stock solution that can be frozen at $-70\text{ }^{\circ}\text{C}$. Weigh 125 mg ovalbumin and solubilize it in 25 mL of the urea/NP-40 mix to make a 5 $\mu\text{g}/\mu\text{L}$ stock solution. It will take a long time to dissolve this chemical, but it must be completely dissolved in the NP-40 to serve as an accurate standard.
2. Prepare the standards used with every assay by making dilutions from the stock solution:

Tube 0	Blank	1 mL of urea/NP-40 mix
Tube 1	5.0 $\mu\text{g}/10\ \mu\text{L}$	100 μL stock + 900 μL urea mix
Tube 2	10.0 $\mu\text{g}/10\ \mu\text{L}$	100 μL stock + 400 μL urea mix
Tube 3	15.0 $\mu\text{g}/10\ \mu\text{L}$	150 μL stock + 350 μL urea mix
Tube 4	25.0 $\mu\text{g}/10\ \mu\text{L}$	250 μL stock + 250 μL urea mix
Tube 5	35.0 $\mu\text{g}/10\ \mu\text{L}$	350 μL stock + 150 μL urea mix
Tube 6	50.0 $\mu\text{g}/10\ \mu\text{L}$	500 μL stock only

These standards may be frozen, thawed, and reused a few times.

3. Prepare the BioRad Protein Assay Dye Reagent by diluting the reagent 1:4 with deionized water and filtering it through Whatman #1 filter paper.
4. Turn on the Beckman DU-7 spectrophotometer at least 30 min before use. Use the single wavelength, visible spectrum lamp.

5. Label duplicate glass test tubes, and add 10 μL sample or standard to each tube. It may be necessary to dilute samples to be within the assay range.
6. Count the number of standard and sample tubes, and make a mix containing 10 μL of 0.1 N HCl and 80 μL deionized water per sample in order to pipette 90 μL per tube.
7. Add 3.5 mL of BioRad Assay Reagent, prepared as described above, to each of the tubes. Use a Vortex to mix the tubes.
8. Allow the tubes to incubate at room temperature for about 10 minutes before reading. The reaction results will remain good for about an hour, so it is not necessary to rush. Pour each solution into a clean disposable cuvette.
9. Read the absorbency at 595.0 nm on the Beckman DU-7 spectrophotometer. Read the standards first, and then read the amount of protein in 10 μL of each sample tube.

1.2.2 NATIVE PROTEINS

1. Prepare standards of bovine plasma gamma globulin from a stock solution of 2 mg/mL (BioRad Cat # 500-0005 in 14.1 mL H_2O) in the following concentrations:
 - 2 mg/mL
 - 1.5 mg/mL
 - 1.0 mg/mL
 - 0.75 mg/mL
 - 0.5 mg/mL
 - 0.25 mg/mL
 - 0.125 mg/mL
2. Combine 20 μL of each standard with 1 mL of BioRad assay reagent (1x) to make the following points on a standard curve:
 - a. 40 μL
 - b. 30 μL
 - c. 20 μL

- d. 15 μL
- e. 10 μL
- f. 5 μL
- g. 2.5 μL

3. Determine a standard curve using 5 of the standard points and 1 blank (1 mL of BioRad reagent and 20 μL of H_2O)
4. Combine 20 μL of unknown sample with 1 mL of BioRad reagent and analyze against the standard curve obtained above.

2 THE ISO SYSTEM FOR FIRST-DIMENSION SEPARATION

2.1 CASTING GELS IN THE ISO APPARATUS

There are two ISO formats, the 7-in. (18 cm) and the 10-in. (25 cm). The selection of ISO format should be based on optimal resolution of the proteins being analyzed. At Argonne, the 10-in. format is used for isoelectric focusing of mouse liver and tissue culture cell proteins, and the 7-in. format is used for BASO-DALTs and isoelectric focusing of microbial proteins. The entire system must be clean and dry.

1. Place a metal retainer on the bottom of the gel tubes.
2. Fill the bottom chamber of the ISO apparatus with tap-distilled water (2 L for 7-in. systems and 3 L for 10-in. systems). Place the Lucite trough on the base of the support stand, and position the rack containing the upper chamber and the gel tubes into the acrylic trough.
3. Prepare the polyacrylamide gel solution by mixing the following in a 150-mL lyophilizer flask. (Note: acrylamide monomer must be handled as a suspected human carcinogen.)

Compound	Amount
Urea	8.25 g
Acrylamide 30%/bis 1.8% solution	2.0 mL
Ampholytes (ranges blended appropriately for different samples such as tissues or microbes)	0.8 mL
Deionized water	6.0 mL

Warm the flask and its contents back to room temperature using hot running water in order to dissolve the urea, because mixing these chemicals creates an endothermic process.

4. Degas the solution briefly using a vacuum pump dedicated for degassing acrylamide solutions. (If the degassing is too long, the urea will come

out of solution; if this happens, warm the flask slightly until the urea goes back into solution.)

5. Add 0.3 mL NP-40 detergent (except with gels for urine proteins, where only two drops should be added) and swirl gently (vigorous swirling introduces bubbles). Then carefully add:

Compound	Amount
Ammonium persulfate (10% solution) (polymerizer)	50 μ L
TEMED (<i>N,N'</i> -tetramethylethylenediamine (catalyst))	5 μ L

6. Pipette the acrylamide solution (approximately 15 mL) into the trough. Carefully layer 3–4 mL of deionized water over the acrylamide solution to bring the fluid level to the top of the trough.
7. Slowly lower the upper chamber/tube assembly with the acrylamide trough into the bottom chamber containing the appropriate volume of water. Allow the acrylamide to rise evenly by displacement in all tubes. Examine the tubes for bubbles. If there are any, use a 1 mL syringe with a cut off yellow pipette tip attached (the wide top end cut off) to suck acrylamide out the top of the tube until the bubble is removed. Allow the fluid to fall back to the proper level.
8. Allow the gels to polymerize for at least 3 hours.

2.2 PREFOCUSING

1. After the gels have polymerized, remove the upper chamber/gel tube assembly with the support stand from the bottom chamber. Empty the water from the bottom chamber; add either 200 or 300 mL (depending on whether you use the small or large chamber) of the 0.85% H_3PO_4 (marked 10X because we make the 0.85% stock solution from the concentrated phosphoric acid [20 ml concentrated into 2 L total] and then dilute that 10-fold) stock solution; and then add enough double-distilled water to fill the chamber to the full mark (2L or 3L).

2. Carefully place the tube stand on its side and hold on to the sides of the metal retainer. Pull off the trough by wiggling it free of the polymerized acrylamide and retainer. Do not bend the metal retainer or stretch the gels in the tubes. Cut with a razor blade between the end of the tubes and the metal retainer. Remove the retainer and rinse the outside of the tubes. Reinsert the upper chamber with attached tubes into the lower chamber containing the acid solution.
4. Degas 200 mL of deionized water. Add 0.4 mL of 10 N NaOH and pour the solution into the upper chamber. Use a 100 μ L Hamilton syringe containing the NaOH solution to displace the air pocket that forms between the top of the gels and the NaOH in the upper chamber. Be careful not to disturb the tops of the gels.
5. Prefocus the 7-in. ISO for 1 h at 200 V or 2 h at 100 V, and the 10-in. ISO setup at 300 V for 1 h.

2.3 ISOELECTRIC FOCUSING WITH CARRIER AMPHOLYTES [ISO]

Using a Hamilton syringe underlay 5 to 25 μ L of sample on top of each isoelectric focusing gel. Larger volumes result in poor resolution and can cause the ISO gel to break. The optimum protein loading level is 100–300 μ g for Coomassie blue detection or 20–60 μ g for silver stain. If isoelectric focusing standards (e.g., Carbamalyte Calibration Kit, Amersham Bioscience, Catalog Number 17-0582-01) are to be run with the samples, add 2–4 μ L to each tube above the sample.

Although the optimal separation time depends on the sample type, most cellular proteins are well separated in the 7-in. ISO apparatus with a run time of approximately 14,000 Vh (e.g., 800 V for 17.5 h), while plasma or amniotic fluid proteins require approximately 12,000 Vh. When using the 10-inch ISO apparatus, the optimum run for tissue and cellular protein is 30,000 Vh.

2.4 ISOELECTRIC FOCUSING WITH IPG STRIPS [IPG]

IPGs are immobilized pH gradient strips that are fixed on a solid plastic support. They must be rehydrated before isoelectric focusing. The apparatus

with corollary equipment is available commercially (e.g., Bio-Rad), and instructions for setup are included.

Add 125-250 μL (7 cm IPG strip) or 300-600 μL (17 cm IPG strip) of the [rehydration buffer](#) to each channel of the isoelectric focusing tray, making sure that each strip is completely wet to prevent uneven rehydration. Do not exceed 30 μL (5–100 μg protein for silver staining, and up to 1 mg for Coomassie blue) volume of sample per IPG strip. Apply mineral oil to each channel containing a strip, covering the entire strip. Put the lid on the tray and place the entire assembly on the Peltier platform. Be sure to align the electrodes of the focusing tray with the color-coded Peltier platform's electrode connections. Program or select the desired method, and start the run.

Perform isoelectric focusing immediately after rehydration. IPG strips may be stored indefinitely at $-70\text{ }^{\circ}\text{C}$ after isoelectric focusing or may be loaded on to second dimension gels. Before running the second dimension, equilibrate the IPG strips with [DTT-equilibration buffer](#) for 10 min and then with [iodoacetamide-equilibration buffer](#) for an additional 10 min. After equilibration, place the IPG strips on top of the second dimension slab gels.

2.5 NON-EQUILIBRIUM pH GRADIENT ELECTROPHORESIS (NEPHGE/BASO)

Proteins with pI values higher than 8.0 do not focus well or may fail to enter isofocusing gels. To study basic proteins, the technique of running a BASO under nonequilibrium conditions was devised as a modification of the NEPHGE (non-equilibrium pH gradient electrophoresis) system ([O'Farrell et al., 1977](#)).

