Announcements

- Chapter 7 Laboratory Reports due by end of day (11:59 PM) today and tomorrow.
- Scintillation counting data have been put on SLACK for B5, B6, & B7. Data for B1, B2, & B3 will be posted by end of the day this Wed. (2/26).
- METABOLISM PROJECT – PART 1 is due by end of the day on Wed. (2/26)
  - Meet and discuss everyone's ideas and come up with a list of top choices.
  - Make a document that lists those as well as the ideas of everyone in the Group
  - NAME the document with your Group Number: "Group # xxxx" and put it in a shared Google folder, sharing it with everyone in the group and Dr. Tolan
  - Send email to Dr. Tolan by the due date a link to the folder or file.

Chapter 8C: In vitro transcription and translation

Objectives

- To learn how to cast and run SDS-PAGE gels.
- To analyze and observe the synthesized proteins by autoradiography.

Procedures

- Run **SDS-PAGE gels** to ***analyze synthesized proteins***
- Cast **SDS-PAGE gels** (running and stacking gel layers)
- Use **autoradiography** to ***observe synthesized proteins***

Transcription and Protein Synthesis

Week 1: Add DNA template and perform transcription/translation using TNT® Wheat Germ Extract.

TCA precipitation

Measure amount of radioactive methionine incorporated into proteins by Scintillation counting.

Week 2: Separate translation products by SDS-PAGE

Dry gel

Autoradiography

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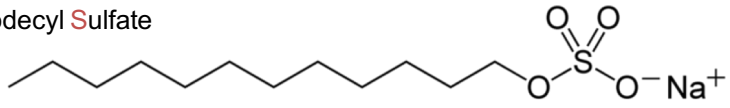
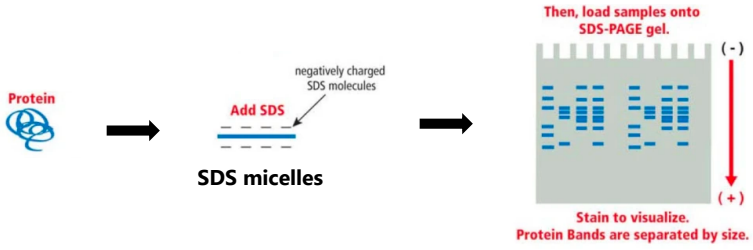
REMINDER: Recall in 421 (Chapter 5)

SDS-PAGE: Denaturing Poly-Acrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate





All proteins will have **same mass/charge ratio**

Proteins are separated based only on **size**

(Image: Edvotek®)

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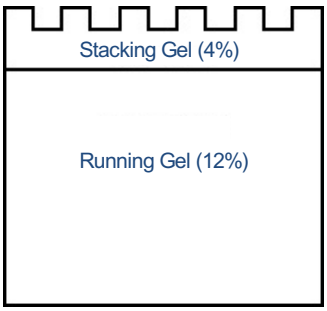
WHY TWO GELS?

Discontinuous Electrophoresis

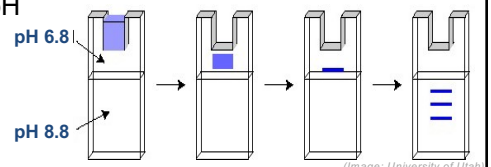
- Two gel layers
  - 1) first for stacking
  - 2) second for separation/sieving

  Each has a different - **pH, pore size**
  - *(See pp. 175-180)*
- **Stacking Gel** – top layer; lower pH (6.8) & lower acrylamide percentage (4-5%)
  - Proteins "stack" into a tight bands due to a voltage gradient set up by glycine in the running buffer undergoing a change in mobility due to the pH changes
- **Running Gel** – bottom layer; high pH (8.8), higher acrylamide percentage
  - proteins resolved based on by size due to "Sieving" effect

Whole gel:



Single well:

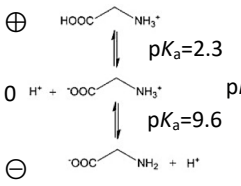


(Image: University of Utah)

Laemmli, UK (1970) *Nature* **227**:680-685.

How does discontinuous SDS-PAGE work?

