Announcements

- What's DUE:
  - Your Chapter 6AB pre-lab write-up is due 30 min before your lab starts
  - Your Chapter 6AB post-lab write-up is due midnight the day of your lab
  - Your Chapter 7 Laboratory Report will be due midnight the day of your lab IN 2 WEEKS week (starting Feb 20).
- Postings:
  - Pre- and post-lab write-up assignments will be released on Mon. at 5:00
  - Laboratory Report assignments will be released at least a week before the first lab's due date. For example, the Chapter 7 assignment will be available before Feb. 12 in not this week.
- Feedback:
  - Discussion quizzes should be graded within a week or less
  - Pre- and post-labs should be graded by M/T for R-F and R/F for M-T
  - Attempts will be made to grade Lab Reports within a 1-2 weeks of their due date.

Announcements

- No labs next week (week starting Thursday Feb. 13 – Tuesday Feb. 18)
- Next Discussion will be Tue. Feb 18 (Monday schedule)
- Be sure to take your **Radiation Safety Training**. MUST be completed by start of your lab (week starting Thursday Feb. 20 – Tuesday Feb. 25)
- Introduction to Creativity Project:
  - Goals (today)
  - Groups (announced by Friday midnight Feb 7)
  - Due dates (Part 1 – Feb 19; Part 2 – March 19; Part 3 – May 4)

Chapter 6: Isolation of Plasmid DNA

Purpose for week 1:

A) Isolate two plasmid DNAs from *E. coli*

B) Determine DNA concentration by two methods

→ *UV absorbance (Cary-60 UV/Vis)*

→ *Gel electrophoresis (agarose gels)*

Chapter 8: Transcription/translation

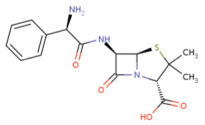
Determine between plasmids A & B which is pGEM3-Rel and which is pGEM4-Rel

- pGEM3-Rel (reverse orientation of ORF)
- pGEM4-Rel (ORF in correct orientation)

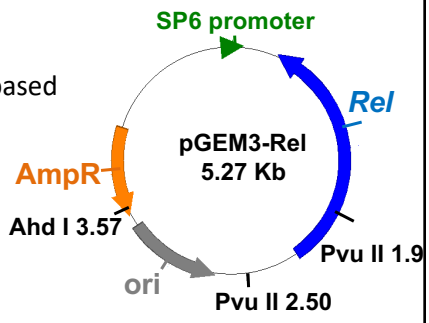
Our Plasmids

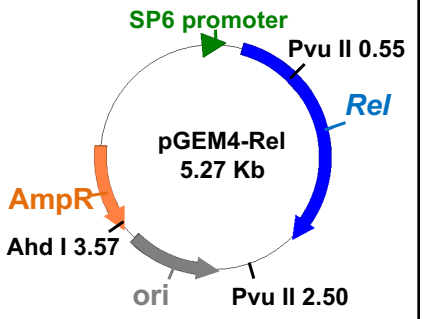
- We are isolating pGEM3- and pGEM4-based recombinant clones from *E. coli*
- Each contains:
  - REL Transcription-factor Gene
  - Phage SP6 Promoter
  - Ampicillin-resistance gene*
  - Origin of replication
- You will need to identify which of your plasmids is which.

