MOLECULAR WEIGHT DETERMINATION ELECTROPHORESIS, LC-MS

Introduction:

Electrophoresis is a commonly used technique in bioanalytical chemistry. In gel electrophoresis, molecules migrate through a gel medium according to their charge. In the case of proteins, charge is proportional to mass and therefore molecular weight resolution is achieved. Mass separation is also achieved by the composition of the gel. These basic principles apply to all types of electrophoresis but there are small differences depending on the molecule of interest.

Electrophoresis is a powerful separation technique, however the technique only gives a crude estimate of molecular weight and identity. It is often used in sequence with other more precise methods. In proteomics, enzymatic digestion is often performed on specific separated protein bands excised from the gel to get a more accurate identification of the protein by mass spectrometry. The benefit of this technique is that the proteins are separated before digestion. The extraction however is a fairly long and laborious technique. The digestion of a single protein will be done. This digestion will also be used for electrophoresis.

MALDI – Matrix Assisted Laser Desorption Ionization is a form of mass spectrometry. Samples are crystalized within a matrix, spotted on a target, ionized by a laser and analyzed by a time of flight (TOF) mass spectrometer.

You will run electrophoresis of several proteins individually and as a mixture. You will analysis of a tryptic digest of bovine serum albumin (BSA) which used LC-MS for analysis.

Prelab:

1. What is reverse pipetting? Why is it a benefit to use this pipetting technique in gel electrophoresis?
2. Briefly describe the basic principles of LC-MS.
3. Why is electrospray ionization useful for protein analysis? Give 2 reasons.
4. How do TOF mass analyzers determine m/z?

SDS-PAGE and In-Solution Digestion

Electrophoresis

1. Bring a water bath (large beaker) to a boil on hot plate.

2. Prepare 1x Tris-Glycine/SDS Running buffer from the 10x stock. You will need 800 ml in total.

3. Prepare protein (diluted in 1X sample buffer) plus sample buffer (Laemmli 4X) and reducing agent beta-2-mercaptoethanol (20X) (B2mE table 1) concentration respectively, as in Table 1.

4. Use the multi-coloured 500 ul centrifuge tubes to prepare the dilutions in Table 1. **Add the reducing agent (B2mE) last and in the fume hood – it is very volatile and has a strong odor! You will need to use micropipette to pipette these volumes.**

Table 1. Preparation of protein samples for gel electrophoresis.

Protein samples*	Initial concentration (mg/ml)*	Sample required (ul)	Sample buffer required (ul)	B2mE required (ul)	Final volume (ul)	Final concentration (ug/ul)
IgG	2	40	15	5	60	1.34
BSA	2	40	15	5	60	1.34
Myoglobin	2	40	15	5	60	1.34
Lysozyme	2	40	15	5	60	1.34
Protein mix	2 (mix 2 of above)	10 (each)	15	5	60	0.25 (each)

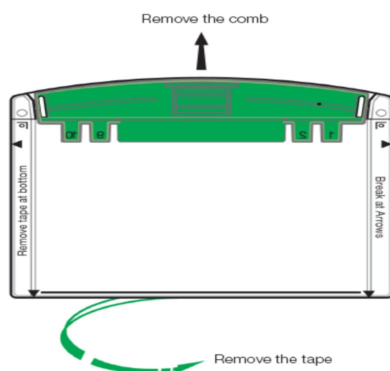
*Prepared in 1x sample buffer (Laemmli). Other proteins may be substituted.

5. The pre-stained Precision Plus protein standard **does not** need to be boiled. You will only need 10 ul and the standard can be loaded directly onto the gel. Load **2 lanes**, 10ul in each lane

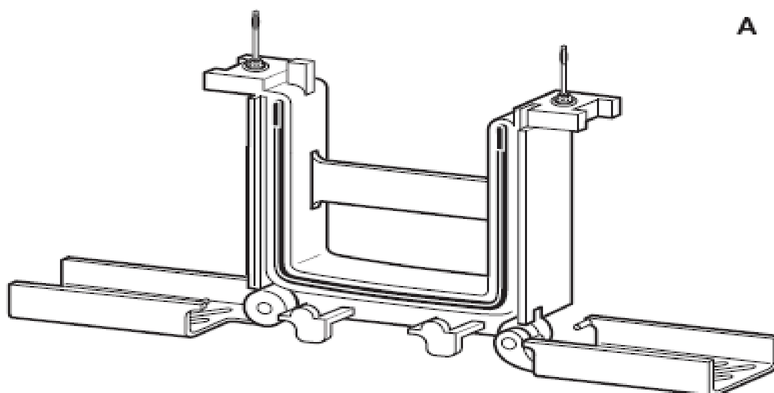
6. Boil the proteins (Table 1) for approximately 5 minutes using a floating rack in a boiling beaker on the hot plate. This will denature the proteins.