Glycine

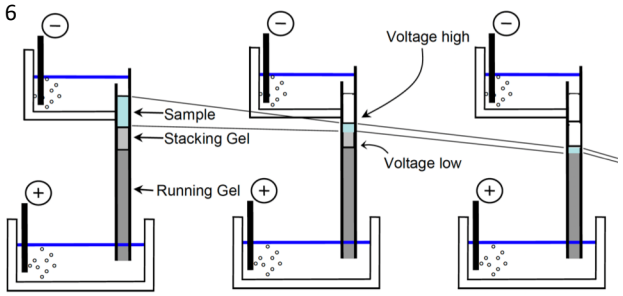


Net charge at 6.8: only very slightly negative (–0.0015)

Net charge at 8.8: –0.15

Stacking gel pH 6.8

Running gel pH 8.8



(slower) **Glycine < Proteins < Dye < Chloride** (faster)

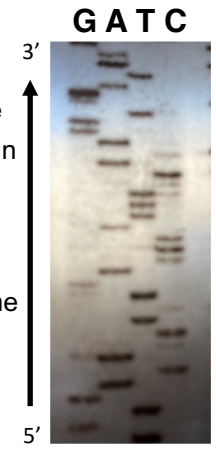
(slower) **Proteins < Glycine < Dye < Chloride** (faster)

Autoradiography

- Technique for determining location of radio-labeled molecules in tissue, chromatograms, and gels
- Allows determination of location of radiation in a sample
- Works by reacting disintegration of a radioactive nuclei in the sample reacting with silver in the X-ray film:

$$\beta^- + AgBr_{(s)} \rightarrow Ag_{(s)} + Br^-$$

- Film is exposed to radioactive sample for a period of time and then developed similar to film photography
- Darker areas of film indicate location of radioactivity in original sample



Fine-grained metal that appears black

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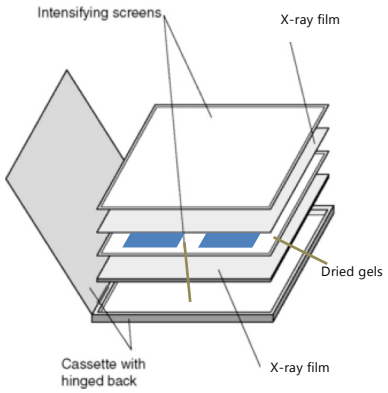
Autoradiography

- Resolution can be improved by:
  - ➢ Decreasing the thickness of a sample
    - ❖ Drying your gel
  - ➢ Decreasing the distance between the film and sample
    - ❖ Clamping the gels and film into a cassette to expose film for several days
- Weak β-emitters typically can only expose 1 side of film
  - ➢ Enhance the signal with **fluorography**
    - ❖ Use fluorescent compound with radioactivity, similar to scintillation counting
  - ➢ Use **intensifying screen**
    - ❖ Fluorescent material placed over film to sandwich sample inside film



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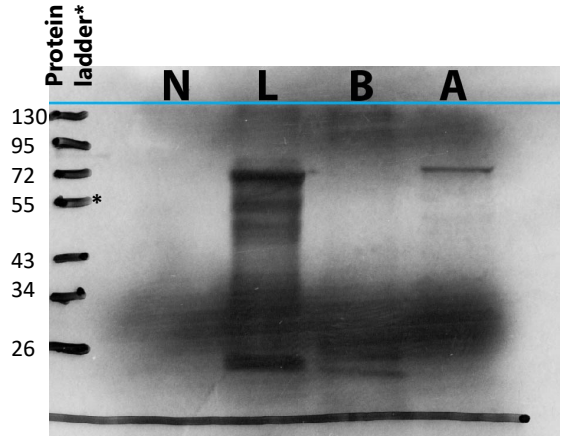

Week-1 cpm counts ***Lane samples***

1,740	N = nuclease-free water (negative control)
23,690	L = SP6-Luciferase (positive control)
2,303	B = Plasmid A
3,650	A = Plasmid B

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Chapter 8: Procedure

- You will be working with radioactive materials
  - Wear plastic aprons and double glove
  - Work on bench coat paper marked with radiation tape
  - Your TFs need to check you and your workspace for radioactivity **BEFORE** you can leave the lab
  - Special waste disposal
    - SDS-PAGE Buffer in radioactive liquid sink
    - Separate disposal of all solids
    - Gel-drying solution (due to methanol)
  - Be very careful with the gel loading tips! They don't have filters in them to prevent radioactivity going into the barrel