*β-lactamase, which hydrolyzes penicillin-like antibiotics



Maps are on p. 209 of the Lab Manual





Overview of Mini-Prep for Plasmid Isolation

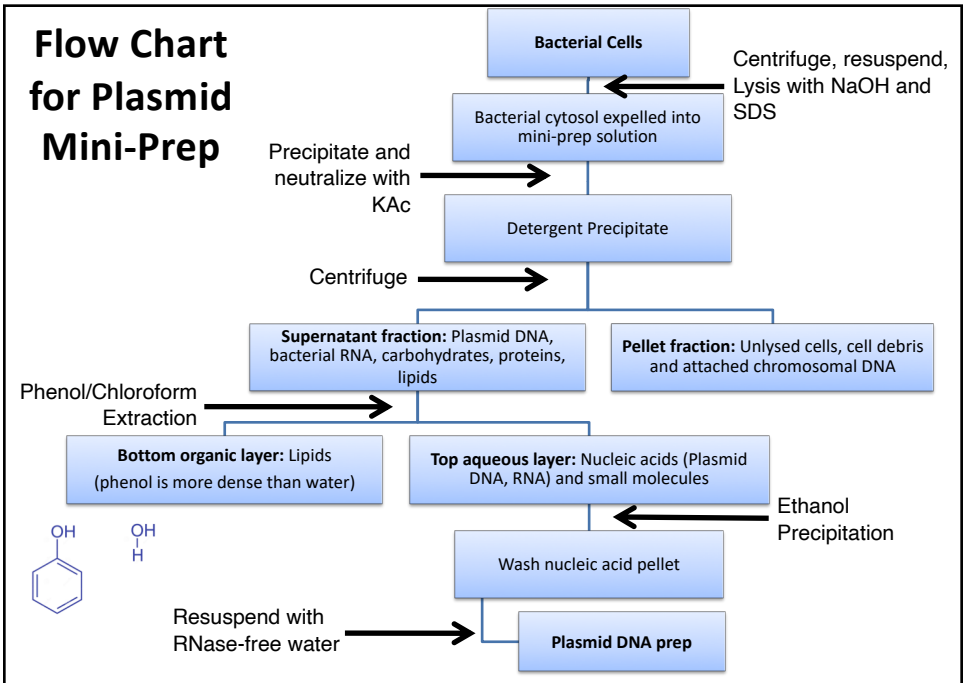
Need to separate nucleic acids from cell membranes and proteins

- **Step 1:** Cell lysis by detergents and pH: SDS and NaOH
  - Detergents will disrupt cell membrane and expels cytoplasm
  - High pH will disrupt interactions and denature macromolecules
- **Step 2:** Addition of potassium acetate: Neutralizes pH and precipitates detergents and high molecular weight impurities
  - Detergents & membrane debris will be pelleted from nucleic acids and proteins
- **Step 3:** Extraction with Phenol / Chloroform: Removes proteins and lipids
  - Separates nucleic acids in an aqueous layer from the lipids in the organic layer and denatured, precipitated proteins at the interface
- **Step 4:** Precipitation of Nucleic Acids using ethanol
  - Nucleic acids (DNA & RNA) are precipitated and recovered by centrifugation

Steps 1 – 4 will allow you to generate "mini-prep" plasmid DNA samples

- **Step 5:** Measure the concentration and calculate yield

Flow Chart for Plasmid Mini-Prep



Characterization of Plasmid Prep:
Determining the DNA Concentration

- **UV Absorbance**
  - Dilute 2 µL of plasmid mini-prep with 998 µl of TE buffer (1:500 dilution)
  - Record $A_{260\ nm}$ values from Cary-60 specs
    - Adjust concentration as necessary so that $0.01 < A_{260} < 1.0$
    - These $A_{260\ nm}$ values are by definition readings for concentration with units of O.D./mL
    - These O.D./mL values are for what was in the cuvette
      - Back-calculate to the concentration of the prep by multiplying by the dilution factor (*i.e.*, multiply by 500 for this example). Example, reading of 0.04 of 1:500 dilution is 20 OD/mL.
- **Gel electrophoresis**
  - Take another 2-7 µL (~0.1 O.D.) of plasmid mini-prep and add electrophoresis sample buffer
  - Load onto agarose gel and separate by size.
  - Stain for nucleic acids and compare to standards of known amounts in ng of DNA

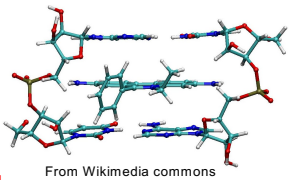
NOTE: These are different units (OD/mL and ng/µL); will need to interconvert them to compare

Agarose-Gel Electrophoresis

- Relative DNA migration rates depends on:
  - Size and conformation (supercoiled versus closed circular)
  - Concentration of agarose in the gel
  - Applied voltage
    - **Your gel will melt if it gets too hot!**
- Recall that all DNA has the same charge-to-mass ratio with a negative charge
  - Your negatively charged DNA will migrate toward the positively charged electrode ***red wire*** (***anode***)