1. Cast the BASO gels in the 7-in. ISO apparatus using the same procedure as for [ISOs](#). However, the BASO gel separation requires the use of only the wide range pH 3–10 ampholyte (e.g., Biolyte 3/10).
2. After the gels have polymerized, remove the upper chamber/gel tube assembly with the support stand from the bottom chamber. Empty the bottom chamber and fill with 2 L of degassed 20 mM NaOH (4 mL of 10 N NaOH diluted to 2L) prepared using degassed deionized water.
3. Clean the gel tubes as described for [prefocusing](#) ISO gels.

- Apply the samples to BASO gels without prefocusing. Using a Hamilton syringe, load the samples directly to the upper gel surface in each tube, and then overlay the samples with 4 M urea, filling the remainder of the gel tube. Fill the upper chamber with 200 mL of 10 mM H₃PO₄ (20 mL of 0.85% H₃PO₄ diluted to 200 mL). The 4 M urea cushion serves to protect the sample proteins from the acid in the upper chamber.
- Reverse the electrodes relative to their use for isofocusing, i.e., so that the positive electrode is attached to the top chamber and the negative electrode is attached to the bottom chamber (red wire to black wire). To run the BASO gels in one day, start the electrophoresis at 400 V for 1 h and turn up the power to 800 V for 4–8 h, stopping the run at approximately 3000–8000 Vh. For convenience, you might also use an overnight run at 300 V for ~20 h. Using shorter run times ensures that proteins with isoelectric points greater than pH 10 are captured in the gels.

2.6 ACIDO ISOELECTRIC FOCUSING

Urine contains very acidic proteins (most acid urinary protein [MAUP] and alpha-1-acid glycoprotein) that will not focus following the ISO protocol for isoelectric focusing gels ([Edwards, Tollaksen, and Anderson 1982](#)). The conditions for ACIDO gels, designed to resolve such acidic proteins, that differ from an ISO run are shown below.

- Acido gels are cast in the 7-in ISO apparatus using the following recipe:

Compound	Amount
Urea	8.25 g
Acrylamide 30%/bis 1.8% solution	2.0 mL
Ampholyte (pH 2.5–4)	1 mL
Ampholyte (pH 3–10)	0.3 mL
Deionized water	5.5 mL

Degas the solution and then add:

Compound	Amount
NP-40	0.3 mL
Ammonium persulfate (10% solution)	90 μ L
TEMED	10 μ L

2. After the gels have polymerized, remove the upper chamber/gel tube assembly with the support stand from the bottom chamber.
3. Clean the gel tubes as described for [prefocusing](#) ISO gels. Empty the bottom chamber and fill with 2 L of water and 3 mL of concentrated H_2SO_4 .
4. Fill the upper chamber solution with 40 mL of degassed deionized water and mix with 1 mL of the pH 3–10 ampholyte. Add this solution to the upper chamber after the gels have polymerized. Remove bubble carefully from each tube using a Hamilton syringe containing the top buffer/ampholyte solution to displace the air pocket that forms between the top of the gels and the upper chamber solution.
5. [Prefocus](#) the setup for 1 h as described.
6. Underlay approximately 20 μ L of sample using a Hamilton syringe and run for 3600–4000 Vh. Electrophoresis can be finished in one day using 700–800 V or overnight using 200 V (18–19 h). After electrophoresis is complete, a white precipitate (protein that did not enter gels) will be visible at the top of each gel.

2.7 ISO/BASO/ACIDO GEL RECOVERY AND EQUILIBRATION

1. Stir the [equilibration buffer](#) at room temperature (if stored refrigerated) until the precipitated SDS goes back into solution, and then dispense 4–5 mL into 5 mL glass screw-top vials.
2. At the power supply, turn down the voltage and then turn off the power. For extra safety, you might take the leads off the power supply. Remove the safety lid and take out the gel tube holder. Drain the upper

buffer solution into the sink and place the gel tube holder on the unloading rack. Use a 1 mL syringe attached to a cut-off pipette tip and filled with deionized water to expel the gels slowly into vials containing the equilibration buffer. When ACIDO gels are unloaded into the [equilibration buffer](#) containing bromophenol blue, an indicator dye, the liquid will change color to yellow or yellow-green. Rinse the gels with equilibration buffer until the equilibration buffer stays blue.

3. Rock the vials gently at room temperature for a maximum of 5 min to minimize protein diffusion; freeze them at $-70\text{ }^{\circ}\text{C}$; and thaw them as needed. Frozen first-dimension tube gels produce higher resolution spots than fresh gels, but they are thinner and somewhat more fragile. Freezing the ISO gels every run keeps the loading conditions consistent. Thus, gels are not sitting thawed with proteins diffusing into the equilibration buffer, because you only take a few out of the freezer at one time. Gels may be frozen indefinitely at $-70\text{ }^{\circ}\text{C}$.

2.8 CLEANING THE ISO SETUP

The glass ISO tubes are cleaned by soaking them overnight in a tank of room temperature chromic-sulfuric acid cleaning solution (e.g., Fisher Catalog No. SC88-212). Use extreme caution when handling chromic acid, as it can cause severe burns. Flush the ISO tubes thoroughly with deionized water and dry by air aspiration. We use the chromic acid cleaning solution for about 6 months, depending on the frequency of cleaning the ISO tubes. If the solution looks green, takes on too much water from the summer's high humidity, or if the ISO tubes start to fall out of the tubes, you must change the cleaning solution then. We put the old solution in a glass bottle to write as waste. Our container holds about $1\frac{1}{4}$ bottles of cleaning solution, and we use it without diluting it.

3 THE DALT SYSTEM SECOND-DIMENSION SEPARATION

3.1 CASTING DALT GELS MANUALLY

Be sure that the entire gel casting system (comprised of a 2 L double-chamber gradient maker, stirring box, DALT casting box, peristaltic pump, and vacuum pump) is clean, dry, and free of polymerized acrylamide.

1. Place the bottom plate with an angle edge in the bottom of the casting box so that the angle is up and toward the solution entry port on the right side of the box.
2. Place a Teflon sheet to serve as a spacer at the back of the box. Stack gel plates into the casting box with the red hinges to the left and vertical, interspersing three "bubble wrap" packing squares cut the same size as the plates between every fifth or sixth plate. Fill the box with 22 plates, and screw on the front cover.
3. Cut and place gel serial numbers (printed on #1 filter paper) in order in front of the gel-casting box.
4. Turn on the aspirator. Run the [blue sucrose underlay solution](#) to the T in the feed line and clamp with a hemostat. Shut off the vacuum line and make sure that the clamp to the vacuum line is closed. Be sure that the gradient-maker lines are clamped off, with one clamp on the heavy (18%) line just as it leaves the gradient maker and the other on the main line just beyond the mixer. Set the gel-casting chamber to a 45° angle from vertical.

NOTE: There will be about 15 min available to pour the plates between the addition of the ammonium persulfate and TEMED and when the gels begin to polymerize, so work fast!

5. Make the appropriate acrylamide working solutions (a 9% solution and an 18% solution) from the stock mixes according to the following table:

Acryl/bis	Buff L10	Buff L20	10% SDS	10% (NH₄)₂S₂O₈	TEMED
(mL)	(mL)	(mL)	(mL)	(mL)	(μL)

For 11 plates:						
9% solution	80	160		2.4	4	70
18% sol'n	240		120	3.6	2	10
For 22 plates:						
9% solution	192	384		5.7	6	80
18% sol'n	440		220	6.6	3.5	10

Degas after mixing the acrylamide and buffer L10 or L20. Then swirl the container gently to mix while adding the ammonium persulfate and TEMED. The amounts of persulfate and TEMED required may vary slightly with different brands of reagents or the temperature of the laboratory.

6. Pour all the light (9%) acrylamide solution into the right side of the gradient maker. Open the line beyond the mixer to fill the mixer and lines up to the gel-casting box. Add the heavy (18%) acrylamide solution to the left side of the gradient maker to the same level as the light acrylamide. Bleed the air bubbles out of the line.
7. Remove the clamp on the 18% line and the clamp above the mixer to allow the solution to run into the "funnel" formed by the V in the casting box.
8. Watch the level of fluid rising in the casting box, and slowly rotate the casting box to vertical as filling is completed. Then drop serial numbers into each gel at the right side. Use a level to check that the plates in the box are completely level.
9. Clamp the line between the vacuum and blue sucrose underlay inlets. Open the line for the [blue underlay solution](#).
10. While the underlay solution is flowing in, open the vacuum line, being sure that the water suction is on. Add water to both chambers of the gradient maker, flushing at least 2 L through the system.
11. After the underlay solution has reached the appropriate level (i.e., almost to the bottom of the glass plates), immediately (and very gently) overlay approximately 1 mL of [Photoflo solution](#) onto each gel.

12. Adjust the underlay solution container up or down so that the blue level remains just below the bottom of the plates without having to clamp the line. This placement allows slightly more displacing fluid to be drawn in as the gels polymerize.
13. Cover the box with plastic wrap. Flush two more liters of water through the gradient maker and tubing. Drain the system and then drain the mechanical mixer by inverting it (the outlet tubes are at the top of the mixer). Allow the gels to polymerize for at least 4 hours.
14. After the gels have polymerized, carefully disassemble the apparatus. Wash the plates carefully with warm water, removing excess acrylamide from the sides and edges of the plates. Rinse the top surface of each gel with tap-distilled water, and set the plates sideways in a dish rack (with the red hinges up) to drain the surface of the gels.

3.2 CASTING DALT GELS WITH COMPUTER ASSISTANCE

The computer-assisted pouring system was designed specifically for use at Argonne and consists of a pair of four-channel peristaltic pumps controlled by a minicomputer. We affectionately named the system Angelique; thus, the buffer was named Angel's buffer. Each pump delivers acrylamide, buffer, TEMED, and ammonium persulfate in constant proportions. The "light" pumps deliver a mixture appropriate for the top (low-percentage acrylamide) end of a gradient SDS slab gel, while the "heavy" pumps deliver the bottom (high-percentage acrylamide) solution. Generally these solutions are about 10% (light) and 17% (heavy) acrylamide. These percentages (and those referred to by the program) refer to the final concentration of acrylamide at the output of the pump, not the acrylamide stock solutions. The pumps are configured to deliver three parts acrylamide solution to one part other (buffer, TEMED, or ammonium persulfate).

