Proteomics laboratory

School of Advanced Medical Sciences and Technologies

Autoimmune Diseases Research Center

"2D Gel Based Proteome Analysis"

List of Buffers

- **Lysis Buffer**

8M Urea (2.88gr), 2% CHAPS (0.12gr), 2% DTT (0.12gr), in 6ml of 5mM Tris-Cl pH7.6.

- **Rehydration Buffer**

8M Urea (2.4gr), 2% CHAPS (0.1gr), 1% Bromophenol blue (10µl). Make up to 5 ml with Milli-Q.

- **Equilibration Buffer**

6M Urea (72.07gr), Glycerol (60ml), SDS (4 gr), 50mM Tris-HCl pH 8.8 (10ml). Make up to 200 ml with Milli-Q.

EQ Buffer 1 – add 0.1 gr DTT in 10cc EQ Buffer (use it fresh).

EQ Buffer 2 – add 0.25 gr Iodoacetamide in 10cc EQ Buffer (use it fresh).

- **Tank Buffer (1X)**

Tris base 25mM (7.57gr), Glycine 192mM (36.048gr), SDS 0.1 % w/v (2.5gr). Make up to total volume of 2500 ml with Milli-Q.

1- Sample Preparation from adherent cells

1-1 Remove media from flask.

1-2 Wash the cells with warmed PBS (37°C).

1-3 Add 2 ml of fresh lysis buffer to a 175 cm^2 culture flask.

1-4 Incubation on ice for 30 min.

1-5 Collect the lysate and transfer to 15 ml tubes.

1-6 Spin at 1500g for 5min at 4°C.

1-7 Transfer supernatant in 2ml microcentrifuge tube.

1-8 Spin at 15000g for 20min at 4°C.

1-9 Carefully remove supernatant and transfer to clean microcentrifuge tube.

1-10 Determine protein concentration and store remaining lysate in -80°C.

2- Steps of 2D Electrophoresis

2-1 Rehydration and Isoelectric Focusing (The First Dimension)

2-1-1 Place samples on ice to thaw.

2-1-2 Remove the necessary number of strips from -20°C freezer.

2-1-3 Determine the amount of rehydration buffer necessary for strip length (~280 µl for each 18 cm strip).

2-1-4 Add 200-300 µg of cell lysate, 7µl of IPG buffer and 0.002 gr DTT to a clean tube. Adjust the volume of 340 µl by rehydration buffer.

2-1-5 Remove plastic cover from strip holder and set aside.

2-1-6 Add rehydration buffer containing sample (from step 2-1-4) to the strip holder.

2-1-7 Remove protective cover from strip before placing gel side down into strip holder starting at the anode (pointed end) and laying strip down to the cathode (blunt end).

2-1-8 Move strip back and forth inorder to spread out rehydration buffer.

2-1-9 Make sure that all bubbles are removed from underneath the strip before adding strip cover fluid (Mineral Oil).

2-1-10 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.

2-1-11 Complete the same process for all strips then place the strip holders on IPGphor system.

2-1-12 Set up protocol for the length strip that is being used, for 18cm strip, rehydrate at 20°C for 12 hours at 60v.

2-1-13 Change the cover fluid and do IEF at 4 °C at total voltage of 40000 Vhr.

2-1-14 Move on to equilibration step or rinse strip with Milli-Q water and place into screw cap tubes for storing in -80°C freezer.

2-2 Equilibration

2-2-1 Prepare 10mL of fresh equilibration buffers 1 & 2 for each strip.

2-2-2 Wash strips with Milli-Q water before placing into equilibration buffer 1. If you are removing strip from -80°C, let tube sit on lab bench to thaw strip. When strip is thawed (strip will be clear) place into equilibration buffer 1.

2-2-3 Incubate in equilibration buffer 1 for 20 minutes at 37°C.

2-2-4 Remove strip and rinse with Milli-Q water before placing into equilibration buffer 2.

2-2-5 Incubate in equilibration buffer 2 for 20 minutes at RT.