Visualization of the Nucleic Acids

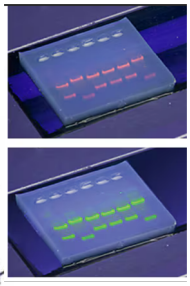
- Tradition uses Ethidium Bromide (EtBr), which is an intercalating agent into double-stranded DNA & RNA

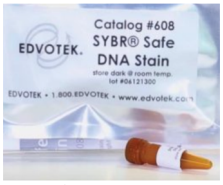


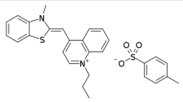
From Wikimedia commons

EtBr as it is a potential mild carcinogen; must take care in handling and use a separate waste stream

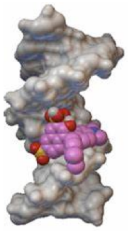
- Will fluoresce under UV light when bound to nucleic acids

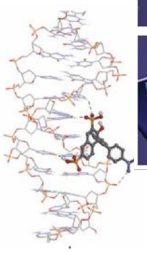






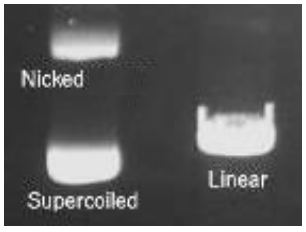
- Newer method uses **Sybr-Safe**, which doesn't intercalate, but still binds and fluoresces.





Observing Plasmid DNA

- A Syber-Safe stain is used to observe DNA
- Multiple forms of Plasmid DNA:
  - **Supercoiled circular DNA**
  - **Nicked circular DNA**
  - **Linear DNA**
  - Our system's migration pattern:
    - **Nicked circular slowest**
    - **Linear**
    - **Supercoiled fastest**



http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/supercoils.jpg

Estimating DNA Concentration from Gels

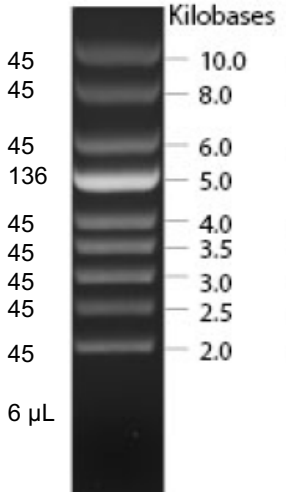
- **Agarose Gel**
  - Each gel will have markers loaded, such as:
    - Supercoiled "size ladder" – to measure **size** of supercoiled DNA sample
    - Another "concentration ladder" – to measure **mass** of supercoiled DNA sample
    - We will use a marker that does BOTH!
  - For measuring DNA concentrations:
    - Compare your sample's signal intensity (most of it should be in the super-coiled band) to a band in concentration ***ladder****
    - Estimate the mass of DNA in your sample in ng (nanograms; 10^{-9})
    - Divide your sample's mass by the ***volume of DNA*** loaded in µL
    - For example: 100 ng of estimated mass divided by, let's say you used 5 µL of DNA prep, = 20 ng/µL

Look at marker tables in p. 196 or 207

*Instructors will tell you the mass in each band per µL loaded

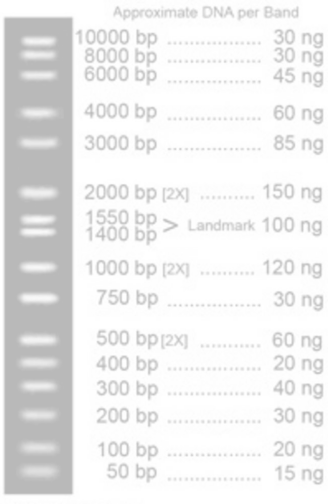
EXAMPLES OF LADDERS

Supercoiled ladder (to measure size)



Look at marker tables in p. 196 or 207

Minnesota ladder (to measure mass)



[1.2% Agarose Gel]

From 10 µL loaded

Please pay attention to how size migration is different

Chapter 6AB Procedures

Workflow for Chapter 6AB:

- Isolate plasmid DNA
- Cast a 1% agarose gel with DNA dye
- Measure nucleic acid concentration with UV-Vis spec
- Prepare samples and gel tank
- Load samples and run gel
- Image gel on UV-gel doc

Make sure to save your plasmid preps for week 2!