The pumps are driven by stepping motors, which move in small, programmed increments (200 steps per revolution). The computer controls these motors by sending electronic pulses to them through power amplifiers. Since each step (1/200 revolution of the pump) has a fixed volume, the total volume of the gradient can be expressed as the number of steps which must be pumped to deliver the necessary liquid to the casting box. About 108,000 steps are required to fill a 7 by 7-in. box, and 175,000 steps to fill an 8 by 10-in. box.

The output of the pumps is combined in the following way: light and heavy acrylamide solutions are combined and delivered to the mixing chamber, while the other six lines (light and heavy buffer and two each for TEMED and ammonium persulfate) are also combined and then delivered to the mixing chamber. This scheme ensures that the ammonium persulfate and TEMED contact the acrylamide only at the mixing chamber (which can be flushed out). Each pump delivers the amount of TEMED and ammonium persulfate necessary to cause slow polymerization of its own concentration of acrylamide, and therefore the amount of these chemicals varies smoothly according to the acrylamide concentration down the gradient.

The output of the mixing chamber is fed by a tube to the casting box to be filled. The end of the feed tube is a plastic 2-mL pipette with a short piece of rubber tubing on the end, which fits snugly into the opening in the sucrose side chamber. When in position, the feed tube causes the gradient material to be pumped directly into the casting box. The sucrose chamber surrounding the feed tube is filled to the correct height (marked on the box) with sucrose so that when the tube is withdrawn after the gradient is pumped, the heavy sucrose follows the gradient down the passage into the box, thereby pushing the acrylamide into the box so it will not polymerize in the tubing leading to the box.

The pumps are controlled by a program that allows either direct control of the pumps (for pumping a given number of steps of either light or heavy solution) or the specification of a gradient which is then computed in real time to determine how many pulses to send to each pump over the course of the gradient. In case of emergency (acrylamide leaking out or a major mistake of some kind), the pumps may be stopped by turning off power to the pump power supplies (the switch marked "Stepping Motor Main Power") on the shelf to the right of the casting bench, or by hitting the red "DC POWER" switch on the control box that controls the program.

A pair of platinum wires is used to sense when the level of liquid in the casting box reaches the desired height. If the "full" option is selected instead of the "constant" volume option, the pumps will continue to deliver, once the gradient is finished, whatever percentage acrylamide was specified for the bottom of the gradient until the liquid reaches the level of the sensor. This procedure allows gels of constant height (run-to-run) to be made even though the casting boxes differ slightly in volume (and thus in the height corresponding to a fixed volume of gradient). It is good practice to use the same casting box for pouring gels that are to be scanned and compared.

An overlay of [0.1% Photoflo in buffer](#) is applied to all the gels following the final pumping of the gradient by using a spray bottle and spraying generously across the top of the gels in the plates so that all the surfaces are completely covered.

Several companies sell a liquid, pre-mixed formulation of acrylamide and bis (37.5:1 ratio), so it is no longer necessary to deal with the hazards of large amounts of crystalline acrylamide. We use liquid acrylamide from Amresco (Solon, Ohio; catalogue no. P0025443) or BioRad (Hercules, California; catalogue no. 161-0149) with equally good results.

On the day prior to casting DALTs:

1. Take a bottle of [Photoflo overlay solution](#) and Angel's buffer solution out of the refrigerator to stabilize at room temperature overnight. Check that there are sufficient amounts of both solutions. Prepare fresh [light \(12%\)](#) and [heavy \(22.46%\)](#) acrylamide solutions, making 1 L of each solution for each box poured. Better results are obtained by using fresh acrylamide solutions, especially when the gels will be silver-stained.
2. Take a bottle of [sucrose underlay solution](#) out of the -20 °C freezer to thaw.
3. Load clean, dry plates into the casting boxes (6 plates, 1 bubble sheet, 6 plates, 1 bubble sheet, 5 plates, 1 bubble sheet, 6 plates) with the rubber hinges to the left. Put the face plate on, but do not tighten the screws. Wedge one or two Teflon spacers at the back of the box if needed. Drop ID number tags into the plates near the red rubber hinges so that the print faces the Teflon spacer. Cover the top of the box with plastic wrap to keep out dust.
4. On the morning DALTs are cast, take the 10% ammonium persulfate and TEMED out of the refrigerator. Prepare fresh [ammonium persulfate](#) and [TEMED](#) working solutions. One box requires 50 mL each of TEMED and persulfate. Pour the leftover stock persulfate in the large waste beaker to help polymerize the waste acrylamide solution.

5. Degas the buffer solution and then the light and heavy acrylamide solutions. The pump is equipped with a small trap in the line to catch any acrylamide solution that may be sucked into the tubing.

The solutions should be in Nalgene #2026-4000 bottles with evacuation caps. Connect each bottle to the vacuum pump by screwing on the designated lid connected to the pump by Tygon⁷ tubing, and turn on the switch. The regulator has been preset to 15-20 mm Hg, and these polypropylene bottles are approved for use with this vacuum. Degas the solutions for approximately 5 min each. Then turn off the vacuum pump, disconnect the vacuum hose, and unscrew the cap.

6. While solutions are degassing:
 - a. Turn on the pump power supply, which is controlled by a small switch box labeled "Stepping Motor Main Power" at the back of the black shelf on the right-hand side of the bench. When the switch is turned on, fans will start running. This is the control switch that will shut down the entire pouring operation in case of an emergency.
 - b. Turn on the switch on the gray panel mounted next to the vacuum chamber, which will give power to the magnetic mixer under the gradient mixing chamber, the peristaltic pump used for overlay, and the vacuum pump.
 - c. Turn on the level detector, which is a small grey box on the shelf above the left side of the bench, by flipping the small main power switch mounted at the bottom of the front face of this box. The main power light will come on. Do not touch any other switches on this box!
 - d. Turn on the red "DC power" button on the lower right corner of the computer control box (on the shelf above the computer terminal).
7. Prime the stepping pump lines by doing the following:
 - a. Prime the Angel's buffer line first (only Angel's buffer is connected at this time) while the next solution (heavy acrylamide) is being degassed. Screw the appropriate cap on the bottle, and make sure the waste flask stopper is in place. Turn on the Angelique vacuum

pump on the platform. With the waste port (the side tube on the magnetic mixer that leads to the vacuum trap) open (as it should be from the previous run's cleanup), pump 28,000 steps of light solution through the system by typing L28000 at the computer terminal).

- b. Tighten the TEMED tubing lines around the MasterFlex pump heads, and clamp the pumps shut. Connect the ammonium persulfate, TEMED, and degassed acrylamide solutions to their appropriate feed lines on Angelique. All tubes should be labeled so they will be inserted into the proper bottle; check all lines to ensure they are oriented to feed in from the left and pump out to the right. Light solutions are then connected to the pump on the right; heavy solutions are connected to the pump on the left. Fill the 1-L beaker (dedicated for acrylamide use only) with distilled water. Close the damper with both the clamp and a hemostat.
- c. Prime the heavy pumps by typing H10000 at the terminal, and then check to see that all four solutions are flowing (particularly the TEMED, as it tends to clog up the pump tubing) and that all air bubbles are moving along in the tubing. Repeat with 10,000 steps of light solutions (type L10000). Always end the priming cycle with light solutions, so that the beginning of the gradient is always light. Check that there are no bubbles in the tubes. Turn off the vacuum pump and close the clamp on the waste line.

8. Prepare the DALT box for casting:

- a. With your fingers, tighten the screws on all casting boxes, checking to see that the sealing gasket made of small Tygon tubing is in the proper track and is not being crushed when the lid is tightened; otherwise, leaks will result.
- b. Insert the gradient feed tube into the sucrose chamber. A liquid-tight seal is required here, made by forcing the tapered feed tube (a 2-mL pipette) covered with rubber tubing into a tapered hole in the sucrose chamber. Load the sucrose chamber with sucrose underlay to the mark on the chamber (premeasured to deliver the correct amount of sucrose).

- c. Insert and tape the level detector probe along the left side of the pouring box, and make sure that the red "high limit" light on the upper right-hand side of the sensor power source is not on; if it is, the sensors are touching and must be separated. Check that the vacuum (waste) line is clamped.

9. Cast the DALT gels. At the computer terminal:

1. Type C7 and press return. (C = cast)
2. Type 9. and press return. (% acrylamide of light solution)
3. Type 17. and press return. (% acrylamide of heavy solution)
4. Type 2 and press return. (number of points in the gradient)
5. Type 10. and press return. (starting value of gradient variable)
6. Type 17. and press return. (percentage of acrylamide at final concentration)

7. Press return.
(Steps 2, 3, 5, and 6 have a period after the number.)
8. Type 108000 and press return. (number of pulses to fill the 7 by 7-in. box)
9. Type 0 and press return. (length of delay desired; 0 = no delay; typing an integer will delay by that number of pulses.)
10. Type f and press return. (f = full; c = constant; the full option allows the box to fill until the solution contacts the level-detection wires; the constant option delivers the given number of pulses.)

11. Press return.
12. Press return. (Note: Steps 11-17 are a checklist to

13. Press return. ensure that appropriate preliminary
14. Press return. steps were completed as described
15. Press return. above. It is useful to read the
16. Press return. questions and verify the steps.)
17. Press return.

The stepping pumps should start at this point and continue until the box is filled to the proper height (according to the position of the sensor). In general, use the "full" option in order to get gels of a reproducible height in the slightly different boxes. Double-check to make sure that no tubing is kinked, especially the heavy feed line of acrylamide.

10. After the box is filled (according to the sensor):
 - a. Remove the gradient feed tube from the sucrose chamber and place it in the beaker of water. Check that sucrose is entering the box.
 - b. Unclamp the vacuum waste line and turn on the vacuum pump, allowing about 50 mL of water through the feed line; this clears the line of polymerizing acrylamide. Remove the feed line from the water and let the vacuum suck all the water from the feed line.
 - c. Spray the overlay solution for about a minute or until the overlay solution covers the tops of the gels. Overlay must be applied immediately or the tops of the gels will be disturbed.
 - d. Remove the level sensor from the box. Cover the box with plastic wrap and allow the box to sit undisturbed for at least 4 h until fully polymerized.
11. Cleanup and shutdown
 - a. Disconnect all solutions from the stepping and peristaltic pumps. Remove the tubes from the TEMED and persulfate bottles, and pour these solutions in the dedicated plastic waste beaker on the bench. Put the tubing in the 1-L beaker of water which will be used for rinsing all tubing.

- b. Take the lid off the buffer bottle; let the fluid in the attached pipette drain down into the buffer bottle; and put the pipette in the 1-L beaker of water. Then remove the lid from the heavy acrylamide and put the pipette line into the water. Pump water through all heavy lines by typing H30000. Make sure that the water gets primed through the buffer line. Remove the heavy acrylamide line from the beaker; let it drain, and store the line, wrapped in a paper towel, on the secondary tray. Put the remainder of the heavy acrylamide into the plastic waste beaker on the bench.
- e. Remove the lid from the light acrylamide bottle, and put the pipette in the attached beaker of water, making sure that there is enough water in the beaker for rinsing. Pump water through all light lines by typing L30000 at the computer terminal. Watch the fluid in the waste flask to ensure it does not overflow into the vacuum pump. Under normal pouring conditions, there should be plenty of room left in the vacuum flask. Remove the light acrylamide line from the beaker; let it drain, and put it on the secondary tray. Repeat for the buffer line. Remove the TEMED and persulfate lines also. Put the remaining light acrylamide into the plastic waste beaker.
- f. Open the damper by removing the hemostat and opening the clamp. Pump all tubing empty (by typing H15000 and then L15000 at the terminal).
- g. Empty the waste flask into the plastic waste beaker on the bench. Rinse the flask with the water remaining in the 1-L rinse beaker and add this water rinse to the waste beaker.
- h. Add approximately 400 μL of TEMED and 3-5 mL of ammonium persulfate to the liquid acrylamide waste. Allow the acrylamide solution in the waste beaker to polymerize for several hours and then dispose of it as general (non-hazardous) solid waste.
- i. Turn off the computer control box by pushing the red "DC power" button, and the button will pop up.
- j. Turn off the pump power supplies (controlling the stepping pumps)

by flipping the switch on the right side of the bench under the bottom black shelf.

- k. Loosen the TEMED tubing from the MasterFlex pump head by unscrewing the plastic screw and physically loosening the tubing so it is not kinked but is loosely curved, and unclamp the MasterFlex pump.
- l. Store remaining buffer and stock TEMED and persulfate bottles at 4 °C, and rinse the diluted acrylamide, TEMED, and persulfate bottles into the waste beaker. Put any extra sucrose underlay solution back in the -20 °C freezer.

3.3 ADDITION OF A STACKING GEL

Since the ISO gel serves the stacking function in the second dimension of 2DE, a stacking gel is not necessary. However, when the DALT gels are used for 1DE, a stacking gel improves resolution. Prepare the [stacking gel recipe](#) and quickly pipette the mix on top of each slab gel.

3.4 LOADING FIRST-DIMENSION GELS ONTO DALT GELS

1. If the first-dimension gels (ISO, BASO, ACIDO) gels are frozen, they should be moved from the -70 °C freezer to a -20 °C freezer about an hour before they are to be loaded. To minimize diffusion of proteins in the gels, thaw out only 4 or 5 vials at a time in a beaker of warm water.
2. Pour the first-dimension tube gel from the vial into a plastic tea strainer, letting the equilibration fluid drain into a beaker. Rinse the top of the gel with diluted Angel's buffer (16 mL diluted to 100 mL). Transfer the tube gel to the top of the DALT loading platform, and position the gel so that the acidic end (bottom of ISO and ACIDO gels or top of BASO gel) is to the left (acidic) side. Note that the razor rough-cut end corresponds to the bottom (acid end) and the smooth tapered end corresponds to the top (basic end) of each tube gel.
3. Place a DALT gel plate on the front of the lectern with the red hinge to the right and the label in the lower right corner. Add a few drops of the buffer to the top of the DALT gel and then roll the ISO gel onto the top of the DALT gel, either with a gloved finger or a spatula.

Smooth the ISO gel into position with a spatula, eliminating any air bubbles. Turn the DALT plate upside down on a towel to drain off any excess fluid before overlaying the gel with agarose.

4. Record the DALT gel numbers on the sample protocol sheet.
5. Overlay each gel with approximately 0.5 mL of melted agarose, removing any air bubbles that form. Allow the agarose to completely solidify before placing the plates in the DALT tank. The plates are run sideways in the tank, so the agarose must hold the ISO gel firmly against the DALT slab gel. Molecular weight standards (e.g., BioRad or Pharmacia) can be incorporated into the agarose overlay.

3.5 RUNNING THE DALT ELECTROPHORESIS SYSTEM

Run each set of gels in a clean DALT tank with fresh buffer. Prepare the buffer in the tank several hours prior to the run to assure adequate cooling (about 4 °C) prior to use.

1. Weigh the DALT tank buffer components and mix on a stirring plate in approximately 4 L of water. Rinse the tank well, and then fill it about half full with water. Pour the concentrated buffer solution into the tank, and add water to the fill mark (26 L). Turn on the circulation pump to mix and chill the buffer, and just before starting the DALT gel run, put the plate spacers back in the tank.
2. After you have loaded the first-dimension gels onto the second-dimension gels, insert the DALT plates carefully between the rubber tank spacers such that the first-dimension gel is along the left side and the red rubber hinge is along the bottom of the tank. Use a slight left-wise sliding motion so that the tank gasket flaps open to the left. Slide the plates firmly to the bottom. Dipping the plates in the tank buffer first simplifies their installation.

NOTE: After the plates are in position, the buffer level should be even with the spacers of the plate and *not above the top of the plates*. Submersion will cause an electrical short circuit.

3. Close the lid on the tank and attach the electrodes. Run the gels at 130-150 V (limited voltage) with the current limited to 0.6 A overnight until the blue tracking dye runs completely off the bottom of the DALT gels (about 16-17 h). These conditions minimize heat generation in the DALT gels.
3. Turn off the power supply and carefully remove the DALT plates from the tank one at a time. Wear gloves when handling the gels.
4. Place the plates one at a time on the unloading lectern, pry them open with a screwdriver, and use a razor blade or spatula to free the gel along the spacers. Carefully peel the gel away from the glass and place it in [Coomassie blue stain solution](#), [silver-stain fixing solution](#), or [transfer buffer](#).
6. Place the box of gels (10 per box for Coomassie blue, 4 per box for silver stain) on a shaker for staining, or proceed with the protein transfer procedure.

3.6 CLEANING THE DALT PLATES

Soak the used plates for several hours in distilled water containing a small amount of SDS. When cleaning, go over all surfaces (including the edges) with a Teflon scrubber. Finally, rinse the plates with water (and, if desired, possibly ethanol). Air-dry them in the open position in a drying rack or stand them on a clean surface in an inverted "v" (Λ) configuration. Do not open the plate books beyond a right angle or the bindings might break.

3.7 DRAINING THE DALT TANK

Pump the buffer down the laboratory drain. Rinse the tank with distilled water, drain, and leave to dry until the next use. This procedure minimizes bacterial growth in the tanks, tubes, and circulation pumps.

4 1D GEL ELECTROPHORESIS

1. Prepare samples with SDS Mix (Modified 1D Blue Mix), because NP-40/urea (Pink Mix) does not run well on the gel.
2. Prepare 1 L of 1 X tank buffer from the [5 X stock](#).
3. We use a Criterion Tris-HCl gel (gradient or linear, depending on protein) for our run (Bio-Rad Catalog Number 345-0037, 8-16% gradient gel). Remove the packaging and discard the plastic comb that keeps the wells in place. Place the gel into the Criterion gel electrophoresis apparatus. Two gels can be run at one time.
4. Fill the top and bottom chambers with tank buffer so the gel does not dry out while samples are being loaded.
5. On each side of the gel, you may load standards in the two smaller-sized wells. To the remaining wells add samples using the same μL volume for each sample well, because different volumes will cause the stained gel to look wavy.
5. Fill both sides of the apparatus with tank buffer, even if only one gel is being run.
6. Attach the lid to the apparatus and plug in the banana plugs to the power supply. Run at 130 V for about $1\frac{1}{2}$ h. Voltage above 130 will cause the gel to become too hot and start to bubble.
7. Turn off the power supply and remove the gel(s) from the tank. Crack open the plastic enclosing the gel, and place the gel in Coomassie blue, silver stain fix, or transfer buffer.

5 PROTEIN DETECTION METHODS

5.1 COOMASSIE BLUE STAIN

1. After removing DALT gels from their plates, place them into [Coomassie blue stain](#) (100 mL per gel, maximum of 10 gels per box) at room temperature and shake overnight.
2. Destain the gels in 20% ethanol (100 mL per gel, maximum of 10 gels per box) for at least 1 h four times, shaking the gels in the final destain solution overnight.
3. One hour before scanning or photography, place the gels in distilled water (100 mL per gel, maximum of 10 gels per box) at room temperature with shaking. Wipe off any stain residue from both surfaces of the gels before scanning or photographing.

5.2 SILVER STAINING

The method described below is a modification of the techniques described in [Guevara et al. 1982](#) and [Giometti et al. 1991](#). Optimal results are achieved when gels are stained directly with silver, although gels previously stained with Coomassie blue can be subsequently silver-stained (backgrounds are darker). Note that "ethanol" refers to 95% (190 proof) ethanol.

1. After removing DALT gels from their plates, place them (maximum of four gels per box) in 50% ethanol containing 1% (v/v) acetic acid and 0.1% (v/v) formaldehyde (250 mL per gel), and shake at room temperature for a minimum of 6 h.
2. Drain the solution and replace with 50% ethanol (250 mL per gel, maximum of 4 gels per box) and continue to shake at room temperature overnight.
3. Drain the gels and rinse in 20% ethanol for 30 min. Rinse an additional 30 min in 20% ethanol containing 5 mg/L of dithiothreitol.
4. For a box containing four gels, mix 940 mL of 20% ethanol with 1.4 mL of 10 N NaOH. In another beaker, dissolve 4.0 g AgNO₃ in 50 mL water. Immediately before use, mix 10.5 mL of NH₄OH in the ethanol

solution. Then with the ethanol solution stirring, add the silver solution. A brown precipitate will form, but should dissolve quickly as the solution is stirred. If the precipitate does not clear, discard the solution and start over. Drain the gels and add the silver solution. Shake at room temperature for 1 h.