Assembling the gel apparatus:

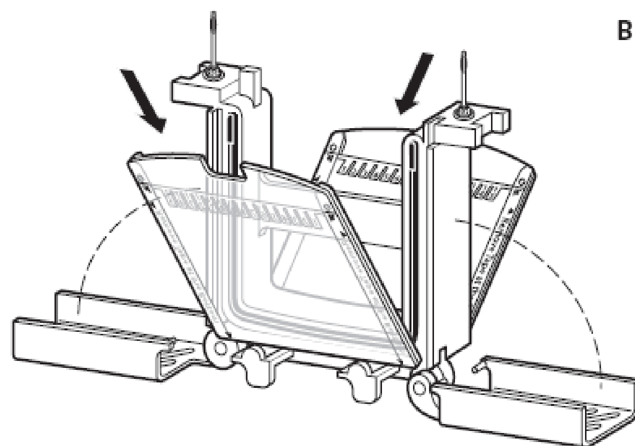
1. Remove the gels from the pouch. Remove the tape at the bottom. Slide the comb out by GENTLY pulling up in one smooth motion. Be careful, since if you damage the stacking gel in this step, those lanes become useless. Rinse the wells with running buffer (1x made as above) using a Pasteur pipette 4 times.



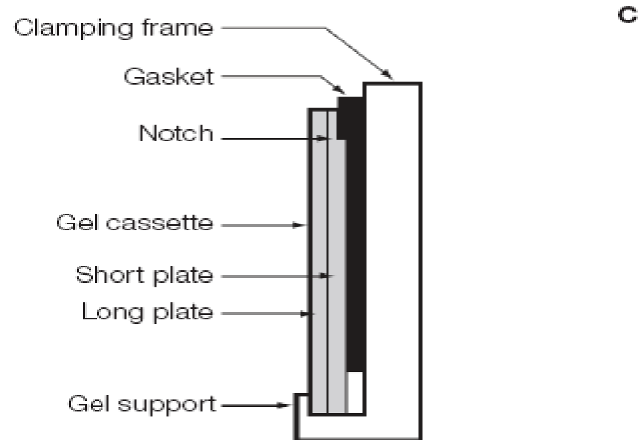
2. Set the gel holder to the open position on a clean, flat surface.



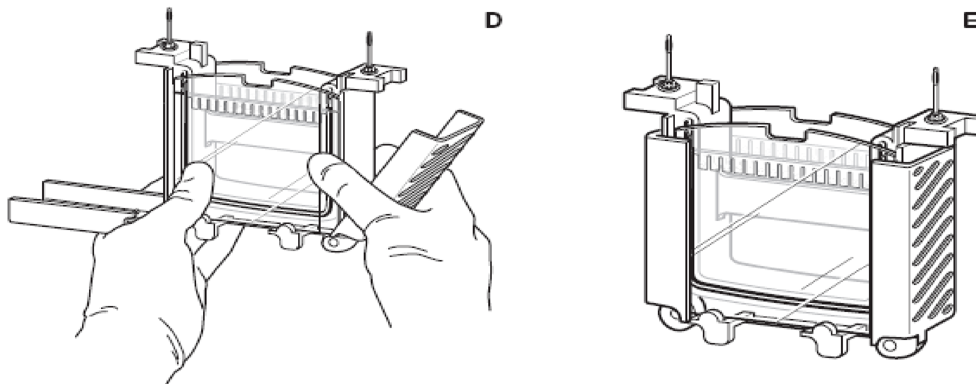
3. Place the cassettes with the wells of the gel facing inward. If using only one gel, the buffer dam will replace the second cassette. Make sure the assembly remains balanced and does not tip over. The cassettes will rest at a 30 degree angle.


B

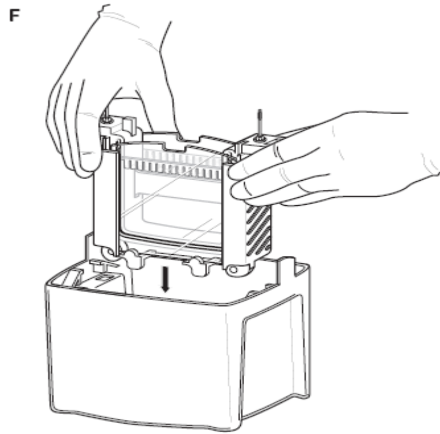
4. Gently push the cassettes together, making sure they rest firmly and squarely against the green gasket at the bottom of the assembly. Align the short plates and check that they align just below the notch at the top of the green gasket.



5. Maintain gentle pressure on each gel cassette. Slide the green arms of the clamping frame one at time over the gels, locking them in place. The wing clamps lift the gel cassettes into the notch, forming a tight seal. Check to ensure everything is square and sitting flat, then pour a small amount of running buffer into the inner chamber to ensure it is not leaking.



6. Place the assembly inside the module. Then fill the outer chamber with the rest of the running buffer, approximately to the line indicating 2 gels.



Loading samples and running the gel:

1. Load 20 ul of denatured protein samples into each well (except standards – use only 10 ul) using reverse pipetting technique and the gel-loading tips provided. Make sure you arrange your samples so that you will be able to tell protein location should the gel get turned a different way while being processed. You may want to load the standard in lanes 1 and 10, and dedicate 2 lanes to each protein.

2. Place the lid onto the gel box. It will snap into place if done correctly

3. Connect the electrodes to the power supply and switch power on. Set the voltage to 150, then press run. There should be a steady stream of air bubbles coming from the solution, if not there is little or no conductivity. Observe the dye front to make sure it begins to migrate. **It should be relatively straight across all the lanes. If not, consult the instructor within 5 minutes of starting the run.**

Removing the cassette and staining the gel:

4 Once the dye front reaches the bottom (after approximately 45-60 minutes), press Stop. Turn the power supply off and disconnect the electrodes. Remove the lid from the apparatus and remove the gel cassette from the assembly.

5 To open the cassette, align the arrow on the opening lever with the arrow on the cassette. Apply downward pressure and the cassette will snap apart.

6 Pull the two plates apart from the top, and gently remove the gel and place in staining reservoir. **BE CAREFUL IT IS REALLY EASY TO RIP THE GEL.** There is a lip at the bottom which is thicker and can be used to lift off if necessary. The gel can be dislodged around the edges by carefully running a pipette tip around the edges between the gel and the cassette.

7 Rinse the gel 5 times with Milli-Q water. Pour in the Imperial protein stain, just enough to cover the gel and gently swirl for a few minutes. You might see bands after about 5-10 minutes, but it might take longer. Determine MW of lysozyme by comparing to standards. Leave samples in the stain overnight.

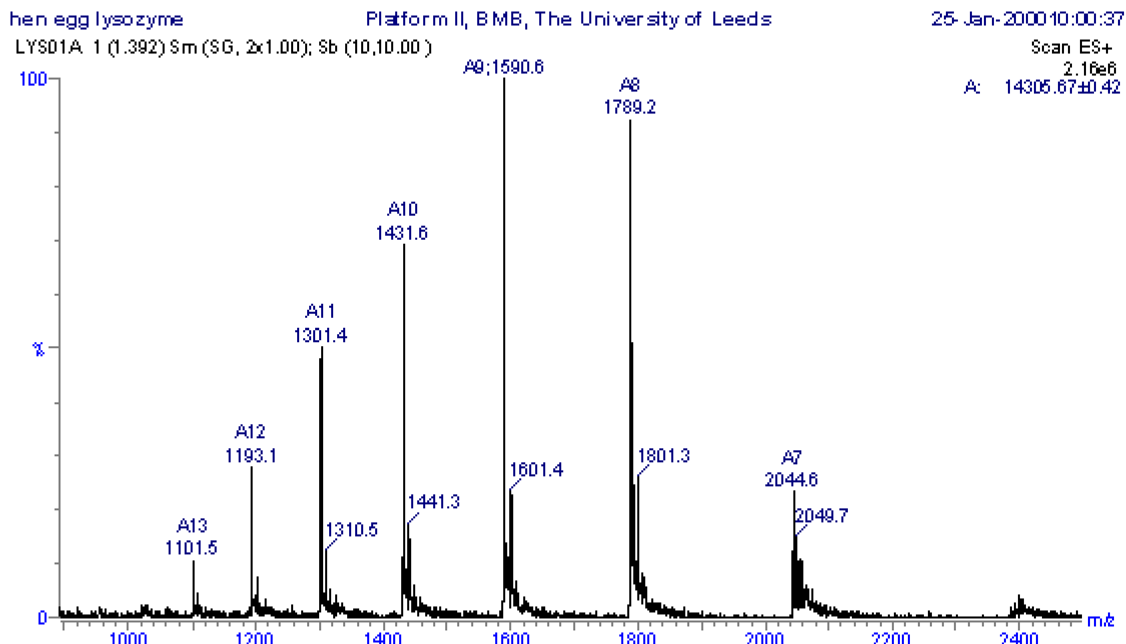
7a. Cover your gel with plastic (saran wrap) and label with your lab slot and names. The lab instructor will decant stain the next morning and replace with Milli-Q water periodically throughout the next day, until the background is clear. A picture will be e-mailed to you, so be sure you leave an e-mail address.

Mass Determination of Lysozyme using LC-MS-TOF

Data will be given to students of LC-MS analysis. The procedure for obtaining data will be part of student lab. Report.

Electrospray MS: Pure lysozyme (Sigma) is analyzed in a solution of 1:1 (*v/v*) acetonitrile to 0.1% aqueous formic acid, diluted to 1 ug/ml and injected into the MS by use of the syringe pump. The instrument is used in full scan, positive mode with a wide scan range of 1000 to 3000 Da.

Samples with molecular weights greater than 1200 Da give rise to multiply charged molecular-related ions such as $(M+nH)^{n+}$ **in positive ionization mode** and $(M-nH)^{n-}$ **in negative ionization mode**. Proteins have many suitable sites for protonation as all the backbone nitrogen atoms could be protonated theoretically, as well as certain amino acid side chains such as lysine and arginine which contain primary amine functionalities. An example of multiple charging, which is practically unique to electrospray ionization, is presented in the positive ionization m/z spectrum of the protein hen egg white lysozyme.



Positive ESI-MS m/z spectrum of the protien hen egg white lysozyme.

The m/z spectrum shows a Gaussian-type distribution of multiply charged ions ranging from m/z 1101.5 to 2044.6. Each peak represents the intact protein molecule carrying a different number of charges (protons). The individual peaks in the multiply charged series become closer together at lower m/z values and because the molecular weight is the same for all the peaks, those with more charges appear at lower m/z values than do those with fewer charges (M. Mann, C. K. Meng, J. B. Fenn, *Anal. Chem.*, 1989, **61**, 1702). The chromatogram and explanation are from an article by Dr. Alison E. Ashcroft, Mass Spectrometry Facility Manager, Astbury Centre for Structural Molecular Biology, Astbury Building, The University of Leeds. (http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm)

The m/z values can be expressed as follows:

$m/z = (MW + nH^+)/n$

where:

m/z = the mass-to-charge ratio marked on the abscissa of the spectrum
MW = the molecular mass of the sample
n = the integer number of charges on the ions
H = the mass of a proton = 1.008 Da

If the number of charges on an ion is known, then it is simply a matter of reading the m/z value from the spectrum and solving the above equation to determine the molecular weight of the sample. Usually the number of charges is not known but can be calculated if the assumption is made that any two adjacent members in the series of multiply charged ions differ by one charge.

For example, if the ions appearing at m/z 1431.6 in the lysozyme spectrum have "n" charges, then the ions at m/z 1301.4 will have "n+1" charges, and the above equation can be written again for these two ions:

$$1431.6 = (MW + nH^+)/n \text{ and } 1301.4 = [MW + (n+1)H^+]/(n+1)$$

These simultaneous equations can be rearranged to exclude the MW term:

$$n(1431.6) - nH^+ = (n+1)1301.4 - (n+1)H^+$$
and so:
$$n(1431.6) = n(1301.4) + 1301.4 - H^+$$
therefore:
$$n(1431.6 - 1301.4) = 1301.4 - H^+$$
and so:
$$n = (1301.4 - H^+) / (1431.6 - 1301.4)$$

hence the number of charges on the ions at m/z 1431.6 = 1300.4/130.2 = 10.

Putting the value of n back into the equation:

$1431.6 = (MW + nH^+)\ n$
gives $1431.6 \times 10 = MW + (10 \times 1.008)$

and so MW = 14316 - 10.08
therefore **MW = 14305.9**

You will be given instructions on the software associated with the LC-MS and get a spectrum of the lysozyme analyzed in full scan mode. Some explanation of the software will be demonstrated. Using the information of the full scan run you will determine the MW of the lysozyme sample and compare this with the MW determined by electrophoresis.

Questions:

1. What are the components of the gel in this gel electrophoresis experiment?
2. Why do proteins separate in gel electrophoresis?
3. What procedure would have been necessary had lysozyme been analyzed using MALDI instead of ESI? Why?
4. What are some advantages of the TOF molecular weight determination?