Announcements

- Quiz on Chapter 5a today, Chapter 5b and 5c quiz next week
- Rest of semester schedule:

	Discussion Dates	Lab Dates	Lab Due Dates
Chapter 5a		Mon. 11/5	
Chapter 5b	11/5 – 11/7	11/7 – 11/12	
Chapter 5c	11/12 – 11/14	11/14 – 11/19	11/28 – 12/3 in lab
Chapter 6a	11/26 – 11/28	11/28 – 12/3	
Chapter 6b	12/3 – 12/5	12/5 – 12/10	12/12, All Sections!
Lab Exam	12/10 – 12/12		

Chapter 5: Structural Characterization of LDH

Purpose:

Learn how to:

1) Pour an SDS Polyacrylamide gel

2) Prepare samples for SDS-PAGE

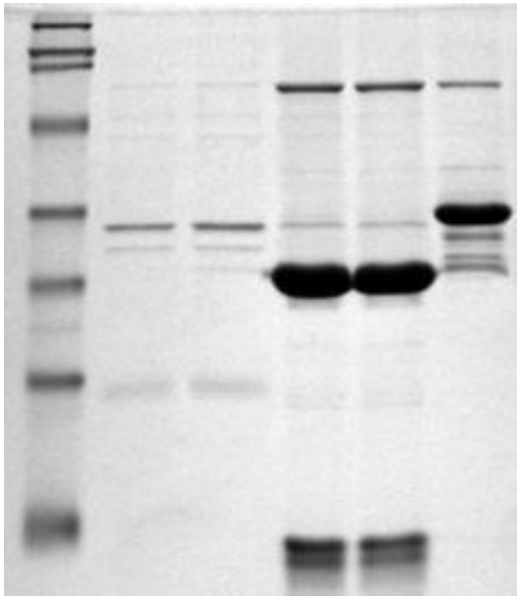
3) Determine purity and subunit MW of your LDH

Chapter 5: Overview

- **Week 1: Gel Filtration Chromatography**
  - Separate LDH from standard proteins by native size
  - Determine Native MW
- **Week 2: Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)**
  - Separate LDH from other proteins by subunit size
  - Determine Subunit MW
- **Week 3: Native Electrophoresis (Zymograms)**
  - Confirm the quaternary structure of LDH from Weeks 1 & 2

Separating Proteins by Electrophoresis

- **Applied electric field separates proteins**
- **Many factors effect rate:**
  - Isoelectric points
  - Titration curves
  - Molecular weight
  - Hydrodynamic properties



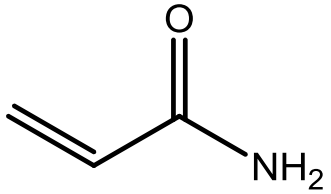
- **Native Electrophoresis**
  - Separation of full protein by size and charge
  - Maintains tertiary and quaternary structure
- **Denaturing Electrophoresis**
  - Denature of protein into subunits
  - Separation of those denatured subunits by size
- **2D Electrophoresis**
  - Separation of protein by pI in first direction
  - Separation by size in second direction

Polyacrylamide Gels

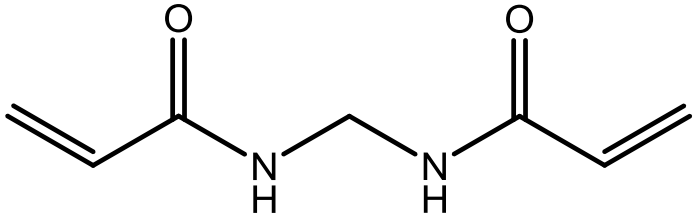
- Matrix for separation
- Acts as homogeneous support to prevent diffusion of proteins out of gel
- Acts as **molecular sieve** slowing the migration of proteins *in proportion to their charge-to-mass ratio*
- Concentration can be varied to separate wide range of proteins
- Speed of separation is related to **electrophoretic mobility**

Polyacrylamide Gel Formation

Acrylamide (monomer)