5. Drain the silver solution and wash the gels three times with 20% ethanol, 20 min per wash.
6. After draining the final ethanol wash, develop the gels for 10 min at room temperature with shaking by adding a solution containing 50 mg citric acid and 0.5 ml formaldehyde in 1 L of 20% ethanol (for four gels). Gently rearrange the gels in the box to ensure even development.
7. Drain the developer solution and add 0.5% acetic acid in water to stop the development. Shake at room temperature for five minutes.
8. Drain the stop solution and wash the gels three times at room temperature with tap-distilled water, 20 min per wash.
9. Photograph the gels after the final 20 min wash.

6 DIGITIZING GELS

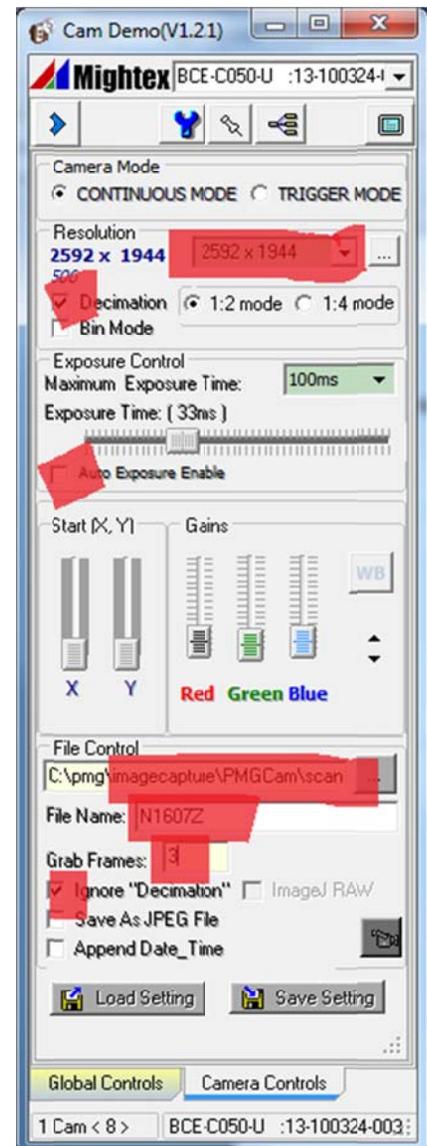
6.1 IMAGE CAPTURE WITH THE MIGHTEX CAMERA

1. Start the Mightex image capture utility:



C:\pmg\imagecapture\Application\BUFCameraApp.exe

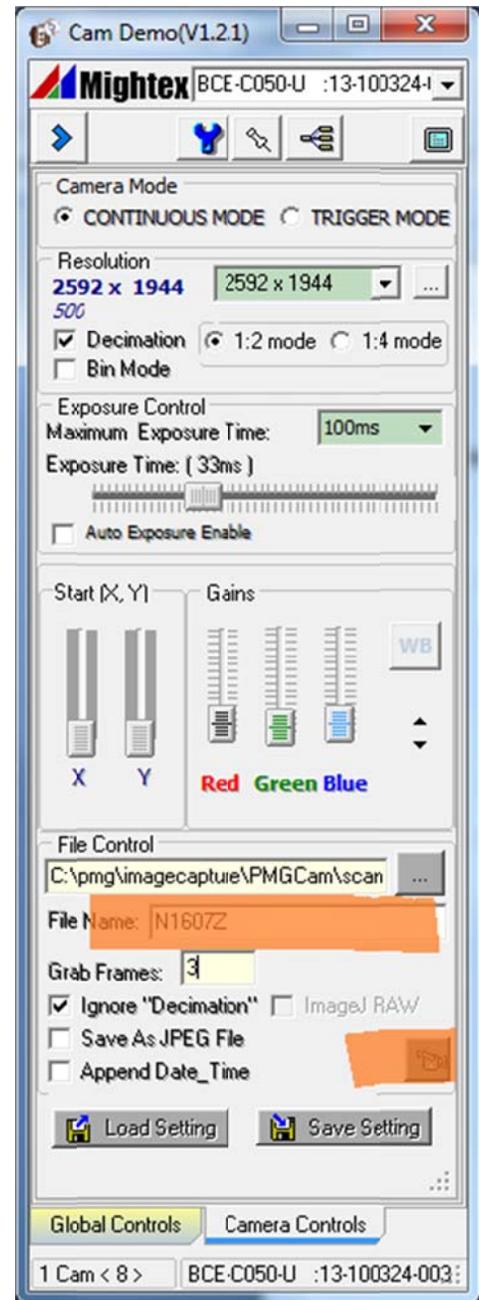
2. Select camera:



ick

gel

age'
ries.
the

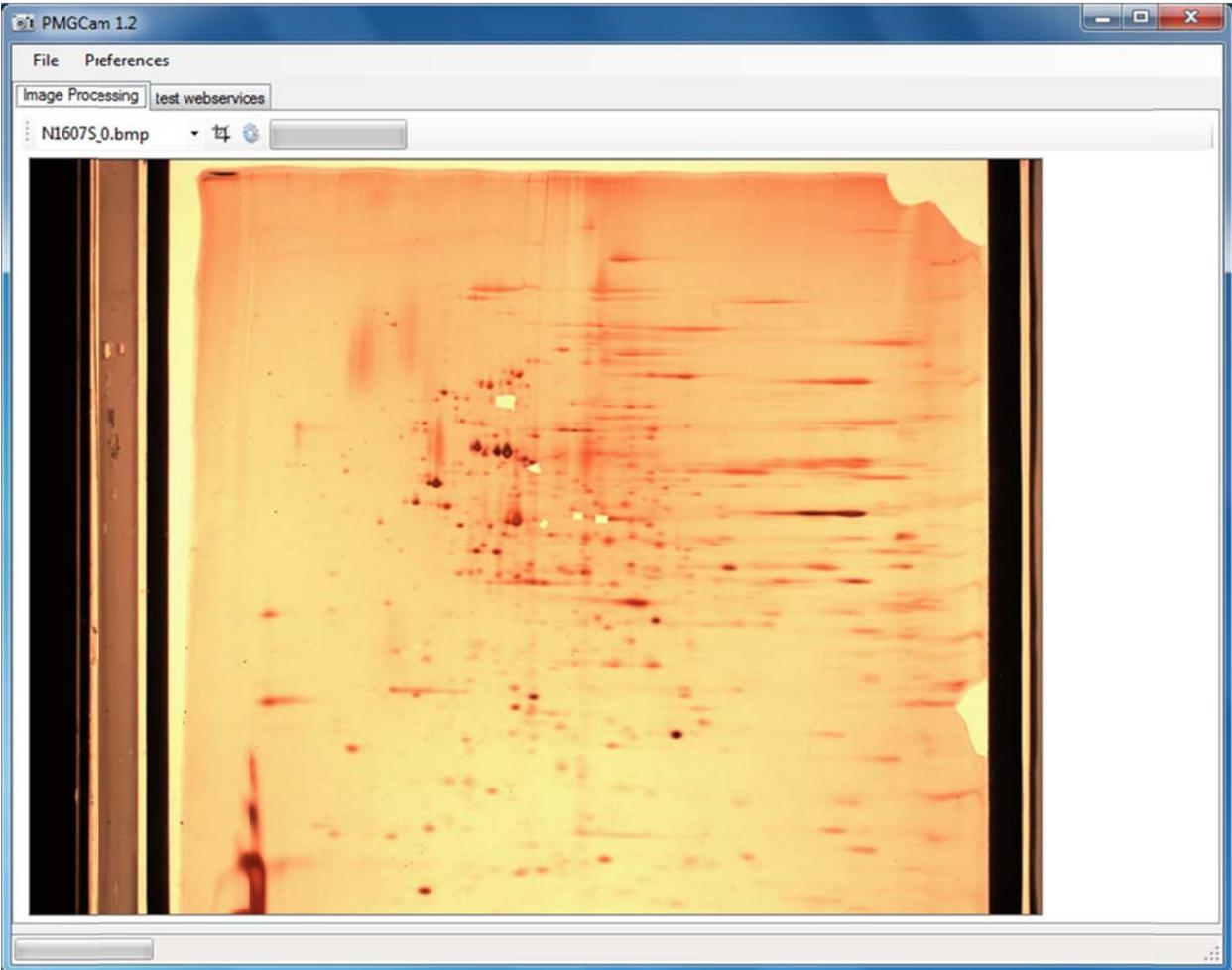


6.2 IMAGE PROCESSING WITH THE PMGCAM APPLICATION

- 1) Start the application: desktop



- 2) Select the area of interest for every image in the pulldown list (select image, draw AOI with mouse, click on 'crop' button: )



- 3) Process images: click on the process button: . A progress is shown on the progress bar. Let process run until a notification informs you it is finished.
- 4) Close application.

7 TRANSFER OF PROTEINS FROM 2DE GELS TO MEMBRANES

Proteins can be transferred out of 2DE gels onto membrane supports for further analysis such as immunoblotting (Western blots) or amino-terminal amino acid sequencing. The membranes of choice are nitrocellulose or polyvinylidene difluoride (PVDF). The following methods are used for PVDF membranes ([Matsudeira 1987](#)) (e.g., Immobilon-P [Millipore Corporation, Bedford, Massachusetts; Catalogue No. IPVH 151 50; 15 ×15 cm] for Western blots; Immobilon-P^{SQ} [Catalogue No. ISEQ 151 50; 15×15 cm] for protein sequencing). The proteins bound to PVDF are stable for an indefinite time on dry membranes stored at -20 °C.

1. Remove DALT gels from plates and equilibrate in a solution containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid ([CAPS transfer buffer](#)) (pH 11.0) and 10% methanol (100 mL per gel, maximum of 6 gels per box) for 5–10 min.
2. Using a fine-tip felt pen, number the dry PVDF membranes to correspond to gel numbers, wet the membranes in 100% methanol for a few seconds, and place them in CAPS transfer buffer. Wet several squares of filter paper (3 pieces per gel) (e.g., Gel Blot paper [Midwest Scientific, Valley Park, Missouri; Catalogue No. 3MWO-1616]) in another box containing the CAPS transfer buffer.
3. Trim each gel to the size of the membrane, and place the gels in fresh CAPS buffer, with the proper orientation, as the identification number will be cut off the gel.
4. Using a semi-dry electroblotting apparatus, place the Mylar[®] mask (with an opening the size of the gels) over the anode electrode surface. Then place three pieces of the wet filter paper squares on the bottom of the electroblotting apparatus. Next put a PVDF membrane (with the number in the lower right corner) on the filter paper. Finally, place the gel, properly oriented, on top of the membrane. To transfer proteins from two gels (the maximum allowed per apparatus for optimal results), add another piece of filter paper, then PVDF membrane, and, finally, another gel.

5. Place two more pieces of wet filter paper on the top of the last gel, place the lid on the apparatus, and turn on the power supply and run for 1.5–2.5 h at 7–8 V.
6. For Western Blots, place the membranes in a non-reactive blocking solution (e.g., 3% BSA or 3% nonfat dry milk or 1% chemiluminescence blocking solution) and leave them overnight in the refrigerator.
7. For detection of proteins by Coomassie Blue, (e.g., Immobilon-P^{SQ} membranes for sequencing),
 - a. Rinse the membranes briefly in deionized water and then saturate the membranes in 100% methanol for a few seconds.
 - b. Place the membranes in a solution of stain containing 0.1% Coomassie Blue R-250, 40% methanol, and 1% acetic acid (approximately 100 mL per two membranes) for 1-2 min.
 - c. Destain the membranes in two to three changes of 50% methanol.
 - d. Rinse the membranes with water, and hang to dry.

8 WESTERN BLOTS

8.1 HRP COLOR DEVELOPMENT

1. If the membrane has been dried prior to staining, rewet in 100% methanol, rinse in water, and equilibrate in [Tris-saline](#) for about 20 min. Otherwise, pour off the BSA blocking solution and use it to dilute the primary antibody (1:100 or greater). Shake the membrane in 50 mL of diluted primary antibody at room temperature for 1 hour.
2. Pour off the antibody (can store frozen at $-20\text{ }^{\circ}\text{C}$) and rinse the transfer membrane with [Tris-saline](#) solution five times to wash off unbound antibody.
3. Shake the membrane in 50 mL of the appropriate peroxidase-conjugated secondary antibody (e.g., if the primary antibody was rabbit anti-human, then use goat anti-rabbit IgG-peroxidase) at room temperature for 1 hour.
4. Discard the secondary antibody solution and rinse the membrane five times with Tris-saline solution to remove excess secondary antibody.
5. Immediately before use, prepare the following two solutions (recipe for two to three transfers):
 - 60 mg of 4-chloro-1-naphthol (HRP color development reagent) (e.g., BioRad, Catalog Number 170-6534 or Sigma, Catalog Number C6788) in 20 mL of methanol.
 - 60 μl of 30% H_2O_2 in 100 mL of cold 20 mM Tris/saline.

Mix the hydrogen peroxide (H_2O_2) solution with the color development reagent at room temperature and immediately pour the mixture over the membrane. Agitate until staining is adequate (blue spots with white or light blue background).

6. Rinse the stained membranes well with tap-distilled water to stop the reaction.

8.2 CHEMILUMINESCENCE

1. If the membrane has been dried, rewet in 100% methanol, rinse in water, and place in 15mL 1 X TBS (tris-buffered saline). Wash the membrane twice in the TBS for 10 min each. After being in the methanol, the membrane will float on top of the TBS and will gradually submerge as the MeOH comes out of it. Avoid mechanical forcing of the membrane to submerge.
2. Discard the washes and incubate the membrane in 30 mL 3% BSA or milk blocking solution for 1 hr (can pause longer at this step, even overnight if left in the refrigerator).
3. Remove 15 mL of the blocking solution from the membrane. To the remaining 15 mL of blocking solution on the membrane, add 15 μ L of the primary monoclonal antibody (thus diluting it 1:1000) and incubate at room temperature for 1 hr with rocking (or 4 °C overnight with rocking).
4. Pour off the antibody and wash the membrane twice in [TBS-Tween20/Triton-X-100](#) (TBSTT) 10 min each to remove unbound primary antibody.
5. Discard washes and incubate for 10 min with 15 mL 1 X TBS (no detergents present prior to incubation with secondary antibody).
6. Shake the membrane for 1 hr at room temperature with peroxidase-conjugated secondary antibody diluted 1:50,000 in the 15 mL 3% blocking solution poured off and saved previously (see #3).
7. Pour off the antibody and wash the membrane 7 times with large volumes of TBSTT for 15 min each. It is important to thoroughly wash the membrane at this point to achieve maximum signal to noise ratios.
8. Drain off the last TBSTT wash, and add 3.5 mL each of Pierce Pico or Femto peroxide solution and enhancer. Incubate at room temperature for 5-10 min. Drain off excess detection reagent and wrap the blot or gel) in plastic wrap or sandwich it between plastic sheets. Remove any bubbles between the plastic and the membrane.

9. Use the digital Kodak Image Station 4000R Pro to scan the membrane. Use a 1 minute exposure for the first scan, followed by subsequent exposures accordingly, and export the image as a jpg file.

9 IN-GEL DIGESTION OF SPOTS OR BANDS FROM GELS

After you have finished running, staining, destaining, scanning, and photographing the gels, you will probably want to cut out spots of interest to identify by a Mass Spectroscopy system. We have our own Mass Spec, but most labs will pre-digest the spots of interest and prepare them for sending to a Mass Spec service facility. Therefore, we include this chapter to help you prepare protein gel spots for Mass Spec identification.

9.1 EQUIPMENT AND SOLUTIONS TO PREPARE FOR IN-GEL DIGEST

Equipment:

Shaker

Vortex

Centrifuge

Gloves

Gel loading pipette tips

Liquid N₂/Air

Aluminum foil

0.5-1.5 mL non-sticking micro-centrifuge tubes

Samples for each group digest:

1. Positive control: 25 pmol transferrin in gel
2. Sample gels
3. Negative control: blank gel piece from the sampled 2D gel

Fresh solutions for Coomassie gels:

10 mM TCEP/100 mM NH₄HCO₃

10 μL 1 M TCEP

100 μL 1 M NH₄HCO₃

890 μL H₂O

55 mM Iodoacetamide/100 mM NH_4HCO_3

10 mg iodoacetamide

100 μL 1 M NH_4HCO_3

900 μL H_2O

Digest Buffer (50 mM NH_4HCO_3 /5 mM CaCl_2)

5 μL 1 M CaCl_2

50 μL 1 M NH_4HCO_3

945 μL H_2O

Trypsin Digest Solution (12.5 ng/ μL trypsin)

10 μL 0.5 $\mu\text{g}/\mu\text{L}$ stock trypsin

90 μL digest buffer

Other solutions:

Milli-Q or deionized H_2O

Acetonitrile

100 mM NH_4HCO_3

100 μL 1 M NH_4HCO_3

900 μL H_2O

25 mM NH_4HCO_3

25 μL 1 M NH_4HCO_3

975 μL H_2O

5% HCOOH (formic acid)

57 μL 88% HCOOH

943 μL H_2O

Fresh Solutions for Destaining Silver Gels:

30 mM $\text{K}_3\text{Fe}(\text{CN})_6$

30 μL 1 M $\text{K}_3\text{Fe}(\text{CN})_6$

970 μL H_2O

100 mM $\text{Na}_2\text{S}_2\text{O}_3$

100 μL 1 M $\text{Na}_2\text{S}_2\text{O}_3$

900 μL H_2O
1:1 mixture of the above (500 μL + 500 μL)

Stock solutions for Destaining Silver Gels:

1 M $\text{K}_3\text{Fe}(\text{CN})_6$
329.2 mg $\text{K}_3\text{Fe}(\text{CN})_6$ (MW 329.2)
1 mL H_2O

1 M $\text{Na}_2\text{S}_2\text{O}_3$
158.1 mg $\text{Na}_2\text{S}_2\text{O}_3$ (MW 158.1)
1 mL H_2O

9.2 IN-GEL ENZYME DIGESTION OF COOMASSIE-STAINED GELS

1. Protein Band or Spot Excision

- Excise the band or spot of interest using a razor blade or scalpel with the gel on a glass plate. The photographed gel on page 35 has 5 spots cut out of the gel.
- Cut the band (or group of spots) into 1 × 1 mm pieces and place into a 0.5 or 1.5 mL tube. Suspend in H_2O

2. Gel pieces-washing

- Add 100 μL acetonitrile. Vortex, and then shake 15 min in the incubator/shaker at 37 °C, unless room temperature is specified. Remove the liquid, which you will do after each step. Discard the liquid into a tube labeled Coomassie Waste.
- Add 100 μL 100 mM NH_4HCO_3 (re-hydration), 5 min. Discard liquid.
- Add 100 μL acetonitrile. Vortex and shake 15-30 min. Discard liquid.

3. Reduction and Alkylation

- Add 50-100 μL 10 mM TCEP/100 mM NH_4HCO_3 . Vortex, and shake

- 20-30 min at room temperature. Discard the liquid.
- QUICKLY add 50-100 μL 55 mM iodoacetamide/100 mM NH_4HCO_3 . Vortex and then shake in the DARK 30 min at room temp. Discard the liquid.
 - Add 100 μL acetonitrile, until the gel spots shrink and turn sticky white, about 15 min. Discard the liquid.
 - Add 100 μL 100 mM NH_4HCO_3 and shake for 5 min. Discard liquid.
 - Add 100 μL acetonitrile and shake for 15 min. Discard liquid.
- Repeat the acetonitrile, NH_4CO_3 , and acetonitrile steps (last 3 lines above) if the blue color persists.

4. In-gel Digest

- Add 25 μL of Trypsin Digest Solution to each tube.
- Add an additional 50 μL Digest Buffer (without trypsin), and incubate overnight.

5. Extraction of Peptides (All incubations at 37 °C)

- Centrifuge 30 seconds. Transfer buffer to 0.5 or 1.5 mL tubes.
- Add 50-100 μL 25 mM NH_4HCO_3 . Incubate 15 min. and centrifuge. Transfer the supernatant to the 0.5 or 1.5 mL tubes.
- Add 50-100 μL acetonitrile. Incubate 15 min. and centrifuge. Transfer the supernatant to the 0.5 or 1.5 mL tubes.
- Add 100 μL 5% HCOOH . Incubate 15 min. Transfer supernatant to the 0.5 or 1.5 mL tubes.
- Add 100 μL acetonitrile. Incubate 15 min. Transfer the supernatant to the 0.5 or 1.5 mL tubes.
- Repeat the above 2 steps if necessary.
- Speedvac the liquid supernatants that have been combined together, until the sample is dry. You may store the dry samples in the refrigerator or freezer if you don't plan on doing the next step immediately.

6. MS Peptide Mapping

- Re-suspend in 10-30 μL 5% HCOOH .

9.3 IN-GEL ENZYME DIGESTION OF SILVER-STAINED GELS

1. Protein Band or Spot Excision

- Wash gel with H₂O twice, 10 min. each.
- Excise the band (or group of spots) of interest using a razor blade or scalpel on a glass plate.
- Cut the gel pieces into 1 × 1 mm pieces and place into a 0.5 or 1.5 mL tube. Suspend in H₂O.

2. Gel De-staining

- Discard the water if the sample is suspended in water. Add 100-400 μL 1:1 solution of 30 mM K₃Fe(CN)₆ and 100 mM Na₂S₂O₃. Incubate 5 min. and discard the liquid into a tube labeled Silver Waste.
- Wash 4 times with 100-400 μL H₂O. Remove the water to the Silver Waste tube.

Then, follow steps 2-6 of the protocol for Coomassie-stained gels.

10 CULTURING AND HARVESTING SHEWANELLA

Obtain a glycerol stock of a bacterial species from a collaborator or a commercial source. If you keep the stocks frozen at $-70\text{ }^{\circ}\text{C}$, they are viable for about a year. The following protocol is for *Shewanella oneidensis* MR-1 and may need to be changed for growth of a different microorganism.

1. Dip a disposable plastic loop into the stock and streak a TSA plate (Tryptic Soy Agar, Difco). Incubate the agar plate at room temperature. All colonies should appear the same, and then you know you have an uncontaminated stock.

Add the remaining 1 mL of stock into 100 mL autoclaved TSB (Tryptic Soy Broth, Difco). Incubate overnight at $28\text{ }^{\circ}\text{C}$, shaking at 100 rpm.

2. Prepare [Shewanella MR-1 Federation Defined Medium](#) (10X), [Aerobic MR-1 Minimal Media](#) (1X), and [Anaerobic MR-1 Minimal Media](#) (1X).
3. From the overnight culture you can make both aerobic and anaerobic cultures. To 100 mL Aerobic MR-1 Minimal Media in a baffled culture flask, add 1 mL of the overnight TSB culture. To 1 L Anaerobic MR-1 Minimal Media, add 20 mL overnight TSB culture. The anaerobic culture must then be nitrogen-purged for 5-10 min before incubation. A larger volume is used to grow anaerobic cultures because cell yield is much lower than with aerobic cultures.
4. Incubate cultures at $28\text{ }^{\circ}\text{C}$ at 150 rpm agitation overnight.
5. The anaerobic MR-1 culture will look like pink lemonade. Test the optical density (OD) at wavelength 600 in each culture. The OD for aerobic cultures is 0.6-0.7 and for anaerobic cells is 0.1-0.2.
6. Harvest cultures by centrifugation at 5000 rpm for 10 min at $4\text{ }^{\circ}\text{C}$. Wash cell pellets 2-3 times with TBS (Tris-buffered saline).
7. Store cell pellets in 1.5 mL microfuge tubes at $-70\text{ }^{\circ}\text{C}$ until use.

11 REFERENCES

- Anderson, N.G., and N.L. Anderson, 1978a. Analytical techniques for cell fractions. XXI. Two-dimensional analysis of serum and tissue proteins: multiple isoelectric focusing, *Analytical Biochemistry* 85: 331–340.
- Anderson, N.L., and N.G. Anderson, 1978b. Analytical techniques for cell fractions. XXII. Two-dimensional analysis of serum and tissue proteins: multiple gradient-slab electrophoresis, *Analytical Biochemistry* 85: 341–354.
- Anderson, N.G., N.L. Anderson, and S.L. Tollaksen, 1979. Proteins of human urine. I. Concentration and analysis of two-dimensional electrophoresis, *Clinical Chemistry* 25: 1199–1210.
- Edwards, J.J., S.L. Tollaksen, and N.G. Anderson, 1982. Proteins of human urine. III. Identification and two-dimensional electrophoretic map positions of some major urinary proteins, *Clinical Chemistry* 28: 941–948.
- Giometti, C.S., M.A. Gemmell, S.L. Tollaksen, J. Taylor, 1991. Quantitation of human leukocyte proteins after silver staining: A study with two-dimensional electrophoresis, *Electrophoresis* 12: 536–543.
- Guevara, J., S. Capetillo, D.A. Johnston, B.A. Martin, L.S. Ramagli, and L.V. Rodriguez, 1982. Quantitative aspects of silver deposition in proteins resolved in complex polyacrylamide gels, *Electrophoresis* 3: 197–205.
- Matsudeira, P., 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes, *Journal of Biological Chemistry* 262: 10035–10038.
- O'Farrell, P.H., 1975. High resolution of two-dimensional electrophoresis of proteins, *Journal of Biological Chemistry* 250: 4007–4021.
- O'Farrell, P.Z., Goodman, H.M., and O'Farrell, P.H., 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins, *Cell* 12: 1133–1142.
- Ramagli, L.S. and L.V. Rodriguez, 1985. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer, *Electrophoresis* 6: 559–563.

12 APPENDICES

A: WORK SAFETY

1. All personnel involved in laboratory procedures must wear lab coats, safety glasses, and gloves. The gloves may be latex, vinyl, polycarbonate, nitrile, heavy rubber, or cotton, depending on the particular procedure or manipulation being performed. Refer to the Argonne Protective Glove Selection Guide for the appropriate choice.
2. No eating, drinking, or chewing gum is allowed in any laboratory. Smoking is not allowed in Building 202 at all.
3. Personnel should wear gloves during washing of dishes and tools, and must wash hands before leaving the laboratory.
4. Disposable pipettes, beakers, tubes, and syringes should be used whenever possible.
5. Personnel must satisfy all Argonne-mandated training requirements for their particular job, based on their Job Hazard Questionnaire.
6. All flammable reagents (e.g., ethanol, methanol, 2-propanol) must be stored in the original containers in a flameproof cabinet.

B: WASTE MANAGEMENT

1. Animal tissue samples are prepared in a solution that solubilizes the proteins before loading them on the ISO. The components of this solution are generally consumed during electrophoresis when samples are analyzed. If excess sample is prepared, it is either stored at $-70\text{ }^{\circ}\text{C}$ or disposed of as liquid chemical waste.
2. Excess human materials (body fluids, tissue pieces) are placed in biohazard bags and taken to the Medical Division for autoclaving and disposal.
3. Hazardous chemical waste is collected in appropriately labeled and numbered polycarbonate bottles, glass bottles, or steel drums, and log sheets are filled out after each entry of waste. When the container is full, a waste form must be filled out and submitted first to Health Physics for approval and then to Waste Management for pickup.

C: RECIPES

Recipe 1. SDS Mix (solubilizing agent)

Compound	Amount	Final Conc.
CHES (2-[N-cyclohexylamino]ethane sulfonic acid)	1 g	0.5 M
SDS (sodium dodecyl sulfate)	2 g	2%
DTT (dithiothreitol)	1 g	1%
Glycerol	10 mL	10%

Adjust pH to 9.5 with NaOH; add water to a final volume of 100 mL.

Recipe 2. NP-40/Urea Mix (solubilizing agent)

Compound	Amount	Final Conc.
Urea	54 g	9 M
Nonidet P-40	4 mL	4%
Ampholyte, 8–10 range (20% w/v stock)	10 mL	2%
2-mercaptoethanol	2 mL	2%

Adjust pH to 9.5 with NaOH; add water to a final volume of 100 mL.

Recipe 3. Urea Mix without NP-40 (solubilizing agent)

Compound	Amount	Final Conc.
Urea	54 g	9 M
Ampholyte, pH 3.5–10 range (20% w/v stock)	5 mL	2%
2-mercaptoethanol	5 mL	5%

Adjust pH to 9.5 with NaOH; add water to 100 mL final volume.

Recipe 4. NP-40/Urea/DTE Mix (solubilizing agent for muscle samples)

Compound	Amount	Final Conc.
Urea	54 g	9 M
Nonidet P-40	4 mL	4%
Ampholyte, 3.5–10 range (40% w/v stock)	5 L	2%
DTE (dithioerythritol)	1 g	1%

Adjust pH to 9.5 with NaOH; add water to a final volume of 100 mL.

Recipe 5. Acrylamide 30%/bis 1.8% (for first-dimension and stacking gels)

Compound	Amount	Final Conc.
Acrylamide (Bio-Rad)	30 g	30%
<i>N,N</i> -methylene-bis-acrylamide	1.8 g	1.8%

Add water to 100 mL. Filter with a 115-mL side-arm filter unit (0.45 μm) attached to the vacuum pump and store the solution in the refrigerator in a bottle marked "Danger: Cancer Hazard".

Recipe 6. 10% Ammonium persulfate

- Dissolve 10 g ammonium persulfate in 100 mL of water.
- Store in the refrigerator in a dark bottle, and use the solution within 1-2 weeks.

Recipe 7. Buffer II (recipe for 375 mL)

Compound	Amount	Final Conc.
----------	--------	-------------

Tris	11.25 g	3%
SDS	0.75 g	0.2%

Add 300 mL water. Stir, and add concentrated HCl until pH is 6.8. Bring volume up to 375 mL.