2-2-6 Remove strip and rinse with Milli-Q water and place strip on its side on filter paper to allow excess water to drain from strip.

2-3 Running the Second Dimension

2-3-1 Gel Preparation:

Acrylamide 30%
Bis-acrylamid 0.8%

Add following reagents to prepare a 15% Acrylamid Gel for a 18 x 20 cm cassette.

H2O	9.40 ml
Tris HCl	10.05 ml
Acrylamid-Bisacrylamid 30%	20.00 ml
SDS 10%	400 µl
APS (Ammonium persulfate) 10%	370 µl
TEMED	10 µl

Total volume	40 ml

2-3-1-1 Prepare gel by removing water from top of gel and wash strip with 1X running buffer.

2-3-1-2 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 by adding warm 1% agarose made with 1X running buffer.

2-3-1-3 Allow the agarose to cool and solidify, which should only take a few minutes, before moving to the electrophoresis apparatus.

2-3-1-4 Add 1X running buffer to the upper and lower buffer chambers and place gel inside apparatus.

2-3-1-5 Run gel for 15 minutes at 10mA per gel then run gel at 25mA per gel for 10 hours (until BPB band reaches bottom of gel).

2-3-1-6 Remove gel from plates and stain.

2-4 Gel Visualization

Silve Srtaining for Mass Spectrometric Analysis

Steps	**Solution per gel**	**Time (min)**
Fix	25 ml acetic acid, 100 ml methanol, 125 ml milli-Q water	15
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Sensitization	75 ml methanol, 10 ml sodium thiosulfate(5%), 17 gr sodium acetate, 165ml milli-Q water	30
Wash	250 ml milli-Q water	5
Wash	250 ml milli-Q water	5
Wash	250 ml milli-Q water	5
Silver Reaction	25 ml of 2.5% silver nitrate (0.625 gr $AgNo_3$ in 25 cc milli-Q water), 225ml milli-Q water (0.25% $AgNo_3$)	20
Wash	250 ml milli-Q water	1
Wash	250 ml milli-Q water	1

Developing	6.25 gr sodium carbonate, 100 µl formaldehyde, 250 ml milli-Q water	
Stop	3.65 gr EDTA, 250 ml milli-Q water	10
Wash	250 ml milli-Q water	5
Wash	250 ml milli-Q water	5
Wash	250 ml milli-Q water	5

2-5 Gel Scanning and Analysis:

After staining, gel will be scanned by appropriate scanner then analyzed by ImageMaster 2D Platinum,version 5.0 software according to manufacture instruction.

2-6 Destaining Silver from the gels:

2-6-1 WORKING SOLUTION

30 mM Potassium Ferricyanide (99 mg of Potassium Ferricyanide in 10 ml Milli-Q).

100 mM Sodium Thiosulfate (248 mg of Sodium Thiosulfate in 10 ml Milli-Q). Mix them together.

Make fresh for use.

2-6-2 steps

2-6-2-1 Add 30-50µl working solution to cover the gel bands and vortex occasionally.

2-6-2-2 Repeat till there is no more brown color left.

2-6-2-3 Rinse with water, twice.

2-6-2-4 Cover the gel with 200 mM ammonium bicarbonate for 20 min. Discard the liquid to waste. Wash with ACN till the gel pieces are opaque.

2-6-2-5 Dry the pieces under vacuum for 30 min.

2-6-3 In-gel Digestion of Protein

2-6-3-1 Solutions:

100 mM Ammonium Bicarbonate (0.79 gr Ammonium Bicarbonate in 100 mL Milli-Q).

10 mM DTT (15.4 mg Dithiothreitol in 10 mL of 100 mM ammonium bicarbonate).

55 mM Iodoacetamide (102 mg Iodoacetamide in 10 ml of 100 mM ammonium bicarbonate).

0.1µg/µl Trypsin (20µg trypsin in 200µl of 25 mM Ammonium Bicarbonate).