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Chapter 8: Procedure

- Scintillation Counting (previous lab):
  - Get results of scintillation counting from week 1
  - Percentage incorporation: 4

$$\text{percent incorporation} = \left(\frac{\text{"60-min precipitated filter CPM"} \times 12.5}{\text{"0-min direct spot CPM"} \times 200 \times 24.5}\right) \times 100\%$$

This is the total ^{35}S incorporated for EACH reaction: get 4 values

This is the total ^{35}S in your reaction

  - Fold stimulation over background: 3

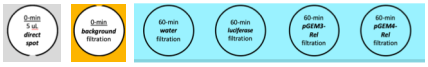
$$\text{fold stimulation} = \left(\frac{\text{"60-min } DNA \text{ precipitated filter CPM"} - \text{"0-min precipitated filter CPM"}}{\text{"60-min } water \text{ precipitated filter CPM"} - \text{"0-min precipitated filter CPM"}}\right) \text{"-fold"}$$

This is the total ^{35}S incorporated for EACH reaction: get 3 values

This is the background ^{35}S after any reaction

This is the background ^{35}S before any reaction

The fold-stimulation calculation are all based on a 2 µL aliquot, so no corrections for volumes needed



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Sample Preparation for SDS-PAGE

- Based on your scintillation counts from Week 1, calculate volume needed for protein gel samples ***before arriving to lab!***
- You will load ">5,000 cpm" but with reactions this year we will try for **75,000 CPM** for each sample
- Sample calculation:

60 min-time point scintillation count of positive control: 25,000 CPM

Therefore, the CPM/µL concentration of sample = $\frac{\text{Scintillation count}}{2\ \mu L}$ ← 2 µL sampling for TCA precipitation

$= \frac{25{,}000\ \text{CPM}}{2\ \mu L} = 12{,}500\ \text{CPM}/\mu L$

Volume to add = $\frac{75{,}000\ \text{CPM}}{\text{Concentration}} = \frac{75{,}000\ \text{CPM}}{12{,}500\ \text{CPM}/\mu L} = 6\ \mu L$

- You could make this into a Table since you have to do the same calc. for all 4 samples
- If you don't have sufficient sample for >50K CPM (<4700 cpm/µL), then use the entire sample that you have (10.5 µL)

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Sample preparation for SDS-PAGE

- Adjust each sample to 21 µL using diH_2O
- Add in 3 µL of 8X Sample Buffer
- Denature samples at 80 °C for 5 min on heat block

Sample calculation:

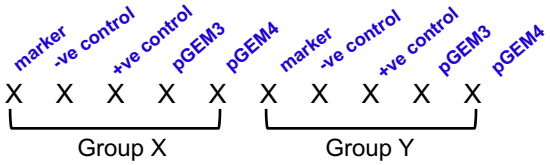
Reagent	Volume
75,000 CPM positive control	6 µL
diH_2O	15 µL
TOTAL Sample volume	**21 µL**
8 X Sample Buffer	3 µL
TOTAL Loading volume	**24 µL**

Do this calculation for all FOUR samples

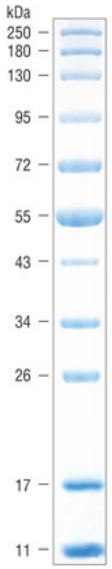
Chapter 8: Procedure

Gel Loading and Running:

- 2 groups per gel!
- NEB ladder is ready to use, no need to denature
- Load 3 µL of ladder
- Use gel loading tips found at the reagents bench
- Record your gel loading scheme in notebook



Page ***267*** in Appendix of lab manual:
M_r of luciferase enzyme is 61 kDa
M_r of REL protein is about 65 kDa



NEB pre-stained broad range ladder (P7718S)

Chapter 8: Procedure

Run Gel

- ***Running gel:*** 75 V through stacking gel,150 V ***until dye reaches approximately 2/3 of the gel***
- Remove gel and nick a corner so you can tell how it was loaded later (!)
- Dispose of running buffer in radioactive waste disposal sink

Casting of Gels

- While your gel is running, prepare new gels for the next class:
  - 0.75 mm-thick Running gel **p. 252**
  - 0.75 mm-thick Stacking gel **p. 253**
- **Turn your newly prepared SDS-PAGE gel to TF for approval!**

Chapter 8: Procedure

- Note the change in amount of TEMED and APS to add is changed in the Tables, but not the text (ignore text)! (Add these last!)
- After you add the running gel solution, layer some isopropanol on top.
- Once running gel layer have solidified, rinse off isopropanol layer with water.
- Add stacking gel layer and a 10-well comb.

Table VII
Running Gel Solutions

Stock Solution	Volume per gel (mL)
1.0 M Tris-HCl, pH 8.8	1.9
40 % (w/v) acrylamide, 1.5% (w/v) bisacrylamide	1.5
10 % (w/v) SDS	0.05
Water	1.5
Ammonium persulfate, 100 mg/mL (10% w/v)	0.05
TEMED (tetramethylenediamine)	0.0035
TOTAL	5.0

Table VIII
Stacking Gel Solutions

Stock Solution	Volume per gel (mL)
1.0 M Tris-HCl, **pH 6.8**	0.63
40 % (w/v) acrylamide, 1.5% (w/v) bisacrylamide	0.56
10 % (w/v) SDS	0.05
0.5 M EDTA, pH 8.0	0.01
Water	3.695
Ammonium persulfate, 100 mg/mL (10% w/v)	0.05
TEMED (tetramethylenediamine)	0.005
TOTAL	5.00

Chapter 8: Procedure

Preparing Gel for Autoradiography

- Put gel in petri dish, wash 3x with DI water (5 min each)
  - ➢ Dispose of washes in radioactive liquids sink
- Wash 1x with gel-drying solution (30% MeOH, 5% glycerol), 10 min
  - ➢ Dispose in special waste for radioactive drying solution
- Bring washed gel to TFs, stretch gel on drying film
- Clamp together all gels between 2 sheets of film
- Dry overnight

Chapter 8: Procedure

Preparing Gel for Autoradiography

- TFs will remove dried gels
- TFs will expose gels to two sheets of X-ray film for **about 1-2 weeks** in a Film cassette that is light-tight
- TFs will then develop film using an automatic X-ray developer, and mark location of gels, tops of gels, and location of standards on the X-ray film
- You will get pictures of X-ray film before the end of spring break
  - ➢ You need to identify size of each of the bands produced in your translated proteins

Announce Concepts Procedure Hazards Tips Clarification End

Hazards and Radioactive waste

- In addition to safety eyewear & lab coats, you must double glove and wear plastic aprons
- Everything you dispose of is considered radioactive (please pay attention when disposing items)
  - SDS-PAGE running buffer goes down only one radioactive liquids sink
  - Gel water "rinsates" goes into the specific radioactive-liquids sink
  - Plastic tubes, pipette tips, hot gloves & aprons goes into solid waste in the back radioactivity room
  - If apron and gloves are **NOT** hot they should go into the trash
- ***The gel drying solution is radioactive AND it contains methanol (can't go down the sink). We have a special liquid radioactive collection glass bottle for this!***

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Chapter 8C Lab Tips

- Always work on radioactive mats on the bench space
- No laptops, phones, calculators, or other unnecessary items should be brought to the bench space.
  - If they are hot, we will confiscate them and seal them away for 10 half-lives.

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My lab partner: **Bro, that radioactive pipette tip just touched your gloves**

Me: **I didn't feel it, it's fine**

The Geiger counter:

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Chapter 8C Pre-lab clarification

- Cpm data will be put on SLACK for each section when we have it
- The pdf will have vial numbers, and corresponding cpm
- ***Calculate and develop your SDS sample preparation strategy ahead of time!***
- Use either the 15-mL or 50-mL conical tube to prepare your running and stacking gel solution
- The SDS-PAGE sample prep uses 8X sample buffer, not 10X

Chapter 8C Post-lab clarification

- Chapter 8 Laboratory Report won't be due until week of Mar 20-25
  - *Template will be on Web site this week*
  - *Don't fall behind on the calculations*

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Chapter 8C

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on Gradescope
  - ✓ Title, purpose and procedures
  - ✓ Remember to include:
  - ✓ Properly calculated volume required for sample preparations (4)
  - ✓ Recipes for both running and stacking gels
  - ✓ Potential loading scheme

At the end of lab, you should have:

- ✓ A sketch of your SDS-PAGE gel loading scheme
- ✓ Electrophoresed samples and turn in washed gel to TF for drying overnight
- ✓ Prepared an SDS-PAGE gel for the next section and turned in to your TFs to check
  (prepared during your electrophoresis step)

Questions?

In-class activity
&
Discussion Quiz