Recipe 8. Equilibration buffer (recipe for 750 mL)

Compound	Amount	Final Conc.
Glycerol	75 mL	10%
Tris	11.25 g	1.5 %
SDS	15.75 g	2%
Dithioerythritol or Dithiothreitol or 2-mercaptoethanol	1.0 g or 1.0 g or 37.5 mL	5%
Bromophenol blue	Trace	Trace

Add ingredients to ~600 mL water, and adjust pH to 6.8. Mix the above ingredients in a convenient dispenser, such as the Repipet Jr. (Fisher Catalog No. 13-687-59B), bring up to volume, and stir for about an hour.

Recipe 9. 27% acrylamide/0.8% bis (for DALT gels)

Compound	Amount	Final Conc.
Acrylamide	300 g	27%
Bis	8 g	0.8%

Mix on a magnetic stirrer and bring up to 1.1 L with water. Filter. If you prefer to use liquid acrylamide instead of working with the powder, use:

Compound	Amount	
Acryl/bis 29:1 ratio	500 mL	
Acryl/bis 37.5:1 ratio	500 mL	

Final concentration will be 33.25% acrylamide and 1.0% bis.

Note: Amresco, BioRad, and Sigma all sell liquid acrylamide and bis.

Recipe 10. Buffer L (recipe for 3 L)

Compound	Amount	Final Conc.
Tris	554 g	13.3%

Add 2 L of water. Add 12 N HCl to pH 8.5–8.6, and bring to 3 L.

Recipe 11. Buffer L10

Compound	Amount	Final Conc.
Buffer L	3 parts	37.5%
Water	5 parts	62.5%

Recipe 12. Buffer L20

Compound	Amount	Final Conc.
Buffer L	3 parts	75%
Glycerol	1 part	25%

Recipe 13. Sucrose underlay solution

Compound	Amount	Final Conc.
Sucrose	35 g	35%
Water	To 100 mL	
Methylene blue	Trace	Trace

Recipe 14. Photoflo overlay solution

Compound	Amount	Final Conc.
Angel's buffer	40 mL	16%
Water	210 mL	84%
Photoflo (or similar surfactant)	0.1 mL	0.1%

Recipe 15. Angel's buffer

Compound	Amount	Final Conc.
Tris base	870 g	29%
SDS	22.5 g	0.75%
Water	~2.1 L	-

Adjust pH to 8.6 with 150-200 mL of 12 N HCl, and add water to 3L.

Recipe 16. Light acrylamide solution (12%, for computer-poured gels)

For 750 mL final volume (1 set):		
Acrylamide (40% commercial premixed acrylamide/bis 37.5:1)	225 mL	12%
Water	502.5 mL	-
Glycerol	22.5 mL	3%

Recipe 17. Heavy acrylamide solution (22.46%, for computer-poured gels)

For 1 L final volume:		
Acrylamide (40% commercial)	561.5 mL	22.46%
Water	308.5 mL	-
Glycerol	130 mL	13%

Recipe 18. Stacking gel mix

Compound	Amount	Final Conc.
Tris	14.4 g	
SDS	0.96 g	
30% acrylamide/1.8% bis solution	140 mL	

Add water to 1 L.

Recipe 19. Running buffer agarose

Compound	Amount	Final Conc.
Tris	3 g	0.3%
Glycine	14.4 g	1.44%
SDS	1 g	0.1%
Agarose	5 g	0.5%

Add water to 1 L. Microwave to dissolve, and freeze in 100 mL portions.

NOTE: Agarose solutions often superheat when they are microwaved, boiling over when disturbed, e.g., when a pipette is introduced, and serious injury can result. Use extreme caution when handling heated agarose.

Recipe 20. DALT tank buffer (26 L)

Compound	Amount	Final Conc.
Tris	78 g	24 mM
Glycine	374 g	0.2 M
SDS	26 g	3.5 mM

Add above components to approximately 4 L distilled water and stir with a magnetic stirrer until dissolved. Pour into the clean DALT tank, and add tap distilled water to the fill mark.

Recipe 21. Coomassie Blue Stain

Compound	Amount	Final Conc.
Coomassie Blue	20 g	0.2%
Deionized water	4.5 L	-
Phosphoric acid	250 mL	2.5%
Ethanol (95%)	5 L	47.5%

Mix the stain and water. Add 250 mL phosphoric acid. Add 5 L 95% ethanol and mix thoroughly for at least an hour. Let the solution stand overnight before use.

Recipe 22. Saline solution

Compound	Amount	Final Conc.
NaCl	90 g	150 mM
Water	10 L	-

Recipe 23. Ammonium persulfate (for computer-poured DALT gels)

"Light" and "heavy" persulfate solutions are so called because they are used, respectively, with the light or heavy acrylamide solutions.

- "Light" persulfate solution": Take 25 mL of the 10% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ solution (recipe 6) and bring to 100 mL with water (final concentration, 2.5% w/v).
- "Heavy" persulfate solution: Take 15 mL of 10% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ solution and bring to 100 ml with water (final concentration, 1.5% w/v).

Recipe 24. TEMED (for computer-poured DALT gels)

Make dilutions immediately before use from the original stock reagent.

- "Light" TEMED solution: Take 230 μL TEMED and bring to 100 mL with water (final concentration, 0.23% v/v).
- "Heavy" TEMED solution: Take 30 μL TEMED and bring to 100 mL with water (final concentration, 0.03% v/v).

Recipe 25. Bovine serum albumin blocking solution

Compound	Amount	Final Conc.
Tris buffer (1 M, pH 7.5)	0.5 mL	10 mM
30% Bovine serum albumin solution	5 mL	3 %
Saline	44.5 mL	150 mM

NOTE: Use sterile procedures when handling bovine serum albumin (BSA) to minimize bacterial contamination.

Recipe 26. Nonfat dry milk blocking solution

Compound	Amount	Final Conc.
Powdered milk	3 g	3%
Tris buffer (1 M, pH 7.5)	1 mL	10 mM
Saline	99 mL	150 mM

Recipe 27. 1 M Tris buffer

Dissolve 12.1 g of Tris in water; adjust pH to 7.5, and make a final volume of 100 mL.

Recipe 28. Tris-saline solution

Add 1 mL of [1 M Tris, pH 7.5](#) to 100 mL of [saline](#) solution.

Recipe 29. Rehydration buffer (for IPGs)

Compound	Amount	Final Conc.
Urea	48.04 g	8 M
SDS	2.0 g	2%
DTT	772 mg	50 mM
Ampholyte	0.5 mL	0.2%
Bromophenol blue	Trace	0.001%
H ₂ O	To 100 mL	

Recipe 30. DTT or Iodoacetamide Equilibration Buffer

Compound	Amount	Final Conc.
Urea	36.036 g	6 M
SDS	2.0 g	2%
Tris Base	4.54 g	0.0375 M
Glycerol	20 mL	20 %
HCl		To pH 8.8
H ₂ O	To 100 mL	

- For DTT Equilibration Buffer, add 200 mg DTT/10 mL Equilibration Buffer just before use.
- For Iodoacetamide Equilibration Buffer, add 250 mg Iodoacetamide/10 mL Equilibration Buffer just before use.

Recipe 31. 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer in 10 % methanol, pH 11.0

- Stir 2.2 g CAPS in 800 mL deionized water.
 - Add 100 mL methanol.
- The pH will be ~ 5.5. Adjust pH to 11.0 with 10 N NaCl, and add water to 1 L.

Recipe 32. Tris buffered saline (TBS), pH 7.5, for chemiluminescence detection

Add 5 mL of [1 M Tris](#), pH 7.5, to 995 mL [saline](#).

Recipe 33. TBS-Tween[®] 20 (TBST)

Dilute 1 mL Tween[®] 20 in 1 L TBS (final concentration 0.1% [w/v]).

Recipe 34. TBS-Tween[®] 20/Triton-X 100 (TBSTT)

Dilute 1 mL of Tween[®] 20 in 1 L TBS (final concentration 0.1% [w/v]).
Add 500ml Triton-X 100 to the TBS with Tween (final concentration 0.05% [w/v]).

Recipe 35. 6 X SDS Mix (Modified 1D Blue Mix)

Makes 40 mL

SDS	2.4 g
1M Tris-HCl, pH 6.8	12 mL
Deionized H ₂ O	1.6 mL
Glycerol	24 mL
2-mercaptoethanol	2.4 mL
Bromophenol blue	trace

Stir until all solids have gone into solution. Store at -70 °C.

Recipe 36. 5 X 1D Gel Tank Buffer

30 g Tris, 144 g glycine and 10 g SDS in 2 L double distilled water.
Refrigerate the stock solution.

Recipe 37. 10X *Shewanella* Federation Defined Media

	<u>1 L</u>	<u>Final Concentration</u>
PIPES Buffer	91 g	3M
Ammonium Chloride, NH ₄ Cl	15 g	29 mM
Potassium Chloride, KCl	1 g	1.34 mM
Sodium Phosphate monobasic	6 g	4.35 mM
pH to 7.0 with NaOH		

Recipe 38. 1X *Shewanella* MR-1 Aerobic Minimal Media

	<u>1 L</u>
10X Defined Media	100 mL
100X Amino Acids	10 mL
1000X Extra Vitamins/Minerals	1 mL
ATCC Vitamins	10 mL (1 vial)
ATCC Minerals	10 mL (1 vial)
100X NaCl	10 mL
Sodium Lactate (60% w/v)	3.5 mL
Deionized H ₂ O to 1 L	

Recipe 39. 1X *Shewanella* MR-1 Anaerobic Minimal Media

	<u>1 L</u>
10X Defined Media	100 mL
100X Amino Acids	10 mL
1000X Extra Vitamins/Minerals	1 mL
ATCC Vitamins	10 ml (1 vial)
ATCC Minerals	10 mL (1 vial)
1M Sodium fumarate	30 mL
Sodium lactate (60% w/v)	3.5 mL

Deionized H₂O to 1 L

Recipe 40. 100X Amino Acids

	<u>100 mL</u>
L-glutamate	0.2 g
L-arginine	0.2 g
L-serine	0.2 g

Recipe 43. 1000X Extra Vitamins/Minerals

	<u>100 mL</u>
Sodium tungstate	15 mg
Sodium molybdate	15 mg