Lysing *E. coli* Cells

- Isolate plasmid DNA
  - Get 2 aliquots of cells transformed with Plasmid A & B
    - In week two, you will use transcription/translation to determine which is pGEM3-Rel and pGEM4-Rel
  - Centrifuge ***1 min*** – remove supernatant
  - Add 100 µl of GTE buffer and vortex to resuspend cell pellet
  - Add 200 µl of NaOH/SDS lysis solution, mix by inversion and ice for 5 min (**do not lyse for more than 5 min**)
  - Add 150 µl of potassium acetate (KAc) solution, mix by inversion
  - Centrifuge for 5 min at top speed – pipette supernatant fraction into a clean eppendorf tube, discard pellet

Isolation of Nucleic Acids

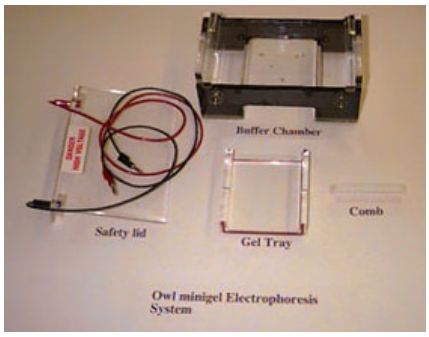
- Plasmid Mini-Prep
  - Add 0.4 mL of 1:1 phenol:chloroform (v/v) to your samples
    - Pull from the bottom layer of the stock bottle; it is saturated with aqueous buffer.
    - **Phenol is toxic, can cause severe burns and throat irritation**
    - **Chloroform is an anesthetic, but can be toxic; damage to organs**
    - ***This step MUST be done in the hood!***
  - Tightly CAP tubes!!
  - Vortex/shake your samples vigorously for 30 sec
  - Centrifuge to separate aqueous and organic phase
  - Transfer top aqueous phase to clean labeled tube
  - **Discard bottom layer and all phenol:chloroform waste directly in the hood. See Instructors.**

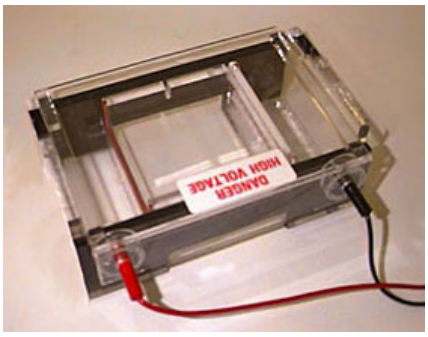
Precipitation of Nucleic Acids

- Plasmid Mini-Prep (continued)
  - Add ***cold*** 100% ethanol to the separated aqueous fraction, mix well
  - Centrifuge for 15 minutes at 17,000 *x g*
  - Remove supernatant, wash pellet with ***cold*** 70% ethanol
  - Centrifuge 1-2 minutes
  - Remove supernatant and air dry – **be careful not to remove pellet at the same time**
  - Add 35 µL **Nuclease-free Water**, vortex/pipet to dissolve pellet
  - **This is your final sample = mini-prepped plasmid DNA**

Agarose-Gel Electrophoresis

- Agarose Gel Electrophoresis – TF's will demo in lab
  - **Prepare Gel:**
    - Prepare casting tray using gel box walls
    - Obtain 50 mL 1% agarose, molten
    - Add 5 µL SYBE-Safe dye
    - Pour into casting tray, add comb, and let solidify





Electrophoresis Samples

- **Sample Preparation:** For Each Plasmid A & B, set up the following

Reagent	Volumes
Plasmid DNA: 0.1 – 0.2 OD units*	2-7 µL
6X Sample Buffer	3 µL
Water	15 µL – (Plasmid DNA µL)
Total Volume	**18 µL**

  - ***You will generate a total of 2 samples (A & B) in your group***
- **Load Gel:**
  - Run gel with another group: 2 samples + standards/group x 2 groups = 6-8 samples per gel
  - Standards for each gel will be pre-aliquoted with sample buffer; load entire volume aliquoted.
    - **Supercoiled DNA Marker and/or**
    - **DNA Mass ladder (Minnesota Molecular)**

*calculate the volume from your OD/mL measurements in the spec.