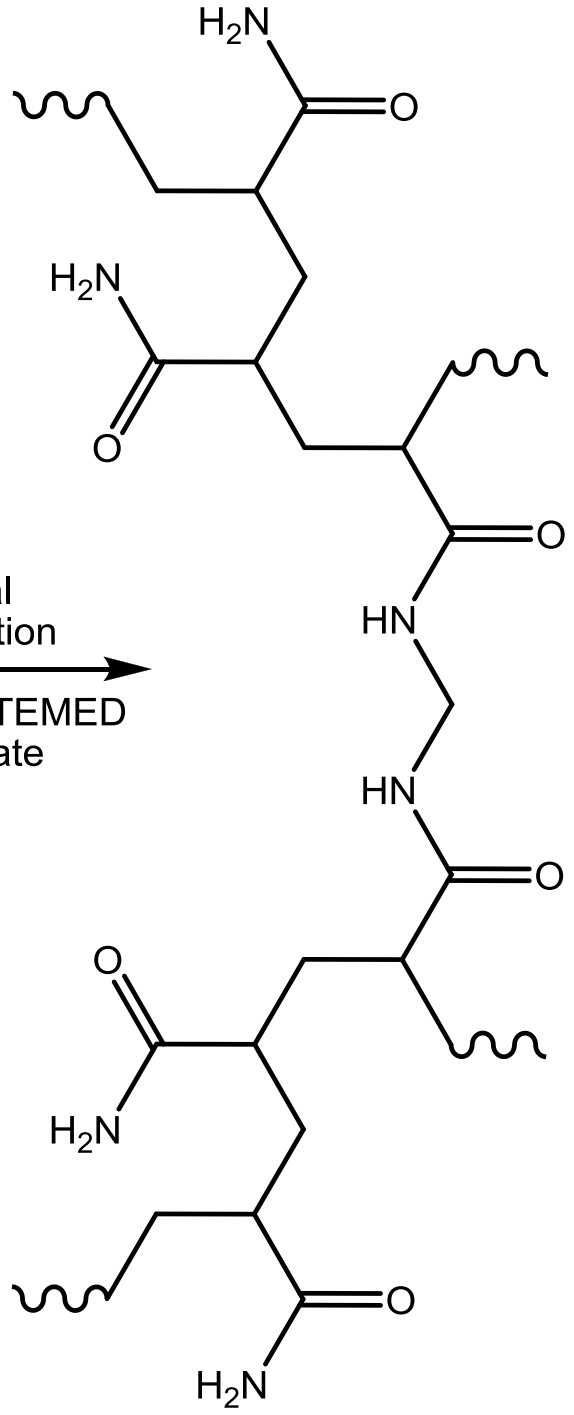
+



N,N'-Methylene-Bis-acrylamine (crosslinker)

Free Radical Chain Reaction

Initiated by TEMED and Persulfate



Polyacrylamide Gel Composition

Need to remove any un-polymerized monomer – it can react with protein functional groups!

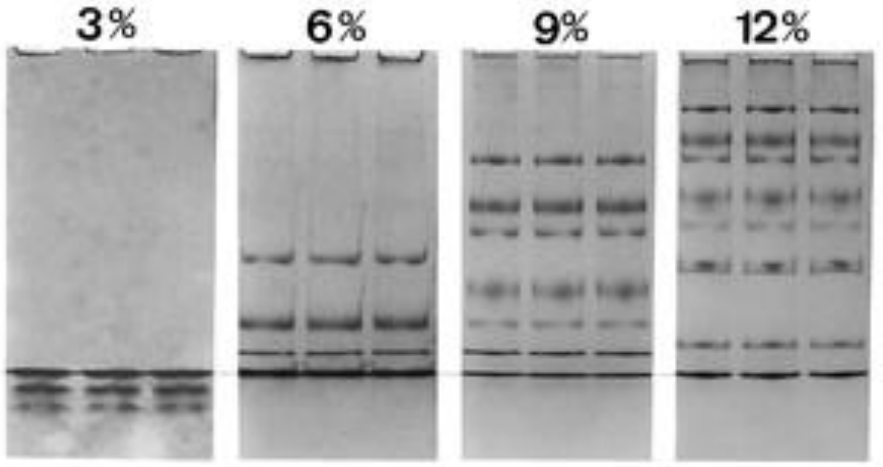
Denaturing Polyacrylamide Gel Electrophoresis – SDS-PAGE

- Method to separate proteins by molecular weight of denatured subunits
- Treat protein with strong denaturant (SDS) and sulfhydryl compound (βME or DTT)
  - Unfolds polypeptide chains into random coils
  - Coats protein surface to give uniform negative charge
  - Prevents disulfide bonds from forming between subunits
- Denatured subunits can be permanently separated with SDS containing buffers
  - Large excess of SDS
  - SDS binds to any protein at 1.4 g SDS/g of protein
    - Gives all proteins same charge/mass ratio

