PBC Day 4 lecture

I. Today in lab
   a. Set up gel box TA demo
   b. Prep samples
   c. Load and run gel
   d. While gel is running, run Bradford (Bio-Rad) Assay on protein samples.
   e. Stop gel, stain with Coomasie
   f. Destain gel
   g. Dry gel (done by Grad TA s)

II. SDS-PAGE
   a. Gel Electrophoresis (GE in SDS-PAGE)
      i. Separates macromolecules in a sample (DNA, RNA, proteins) by forcing them through a porous matrix via an electrical current. What other technique have we used that separates thing by pushing them through a porous matrix? Answer: Gel filtration (PD-10 column).
         1. Electrophoresis uses a current across a voltage differential to move charged molecules.
         2. PD-10 just used lots and lots of buffer and bad gravity.
      ii. Typical Porous Matricies
         1. Agarose this will be used in RDM
         2. Polyacrylamide (PA in SDS-PAGE) used in PBC. Matrix formed by polymerizing acrylamide and bis-acrylamide. Concentration of acrylamide and acrylamide:bis-acrylamide ratio control pore size. Increase [acrylamide] equals smaller pores. Higher acrylamide:bis-acrylamide equals larger pores.
      iii. Acrylamide gels can be used to separate nucleic acids (DNA, RNA) or proteins.
         1. DNA have constant charge/mass ratio each nucleotide contains a phosphate group and therefore a negative charge.
         2. Protein doesn t not have a constant charge/mass ratio. Additionally protein tertiary structure does not allow us to correlate volume of protein to length of it polypeptide chains.
         3. Consequentially, we cannot accurately separate native proteins based on size.
      iv. Sodium Dodecacyl Sulfate (SDS in SDS PAGE)
         1. Aliphatic carbon tail with charged sulfate group strong ionic detergent.
         2. Denatures proteins. How? Stabilizes hydrophobic groups, which disrupts the hydrophobic effect, which helps maintain protein tertiary structure. Fixes the folding problem.
         3. Binds in a 1:2 ratio with peptide bonds in protein overwhelming the native charge of the protein. Gives protein constant charge/mass ratio as with DNA. Protein now separates by number of residues in polypeptide chain (molecular weight).
      v. Other components of sample prep buffer
         1. Glycerol causes sample to sink to the bottom of the well (think of into lab).
         2. β-mercaptoethanol (BME) strong reducing agent. Obliterates disulfide bridges holding protein together.
         3. Brophenol Blue loading dye Makes sample easy to see while loading. Also, runs faster than proteins in the sample, and indicates when to stop running gel.
         4. Tris pH buffer
         5. Heat helps in protein denaturation
      vi. Laemelli SDS-PAGE gives greater resolution of protein bands in gel reduces amount of overlap in protein bands.
         1. Uses two gels
            a. Stacking gel 4-5% acrylamide, pH 6.8. Pores large enough to not significantly impede protein movement and cause separation of bands.
            b. Separating gel 7.5% acrylamide, pH 8.8. Pores small enough to separate proteins.
         2. Running Buffer
            a. Glycine Amino acid. Has isoelectric point just below pH 6.8.
            b. Cl- ions in copious amounts.
            c. Tris pH buffer

3. How a stacking gel stacks.
    a. At pH 6.8, glycine ions, being only slightly negatively charged overall, migrate slowly through stacking gel and impede the movement of the protein. Cl- smooshes in protein from behind giving a narrow band of protein.
    b. At pH 8.8, glycine has about _ of a negative charge overall (being close to the pKa of the amino group) the same as an amino acid residue in a protein. Because it is merely a single amino acid, it will run much much fast through the gel than the proteins in the sample which are composed of multiple proteins (Gets out of the way). Protein now free to separate out as normal.

vii. How do we see our protein bands
1. Use Coomasie Blue binds non-specifically to negative and hydrophobic residues in protein.
2. Turns protein bands blue color allowing them to be visually monitored.

viii. RM-relative mobility
1. rM=(distance of protein band migration)/(distance of dye migration)
2. rM is directly proportional to log(MW of protein)
3. Compare rM of protein to rM of standard proteins of known MW to find RM of protein of interest.

III. Bio Rad assay
- a. Quantitative Coomasie staining. Quick and dirty way to determing protein concentration of sample.
- b. Uses Coomasie and phosphoric acid
    - i. Coomasie is protonated brown color
    - ii. When Coomasie binds to protein becomes deprotonated and turns blue.
    - iii. Deprotonated form also abosorbs at 595nm colorometirc assay for protein concentration. (Compare to β-gal assay). Reaction theoretically stops after about 5 minutes when all Coomasie binding sites on protein are saturated color stable for about 30 minutes.
- c. Use protein concentration in specific activity calculations.
    - i. Specific activity (S.A.) = (Units of β-gal activity) / mg protein.
    - ii. A high specific activity indicates a high amount of β-gal relative to other proteins in the sample thus purer β -gal.