2D Gel Electrophoresis Protocol

Lysis Buffer

7M Urea
2M Thiourea
4% CHAPS

Add fresh: 20mM Spermine
20mM DTT
1mM PMSF

Rehydration Buffer

8M Urea
2% CHAPS

Add fresh: 20mM DTT
0.5% IPG Buffer
trace amount of bromophenol blue

Equilibration Buffer

6M Urea
30% Glycerol
2% SDS
50mM Tris-HCl pH 8.8

EQ Buffer 1 – add fresh 1% DTT
EQ Buffer 2 – add fresh 2.5% Iodoacetamide

Sample Preparation

1) Remove media from flasks.
2) Add 4mL of trypsin to each flask and place back in incubator for 2-3 minutes.
3) Remove flasks from incubator and add 4mL of media to each flask to inactivate trypsin.
4) Collect cells into 15mL conical tubes and spin @ 3K RPM for 5-10 minutes at RT.
5) Remove supernatant from conical tubes and add 5mL of 1X PBS to each tube.
6) Resuspend cells in 1X PBS in order to remove excess trypsin and media.
7) Spin @ 3K RPM for 5-10 minutes at RT.
8) Remove supernatant from conical tubes and add 1mL of fresh lysis buffer to each tube.
9) Resuspend cells in fresh lysis buffer then place tubes in refrigerator at 4°C for 2-4 hours.
10) Transfer cell lysates into 1.5mL microcentrifuge tubes (Beckman tubes) and spin at 40K RPM for 1 hour at 4°C.
11) Carefully remove supernatant and transfer to clean microcentrifuge tube.
12) Determine protein concentration and store remaining lysate in -80°C freezer.

Isoelectric Focusing/The First Dimension

1) Prepare 2.5mL of fresh rehydration buffer for each type of DryStrip.
2) Place samples on ice to thaw and collect 1.5mL microcentrifuge tubes for sample dilution.
3) Remove the necessary number of DryStrips from -20°C freezer.
4) Determine the amount of rehydration buffer necessary for strip length.
5) Add 200-300ug of cell lysate to clean microcentrifuge tubes and add the difference between the total volume and the volume of sample using fresh rehydration buffer.
6) Remove plastic cover from strip holder and set aside.

7) Add rehydration buffer containing sample to the strip holder at the anode (pointed end of the holder).
8) Remove protective cover from DryStrip before placing gel side down into strip holder starting at the anode (pointed end) and laying strip down to the cathode (blunt end).
9) Move strip back and forth in order to spread out rehydration buffer.
10) Make sure that all bubbles are removed from underneath the DryStrip before adding DryStrip Cover Fluid (Mineral Oil).
11) Add cover fluid from one end until it reaches the middle of the strip holder then add from the opposite side so that fluid meets in the middle.
12) Complete the same process for all strips then place the strip holders on IPGphor system.
13) Set up protocol for the length strip that is being used.
14) For 13cm strip:
    Rehydration at 20°C for 12 hours
    50uA per strip
    500V for 1 hour
    1000V for 1 hour
    8000V for 3-5 hours
15) Move onto equilibration step or rinse strip with MilliQ water and place into screw cap tubes for storing in -70°C freezer.

Equilibration

1) Prepare 15 mL of fresh equilibration buffers 1 & 2 for each strip.
2) Wash strips with MilliQ water before placing into equilibration buffer 1. If you are removing strip from -70°C, let tube sit on lab bench to thaw strip. When strip is thawed (strip will be clear) place into equilibration buffer 1.
3) Incubate in equilibration buffer 1 for 15 minutes.
4) Remove strip and rinse with MilliQ water before placing into equilibration buffer 2.
5) Incubate in equilibration buffer 2 for 15 minutes.
6) Remove strip and rinse with MilliQ water and place strip on its side on filter paper to allow excess water to drain from strip.

Running the Second Dimension

1) Prepare gel by removing water saturated butanol from top of gel and washing with 1X running buffer.
2) Cover top of gel with 1X running buffer and lay strip across the top of the gel making sure that the gel is lying flush with the gel and remove any bubbles between the strip and the top of the gel.
3) Remove the running buffer and add warm 1% agarose made with 1X running buffer.
4) Allow the agarose to cool and solidify, which should only take a few minutes, before moving to the electrophoresis apparatus.
5) Add 1X running buffer to the upper and lower buffer chambers and place gel inside apparatus.
6) Run gel for 15 minutes at 10mA per gel then run gel at 25mA per gel for 4-6 hours (until BPB band reaches bottom of gel).
7) Remove gel from plates and stain.