SDS-PAGE

- Discontinuous (Disc) electrophoresis
  - Layers of gel for better separation based on pH changes (p. 165-8)
- **Running Gel** – bottom layer, high pH (8.8), higher % acrylamide
- **Stacking Gel** – top layer, lower pH (6.8), lower % acrylamide, where the wells are located
- Stacking layer for loading samples & stacking condition, running layer for separation

- Different acrylamide percentages in gel affect separation



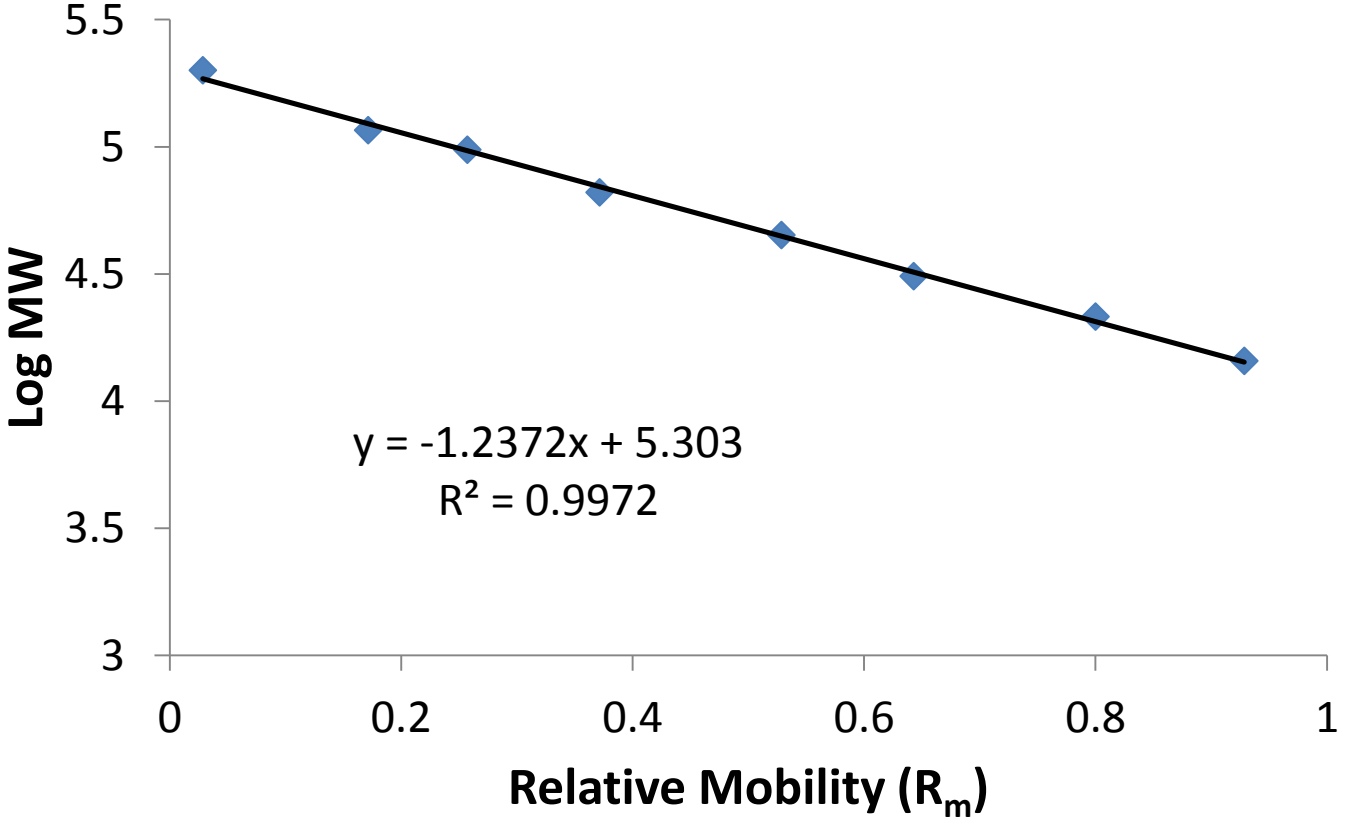
- **Higher % gel** – better separation of small proteins
- **Lower % gel** – better separation of large proteins