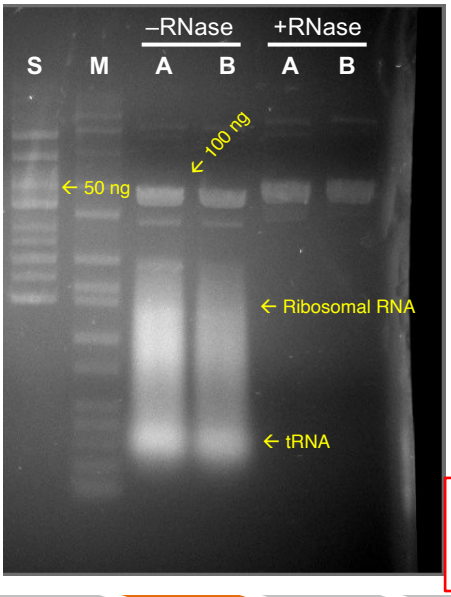
Electrophoresis Procedure

- Agarose Gel Electrophoresis
  - **Run Gel:**
    - What is the charge on DNA? Which direction will it run?
    - Run gel at 100 - 150 V until dyes separate
    - If you run the gel faster it will **MELT!**
  - **Image Gel:**
    - Take picture of agarose gel on gel dock. Instructors will demonstrate.

Determination of DNA Concentrations

- Before you ran your gel – Measure concentration by **UV-absorbance**
  - Take your OD/mL and convert these O.D./mL measurements into mg/mL of DNA
  - **1.0 O.D./mL = Amount of nucleic acid that gives A_{260} = 1 in 1 ml**
- **For DS DNA**: 1.0 O.D./mL = 50 µg/mL
  - 20 O.D./mg DNA
- **For RNA:** 1.0 O.D./mL = 40 µg/mL
  - 25 O.D./mg RNA
- EXAMPLE: from before, if you measured 20 OD/mL, this would be 20 OD/mL ÷ 20 OD/mg = 1 mg/mL, or 1 µg/µL, or 1000 ng/µL.
- Compare this to the estimated **concentration from the gel in ng/µL.**

Visually removing bacterial RNAs



- Bacterial RNAs are released into purification along with plasmid DNA
- 260 nm wavelength on the UV/Vis detects the nucleobases, which are present in both DNA and RNA
- Your UV/Vis-based DNA concentrations will be significantly greater than your gel-based estimations. If you used 0.1 OD for your samples (5 µL from your 20 OD/mL prep), then estimate 100 ng/5µL = 20 ng/µL.
- We would remove *E. coli* RNAs for the rest of Chapter 6 to prepare for digestion of DNA fragments.

S = Supercoiled size ladder
M = Concentration ladder/marker
A = plasmid A
B = plasmid B

Announce | Concepts | Procedure | Hazards | Tips | Clarification | End

Chapter 6AB/8 Week 1:

Before the lab period, you should have:

✓Completed your prelab
  ✓Title, date, introduction, procedures, ***a flowchart*** of your plasmid isolation procedure, conversion formulas

At the end of lab, you should have:

✓Isolated your plasmid DNA (**save DNA preps for week 2**)

✓Ran your samples on an agarose gel

✓Stained and imaged your gel

Announce | Concepts | Procedure | Hazards | Tips | Clarification | End

Hazards

Phenol/chloroform: work in hood, PPE, care to cap tubes, watch if any liquid on gloves, use 95% ethanol to wash any off

Electrophoresis: don't stick fingers in chamber, power off when handling electrodes, power off is spilled

Tips

- Double check calculations; both do them and compare
- Use one notebook with steps in protocol and check off as each step is done (record any changes with pre-lab)
- One student can do UV/VIS while other prepares the agarose gel

Changes from the Lab Manual

1) Not doing part C, only A & B
2) Spin initial cultures longer than 20 sec → **1 min**
3) Not using TE to re-dissolve final DNA prep; USE **Nuclease-free water**
4) Not using RNase!
5) Using Sybr-Safe, not ethidium bromide

Questions?

Discussion Quiz #2

In-class activity