1-D Polyacrylamide Gel Electrophoresis

Gel type	Sample volume(µl/well)	Total well volume(µl/well)
Mini Gels 0.75 mm, 10 well	2 to 5	25
Large Gels 0.75 mm, 10 wells	10-140	150

Sample buffer
0.062 M tris-HCl, pH6.8
2% SDS
0.01% Bromophenol Blue
10% Glycerol
5% 2-mercaptoethanol

Upper buffer

10% SDS	10 mL
250mM EDTA	4 mL
Reservoir buffer	100 mL
Nanopure water	add enough to 1 L mark

Lower buffer

Reservoir buffer	100 ml
Nanopure water	add enough to 1 L mark

Store buffers in refrigerator.

Sample Preparation
1. Prepare the sample with the sample buffer to a concentration of 1 µg/µl.

2. Heat at 100 °C for 2-3 min in a boiling water bath.

Sample loading
1. Remove the comb from the cassette by sliding it slowly with a steady motion straight up. Do not distort or tear any wells.

2. Flush the wells with water to remove residual acrylamide

3. For glass cassettes leave the tape on the side of the gels.

4. Use permanent marker to draw the bottoms of the wells. That will make it easier to load.

5. Insert these plates on the electrophoresis unit.

6. Pour the upper and the lower buffers

7. Load the appropriate amounts of samples. Load sample buffer in any wells that do not contain samples.

Gel Visualization

Copper Staining

1. Prepare the Copper stain by dissolving 4 g of $CuCl_2$ in 100 mL of water.

2. Wash the gels with nanopure water.

3. Pour the Cu stain on the gel. Make sure that the gel is completely submerged.

4. Rock for 5 minutes.

5. Pour the stain into the waste and wash the gel with water to remove excess copper.

6. Store the gel in water in the refrigerator.

Zn Staining

1. Dilute Imidazole (Solution A) 1:10 with water, mix thoroughly. Dilute Zinc Sulfate (Solution B) 1:10 with deionized water, mix thoroughly. Gels will require 100-150 mL so that they are compeletely immersed.

2. Place the gel in a staining container and add Imidazole, solution A. Rock at room temperature for 10 min.

3. Decant Solution A, and add the dilute Zinc sulfate, solution B. Make sure the gel is completely covered to ensure even staining. Rock at room temperature for 30 sec approx while the gel develops.

4. Decant solution B, and add 100 mL of water. Rinse for 3-5 min rocking at room temperature

5. Decant water and replace with fresh water. The gel can be stored like this for days.

Silver Staining for Mass Spectrometric Analysis

Step	Solution per gel	Time(min)
Fix	25 ml acetic acid, 100ml methanol, 125 ml water	15
Fix	25 ml acetic acid, 100 ml methanol, 125 ml water	15
Sensitization	75 ml methanol, 10 ml sodium thiosulfate(5%), 17 g sodium acetate, 165 water	30
Wash	250 ml water	5
Wash	250 ml water	5
Wash	250 ml water	5
Silver	25 ml silver nitrate(2.5%), 225 water	20
Wash	250 ml water	1
Wash	250 ml water	1

Develop	6.25 g sodium carbonate, 100 ul formaldehyde 250 ml water	
Stop	3.65 g EDTA 250 water	10
Wash	250 ml Water	5
Wash	250 ml Water	5
Wash	250 ml Water	5

Destaining Silver from the gels
WORKING SOLUTION
30 mM Potassium Ferricyanide
99 mg of Potassium Ferricyanide in 10 ml water
100 mM Sodium Thiosulfate
248 mg Sodium Thiosulfate in 10 ml water
Mix them together. Make fresh for use.

Add 30-50uL working solution to cover the gel bands and vortex occasionally.
Repeat till there is no more brown color left.
Rinse with water , twice.
Cover the gel with 200mM ammonium bicarbonate for 20 min. Discard the liquid to waste.
Wash with ACN till the gel pieces are opaque.
Dry the pieces under vacuum for 30 min

Alternative Version: Silver Staining for Mass Spectrometric Analysis
(Use 5 gel volumes of each reagent for staining)

Step	Solution per gel	Time(min)
Fix	5% acetic acid; 45% methanol; 50% water	90
Wash	water	10
Wash	water	10
Sensitization	0.02% sodium thiosulfate	3
Wash	water	0.5
Wash	water	0.5
Silver	0.1% silver nitrate	30
Wash	water	0.5
Develop	0.02% formaldehyde/2.5% sodium carbonate (bands suitable for MS analysis will appear within 30-60 seconds)	2
Stop	1% acetic acid	10

Wash	water	20
Wash	water	20

Alternate Version: Destaining Silver from the gels
WORKING SOLUTION
30 mM Potassium Ferricyanide
99 mg of Potassium Ferricyanide in 10 ml water
100 mM Sodium Thiosulfate
248 mg Sodium Thiosulfate in 10 ml water
Mix at 1:1 ratio. Make fresh for use.

Cover the band in working solution and vortex until brown color removed (5 minutes)
Remove the solution and wash three times with 200 µL water.

In-gel Digestion

100 mM Ammonium Bicarbonate
0.79 g Ammonium Bicarbonate
Make up to 100 mL with water

10 mM DTT
15.4 mg Dithiothreitol
Make up to 10 mL with 100 mM ammonium bicarbonate

55 mM Iodoacetamide
102 mg Iodoacetamide
Make up to 10 ml with 100 mM ammonium bicarbonate

0.1 µg/µl Trypsin
200 µl of 25 mM Ammonium Bicarbonate
20 µg trypsin

Excise gel bands prior to in-gel digestion

Digestion
1. Add enough volume of DTT to the gel pieces to cover.
2. Reduce for 30 min at 56 °C
3. Cool to room temperature.
4. Replace DTT with iodoacetamide solution with occasional vortexing.
5. Alkylate for 30 min in dark with occasional vortexing.
6. Wash the gel pieces with 50-100 µl ammonium bicarbonate, for 10 min.
7. Dehydrate with ACN. Vortex for 10 min. Discard the liquid.
8. Re-swell by addition of ammonium bicarbonate again. Vortex for 10 min
9. Shrink again with ACN. Vortex for 10 min. Discard liquid. Dry the gel pieces for 10 min.
10. Add 1-10 µg of trypsin(according to the spot color)

11. Incubate for 18 hrs at 37 °C.

*Extraction (Note, this extraction protocol is **not** suitable for hydrophobic proteins)*

1. Pool the digested liquid.
2. Extract with 25 mM ammonium bicarbonate. Pool it in the same tube.
3. Extract with 5% Formic Acid/50% Acetonitrile three times pooling all liquids.
4. Concentrate final product to 20 µl.

Desalt

Use C18 Zip tips.

MALDI-MS

1. Make a saturated solution of 10 mg α-cyano-4-hydroxy-cinnamic acid in 1 mL 60%ACN/0.1%TFA. Vortex and centrifuge. Use the supernatant only.
2. Mix the analyte and the matrix in the ratio of 3:1 – 6:1 (v:v). Vortex. Use 1 µl of this to spot on the target plate.
3. For calibration, mix 1 µl of Angiotensin II and 1µl of ACTH. Add 6 µl of matrix. Vortex. Spot 1 µl onto the target plate.
4. Follow instrument instructions for obtaining mass spectra.

Database Searching

1. Once you have acquired your mass spectral data, you will need to search the experimental peptide masses against database values. The first step is to go to one of the database front-end sites. Any of the following are suitable and are listed only in alphabetical order:

   Mascot
   http://www.matrixscience.com/home.html
   MOWSE
   http://www.seqnet.dl.ac.uk/Bioinformatics/Webapp/mowse/
   Peptide Search
   http://www.mann.embl-heidelberg.de/Services/PeptideSearch/
   Protein Prospector
   http://prospector.ucsf.edu/
   Prowl
   http://prowl.rockefeller.edu/

2. After reaching the front-end interface, follow the guidelines for the particular site for entering your information. Below are some useful hints for database searching:

   For MALDI data, you generally should search against one of the following two protein databases:

   Swiss Prot (This site is fast but not comprehensive)

NCBI (This site is slower but has more information)
If you think you have a "novel" protein sample, choose the NCBI database; for standard systems the Swiss Prot site is fine.

The EST database can also be searched, if necessary. (Typically can be searched more effectively when one has tandem MS sequence information.)

Some of the parameters you'll be asked to input include:

Mass Tolerances are determined by the quality of instrumentation and data; err on the side of caution (normally ± 0.1 Da); if data acquired by another party, make sure they tell you appropriate mass tolerances. Also remember that the better the tolerance (i.e., the smaller the value), the higher the specificity. You'll be able to narrow down your "hits" to a few proteins – the key is to make sure your protein is one of those hits.

Missed cleavages should typically be set to 1 (if you've properly denatured your protein and performed a thorough digestion). If you notice that many of the mass-to-charge values that you submit to the database are "missed" when your protein is identified, you can increase this to 2 or higher and see if those "missing" values are due to incomplete digestion.

Modifications can take a number of forms. In almost all cases you will be alkylating the Cys residues so include this modification. If you have someone else perform the digestion, ask them if any other modifications should be included. You can have the database look for particular post-translational modifications (e.g., phosphorylation) but beware! – this usually results in a greater number of possible protein choices and a standard MALDI experiment on gel-separated proteins is not an effective methodology for identifying the presence of post-translational modifications.

Whenever possible, the quality of the search results and speed of the search will be improved if you can limit the database to search only particular **organisms**. Nearly all interfaces allow this option so use it to your advantage.

As with any computer-generated information, your background and knowledge will be your best guide regarding the "suitability" of identified proteins. If the search results identify a protein as the top candidate that you know (or strongly feel) isn't present, then make sure to view all of the candidate proteins or modify the search parameters appropriately.