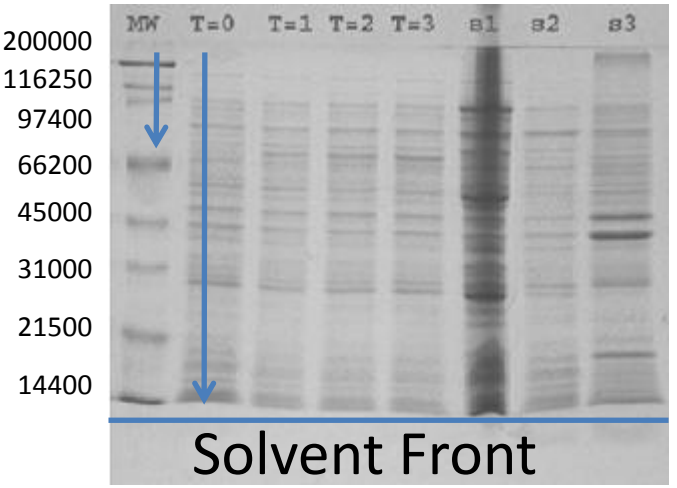
Relative Mobility

Linear relationship between mobility of a protein and Log MW

- Measure distance from top of gel to solvent front
- Measure distance from top of gel to protein band interest

R_m = Distance to protein band on interest/ Distance to Solvent Front





Protein Standards used in Lab 5 on p. 156 of Lab Manual

Procedure: Chapter 5-Week 2

- Prepare Samples
- Assemble Gel Apparatus
- Load Samples
- Run Gel
- Pour Gels (for the next lab section)
- Staining and Visualization

Procedure: Chapter 5-Week 2

- **Prepare Samples**
  - Crude Extract – 30-50 µg
  - 3P-Dialyzed – 15-30 µg
  - Purified LDH – 2-5 µg
  - SDS-PAGE Standards –
    5 µl aliquot, ready to load, 1 per gel
- Total Sample Volume = 20 µl
  - 2 µl 10X Sample Buffer (thick blue liquid)
  - Up to 18 µl of sample to put at the appropriate concentration of protein
  - Water to bring total volume to 20 µl
- Denature samples 1-2 min at ~ 90°C

❖Use concentration from Dye-Binding from Chapter 3!

❖Calculate volumes before you come to the lab!

Standards:
Bio-Rad Broad Range
See Table p. 156
Marked with ¶ Symbol

Procedure: Chapter 5-Week 2

- **Assemble Gel Apparatus:**
  - 2 groups/gel, 2 gels/gel box
  - Pictures p. 135-136 and demo!
- **Load Samples:**
  - Use gel loading tips
  - Yellow loading guides can help line up wells
  - Write down your loading scheme!
- **Run Gel:**
  - 50 V until through the stacking gel, 200V after that
  - Run until blue tracking dye reaches bottom of gel
  - Total time ~ 1 hr

Procedure: Chapter 5-Week 2

- **Pour Gels:**
  - Put together short plate and spacer plate (1.5 mm)
  - Lock together with casting clamp and stand (pictures p. 133)
  - Check plates for leaks with water
  - Prepare Running Gel – Recipe p. 134 **(100 µl APS)**
    - Pour or pipet into plate, save extra and see when it polymerizes
    - Overlay with isobutanol
  - Prepare Stacking Gel – Recipe p. 134 **(40 µl APS)**
    - Pour or pipet into plate, save extra and see when it polymerizes
    - Insert comb to form wells
  - Put poured gel in buffer at 4°C for the next section

Procedure: Chapter 5-Week 2

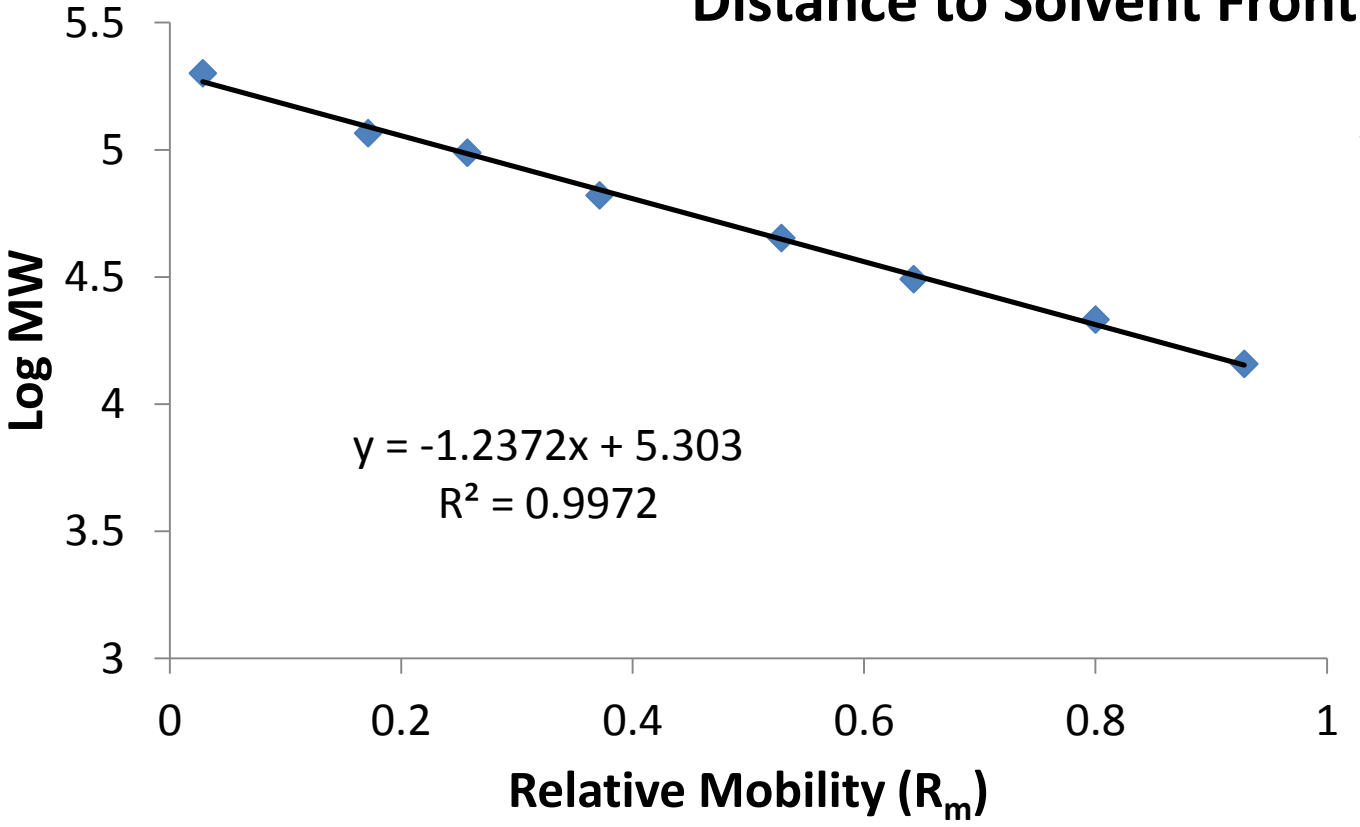
- **Staining and Visualization:**
  - Remove gel from plates
  - Nick a corner so you know the orientation
  - Stain with Coomassie Brilliant Blue R-250
  - Destain with 1:5:5 Acetic Acid/Methanol/Water solution
  - Image on gel doc
  - Calculate R_m values for standards and unknown protein bands
  - Make graph of Log MW vs R_m

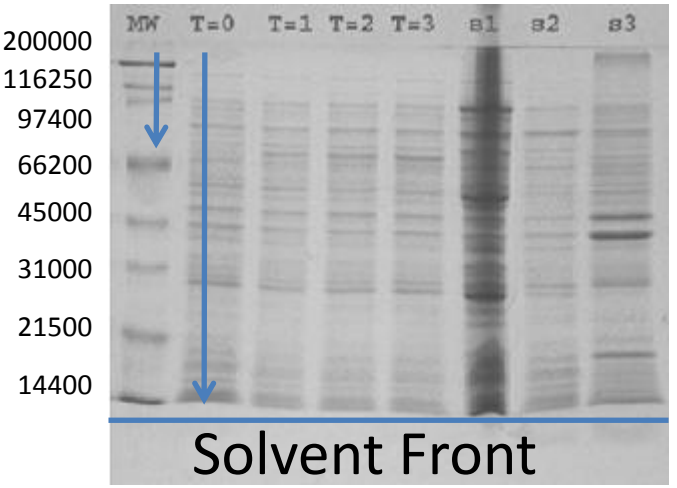
Calculate R_m Values
Make Plot of Log MW vs. R_m

Linear relationship between mobility of a protein and Log MW

- Measure distance from top of gel to solvent front
- Measure distance from top of gel to protein band interest

R_m = Distance to protein band on interest/ Distance to Solvent Front





Protein Standards used in Lab 5 on p. 156 of Lab Manual