2-6-3-2 steps:

2-6-3-2-1 Excise gel bands prior to in-gel digestion

2-6-3-2-2 Add enough volume of DTT to the gel pieces to cover.

2-6-3-2-3 Reduce for 30 min at 56 °C.

2-6-3-2-4 Cool to room temperature.

2-6-3-2-5 Replace DTT with iodoacetamide solution with occasional vortexing.

2-6-3-2-6 Alkylate for 30 min in dark with occasional vortexing.

2-6-3-2-7 Wash the gel pieces with 50-100 µl ammonium bicarbonate, for 10 min.

2-6-3-2-8 Dehydrate with ACN. Vortex for 10 min. Discard the liquid.

2-6-3-2-9 Re-swell by addition of ammonium bicarbonate again. Vortex for 10 min.

2-6-3-2-10 Shrink again with ACN. Vortex for 10 min, discard liquid, dry the gel pieces for 10 min.

2-6-3-2-11 Add 1-10 µg of trypsin (according to the spot color).

2-6-3-2-12 Incubate for 18 hrs at 37 °C.

2-6-3-2-13 centrifuge and collect the supernatant for analysis by Mass spectrometry.

2-7 Mass spectrometry:

2-7-1 Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios. In a typical MS procedure:

2-7-1-1 A sample is loaded onto the MS instrument, and undergoes vaporization.

2-7-1-2 The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions).

2-7-1-3 The ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields

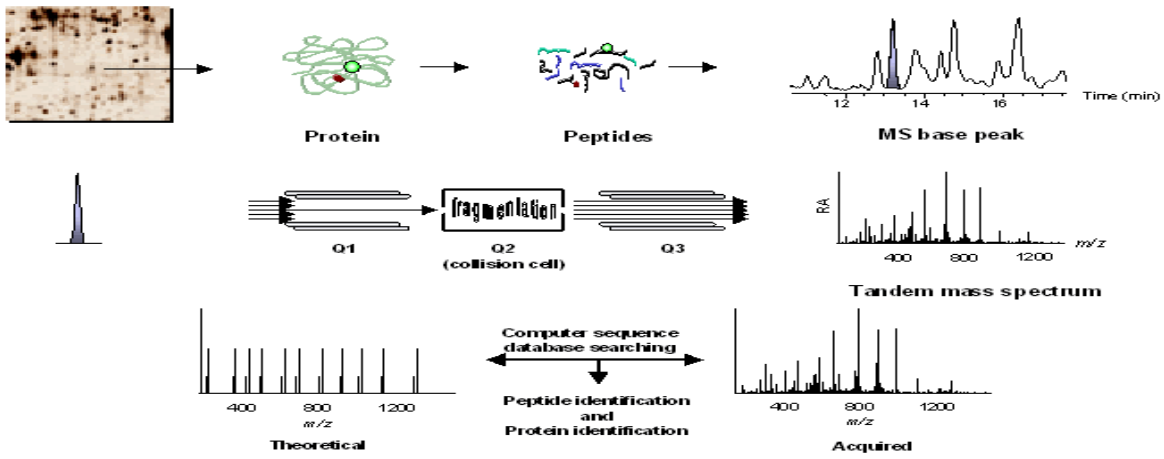
2-7-1-4 The ions are detected, usually by a quantitative method.

2-7-1-5 The ion signal is processed into mass spectra.

MS instruments consist of three modules:

- An *ion source*, which can convert gas phase sample molecules into ions (or, in the case of electrospray ionization, move ions that exist in solution into the gas phase).
- A *mass analyzer*, which sorts the ions by their masses by applying electromagnetic fields.
- A *detector*, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present.

The technique can be used to identify unknown compounds, determining the isotopic composition of elements in a molecule, and quantifying the amount of a compound in a sample. MS is now in very common use in analytical laboratories that study physical, chemical, or biological properties of a great variety of compounds.



Mass spectrometry Overview

2-7-2 Database Searching

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/

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 (Matrix Assisted Laser Desorption Ionization) 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.

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

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.

2-7-3 Mass spectrometry result: