7.002

Fundamentals of Experimental Molecular Biology

Lab Manual

Spring 2025

Lab Objectives Throughout the Semester

The steps that you will perform in lab will help elucidate what amino acids in ClpX are important for ATPase activity. We have divided the protocol into specific objectives to complete this goal:

1. **Reagent preparation**
   You will prepare some of the reagents and tools that you will need for later lab procedures. This will include making buffers and designing primers.

2. **Mutagenesis of the *clpX* gene**
   You will change the sequence of the gene that encodes the ClpX protein.

3. **Expression and purification of mutant ClpX**
   You will induce bacterial cells to produce a mutant ClpX protein from the gene that you mutated in Objective 2. You will then separate the protein from the rest of the bacterial cell material.

4. **Analysis of mutant ClpX protein purification**
   You will confirm you purified ClpX and check to see if there are contaminating proteins in your purified material.

5. **Analysis of mutant ClpX ATPase activity**
   You will test whether the mutant ClpX protein hydrolyzes ATP more efficiently, less efficiently, or with similar efficiency to the wildtype (unmutated) ClpX.

Lab Schedule

Day 1: **Lab Objective 1: Reagent preparation**
1.1: Sterile media preparation
1.2: Primer design

Day 2: **Lab Objective 1 (continued): Reagent preparation**
1.3: Buffer and gel preparation

Lab Objective 2: Mutagenesis of the *clpX* gene
2.1: Preparation of "Round-the-horn" PCR mutagenesis reactions

Day 3: **Lab Objective 1 (continued): Reagent preparation**
1.4: LB agar plates preparation

Lab Objective 2 (continued): Mutagenesis of the *clpX* gene
2.2: Gel analysis of PCR mutagenesis products
2.3: Purification of PCR mutagenesis products
2.4: Digestion, phosphorylation, and ligation of purified PCR products

Day 4: **Lab Objective 2 (continued): Mutagenesis of the *clpX* gene**
2.5: Transformation of *E. coli* DH5α with PCR ligations

Day 5: **Lab Objective 2 (continued): Mutagenesis of the *clpX* gene**
2.6: Miniprep of putative mutant *clpX* plasmid DNA from bacteria
2.7: Preparation of *clpX* miniprep DNA for sequencing
2.8: Analysis of *E. coli* DH5α PCR transformations

Day 6: **Lab Objective 3: Expression and purification of mutant ClpX**
3.1: Analysis of sequencing data
3.2: Transformation of *E. coli* LOBSTR BL21(DE3) with mutant *clpX* plasmids
3.3: Make purification buffers

Day 6.5: **Lab Objective 3 (continued): Expression and purification of mutant ClpX** 3.4: Expression of ClpX mutant protein & collection of cell pellet

*** **Note**: Part 6.5 will be performed by the 7.002 teaching staff

Lab Schedule

Day 7: **Lab Objective 3 (continued): Expression and purification of mutant ClpX**
- 3.5: Analysis of *E. coli* LOBSTR BL21(DE3) transformations
- 3.6: Purification of mutant ClpX ATPase protein
- 3.7: Determination of purified mutant ClpX protein concentration

Day 8: **Lab Objective 4: Analysis of ClpX protein purification**
- 4.1: Run samples on two SDS-polyacrylamide gels
- 4.2: Western transfer one SDS-polyacrylamide gel
- 4.3: Coomassie-stain second SDS-polyacrylamide gel

Day 9: **Lab Objective 4 (continued): Analysis of ClpX protein purification**
- 4.4: Rehydrate Western blot, block and probe with primary antibody
- 4.5: Destain Coomassie-stained SDS-polyacrylamide gel
- 4.6: Wash and detect Western blot

Day 10: **Lab Objective 5: Analysis of mutant ClpX ATPase activity**
- 5.1: Prepare ATPase reaction buffer
- 5.2: ATPase activity of mutant ClpX and wildtype ClpX
- 5.3: Generate a standard curve

DAY 1

Lab Objective 1: Reagent preparation

Part 1.1: Sterile media preparation

Overview

Throughout the semester, you will grow *E. coli* in liquid media in test tubes or in a petri dish on agar. It is important that the media the *E. coli* grow in be sterilized, to ensure that we are only growing the **desired** *E. coli* strain and no other contaminating microbes. To sterilize the media, you can use an autoclave, a device that maintains a **high temperature** and **pressure** in order to kill unwanted microbes. Alternatively, you can pass the media through a filter to physically separate any microbes from the solution.

Today, you will prepare the following solutions and plates:

- Ultrapure dH_2O
- Luria-Bertani Broth (LB)
- Terrific Broth (TB)

Materials

- (1) 100-mL glass bottle for LB Broth autoclave and storage
- (1) 250-mL filter apparatus (0.22 µm) for TB media
- (1) 150-mL filter apparatus (0.22 µm) for dH_2O
- (1) 1-L flask for mixing TB media before filtering
- (1) 250-mL graduated cylinder
- (1) Stir bar
- Bottles of autoclaved dH_2O
- Tryptone (powder)
- Yeast extract (powder)
- Sodium chloride (crystals)
- Bacto-Agar (powder)
- Overnight Express Instant TB Medium (powder)
- 40% glycerol (liquid)
- Weigh station with balance

Hazardous Chemicals Used: None

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Procedure

Ultrapure dH_2O

Ultrapure water and the commonly used deionized water are not the same. The "d" in front of the H_2O stands for *deionized*, meaning any charged ions have been removed. The water from the white taps at the sinks is dH_2O and has a resistivity of 18.2MΩ-cm. You will prepare an aliquot of ultrapure water that has been filtered and autoclaved. An autoclave is a device that maintains a **high temperature** and **pressure** in order to kill unwanted microbes. Passing the autoclaved water through a filter again can ensure high quality water in aliquots.

1) The staff will autoclave a large flask of dH_2O for each bay.

2) You will use vacuum filtration to ensure sterilization in your aliquot of the autoclaved dH_2O. The filter will allow the dH_2O to pass through, while trapping any dead bacteria or fungi or particles that are too large to pass through the pores of the filter. Unwrap a 150mL plastic filter with bottle attached.

3) Pour autoclaved dH_2O from the flask into the top portion of the filter apparatus (filling it most of the way) and cap the filter.

4) Attach the arm of the filter apparatus to the vacuum line, and turn on the vacuum.

5) Allow all of the liquid to pass through the filter into the bottle. Check to make sure you have >150 mL of sterile water in the bottle. If not, you can pour a little more water into the top of the filter apparatus.

6) Unwrap the bottle screw-cap, and leave it screw-side down on your bench, being careful not to touch the inside of the lid. Turn the vacuum off, unscrew the filter from the bottle, and quickly cap the bottle, minimizing the time that the bottle is open to the air.

7) Discard the filter in the regular trash.

8) Using a piece of lab tape, label the bottle with "Ultrapure dH_2O", your lab bench number, and the date. Store the bottle in your cupboard at room temperature.

Lysogeny Broth (LB)

Lysogeny Broth (LB) is a standard media for growing bacteria in the lab.

The standard recipe for 1L of LB is:

10 g	tryptone (partially hydrolyzed proteins)
5 g	yeast extract (yeast cells that have been broken open)
10 g	sodium chloride (a salt)
1 L	dH_2O

1) You will not need a full liter of LB. To save on reagents, you should only make 50 mL of LB. Calculate how much of each item you will need. Check the calculation with your TA.

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2) Use a piece of lab tape to label a 100-mL glass bottle with “LB Broth”, your lab bench number, and today’s date.

3) Use the balance to measure the appropriate amount of each solid chemical and transfer to the glass bottle. Rinse the spatula with dH2O at the sink and dry with a paper towel.

4) Fill a graduated cylinder with the appropriate amount of dH_2O, then transfer the water to the 100-mL bottle. (Note that bottle markings do not accurately measure volumes.)

5) *Loosely* cap the 100-mL bottle and bring the LB to an instructor at Bench C. (Normally it is important to mix your solutions thoroughly, but this solution will mix as it is autoclaved.) Obtain a piece of autoclave tape from the instructor to secure the cap.

6) Once autoclaving is complete, allow the bottle to cool to touch, screw the cap on completely and store in your cupboard at room temperature.

Terrific Broth (TB)
Terrific Broth (TB) is a bacterial growth media that is very nutrient rich, and it is often used for growing bacteria that are over-producing a protein of interest. The 7.002 version of TB has been specially formulated for autoinduction. In autoinduction, bacteria will first use the sugar glucose as an energy source, which allows the bacteria to divide until a sufficient number of bacteria exist in the culture. Once the glucose is used up, bacteria will switch to lactose, which will induce the bacteria to produce the protein of interest (mutant ClpX). The mechanism behind this induction will be explained in more detail later in the course.

For convenience, we order a pre-mixed powder of TB media (“Overnight Express Instant TB Medium” from Novagen).

The recipe for 1L of TB media is:

60 g Overnight Express Instant TB Medium
25 mL 40% glycerol
1 L dH_2O

1) You will make 250 mL of TB media. Calculate the amount of each reagent you will need. Check the calculation with your TA.

2) Obtain a 1000-mL flask and a stir bar. Add the appropriate amount of autoclaved dH_2O (not the white tap at the sink) to the flask.

3) Weigh out the appropriate amount of TB powder, and add it to the flask. Rinse the spatula with dH2O at the sink and dry with a paper towel.

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4) Using a 10-mL glass pipette, measure the appropriate volume of glycerol. Note that when pipetting viscous liquids like glycerol, you should pipet slowly, and pause to let the liquid settle before removing the pipet from the bottle. When ejecting the liquid, eject slowly. You may need to let the liquid settle a few times, while ejecting. Once most of the glycerol has been ejected, pipet up and down using the water in the flask to transfer the remaining glycerol in the pipette.

5) Place the flask on a stir plate and allow it to stir until thoroughly mixed.

6) Note that you will not autoclave the TB media, as some ingredients in the media are heat-sensitive. You will use vacuum filtration to sterilize the TB media instead. Unwrap a 250-mL plastic filter with bottle attached.

7) Pour the well-mixed TB media into the top portion of the filter apparatus and cap the filter.

8) Attach the arm of the filter apparatus to the vacuum line, and turn on the vacuum.

9) Allow all of the liquid to pass through the filter into the bottle.

10) Unwrap the bottle screw-cap, and leave it screw-side down on your bench, being careful not to touch the inside of the lid. Turn the vacuum off, unscrew the filter from the bottle, and quickly cap the bottle, minimizing the time that the bottle is open to the air.

11) Discard the filter in the regular trash.

12) Using a piece of lab tape, label the bottle with “TB media”, your lab bench number, and the date. Leave the bottle on the bench in the 4°C cold room.

DAY 1

Information about using the Autoclave
A STAFF MEMBER WILL RUN THE AUTOCLAVE

Autoclaves are used to sterilize equipment and media by holding them at a high temperature (around 132ºC) and pressure that micro-organisms cannot survive. Note that not all items and reagents can be autoclaved (for example drugs or some types of plastic), so be sure to check that an item can be autoclaved before doing so.

Important Autoclave Reminders:

- Make sure that all items to be autoclaved are placed in an autoclave bin. They should not be put directly in the autoclave.

- Autoclave tape should be used to help ensure that the autoclave has reached proper temperature for sterilization. Once the autoclave reaches the correct temperature, the tape will develop black stripes or change color. You should keep the tape on the items when you store them to indicate that the contents are sterile.

- Make sure any liquids are in glass bottles and do not exceed the suggested bottle volumes.

- If the autoclave door is closed, check that the previous cycle is complete AND that the pressure gauge has reached 0 before opening the door.

- To open the door, turn the handles counter-clockwise. Stand back when opening the door, as hot steam may still be present.

- Always assume that the inside of the autoclave and its contents are hot. Wear heat-protective gloves when adding or removing items from the autoclave.

- Liquid media and solids should be autoclaved separately.

Using the autoclave:

- Open the door and place your bin of items on the rack in the autoclave.

- Close the door and turn the handles clockwise as tightly as you can. It is important that the handles be turned as far as possible to create a seal.

- Follow the instructions on the autoclave for selecting the settings on the screen.

- For dry goods/glassware:
    - Use the gravity setting for 20 min

DAY 1

- For liquids:
    - Double check that the caps are only loosely on the bottles. It is essential that the steam be able to vent from the bottles, so that the bottles do not break.
    - Use the liquid setting for 20 min. The liquid setting will have a slower exhaust (release of the steam) at the end of the cycle, which helps to prevent liquid spill-over.

- Start the autoclave.

- The autoclave will take about 10-20 minutes longer than the cycle length to sterilize your items and exhaust the steam. Do not leave items in the autoclave for too long after the cycle has completed, as there is still some steam present that can damage the autoclave bins and other items over time.

- When the sterilization is complete, ensure that the pressure gauge has reached 0 before opening the door.

- To open the door, turn the handles counter-clockwise. Stand back when opening the door, as hot steam may escape rapidly as the door opens.

- Use gloves to remove the bin, and transfer the bin to a bench or cart.

- Make a sign to put on top of the bin to warn others that the items are hot. Allow the items to cool before storing or using.

- If you are using liquid items, be sure to tighten the caps AFTER cooling and BEFORE long-term storage.

- If anything spilled in the autoclave, ask the course staff for help cleaning up.

DAY 1

Part 1.2: Primer design

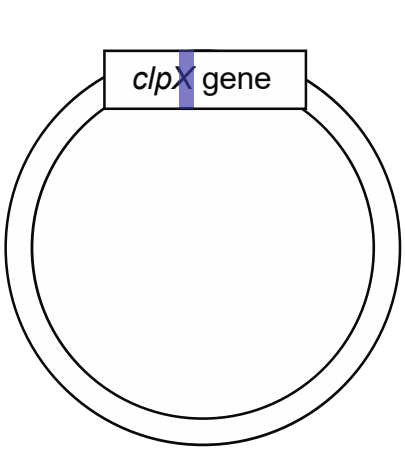
Overview

You will be creating a mutant ClpX protein to determine whether a particular amino acid is important for ClpX's ATPase activity. You should be familiar with the structure of ClpX from your pre-lab activities. Which two residues are you interested in mutating?

Residue of Interest 1: _____ Residue of Interest 2: _____

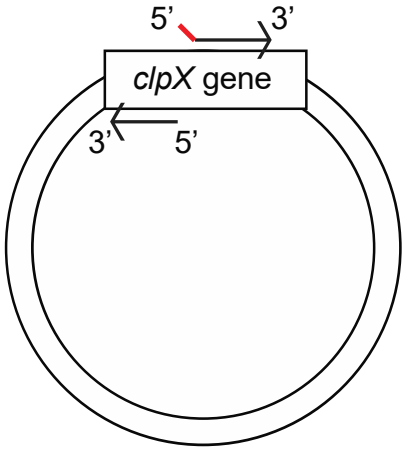
Today you will discuss these residues of interest and choose one amino acid to mutate. Groups under the same TA will choose the same amino acid for mutation. Each group will mutate this amino acid to a new, different residue.

You will then select or design a pair of DNA primers that will allow you to incorporate the desired mutation into the *clpX* gene. Remember, a primer is a short (18-30 bp) stretch of single-stranded DNA that will bind to a complementary region of a template DNA molecule (in this case the *clpX* gene). DNA polymerase will add nucleotides to the primers, producing a copy of the template DNA between the primers. **If there is a mutation in the primer, the mutation will appear in the new DNA copies as well.**



Circular, double-stranded DNA template containing the wildtype *clpX* gene.

The codon of interest is highlighted in blue.



Forward and reverse primers. The 5' ends of the primers are adjacent; all DNA in between will be replicated, which in this case includes the entire piece of circular DNA.

The forward primer contains a mutation at the 5' end (red) that will change the codon of interest within *clpX*.

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Procedure

1) Discuss with your neighboring groups which residue you would like to change.

2) All groups working under the same TA should choose to replace the same amino acid residue. However, each group should choose a different and unique amino acid and have an overall hypothesis for how the change will affect ATPase activity. You should consider the different chemical characteristics of the amino acid side chains as you make your decisions (amino acid structures are in the Appendix).

3) You now need to design primers to incorporate a mutation at the desired residue. You will want to view the *clpX* sequence and codon chart in the Appendix when selecting and designing your primers.

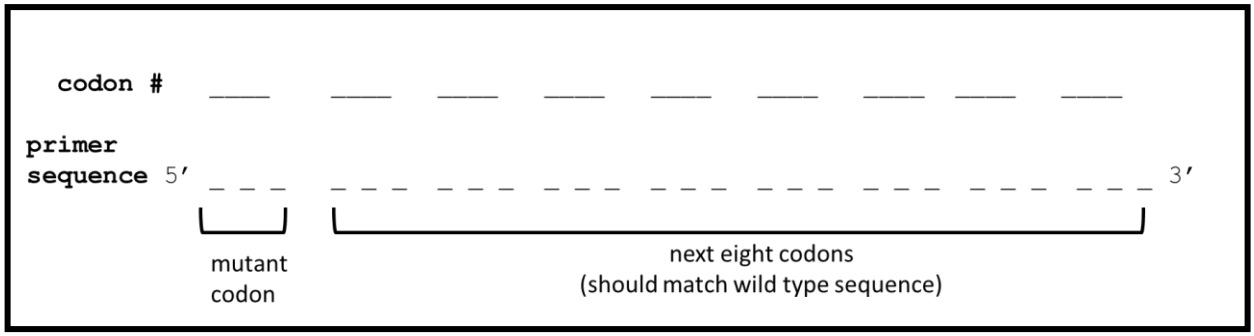
   a) You may select primers from the 7.002 collection that will mutate the residue of interest. Some of the available primers are listed in the Appendix. Follow the design exercise below and compare your primers sequences to the list in the Appendix.

   OR

   b) You may design primers from scratch that will allow you to introduce a new mutation into the *clpX* coding sequence.

   Design the Forward Primer:

   1. Find the codon that you would like to mutate in the *clpX* sequence at the end of the manual. Note that the coding strand of DNA is shown in this sequence. Write the codon # in the first blank in the figure below.



   2. Fill in the subsequent codon #s.

   3. Determine what the mutant codon should be. Write this codon in the first three blanks of the primer sequence.

   4. Fill in the next eight wildtype codons in the remaining blanks of the primer sequence.

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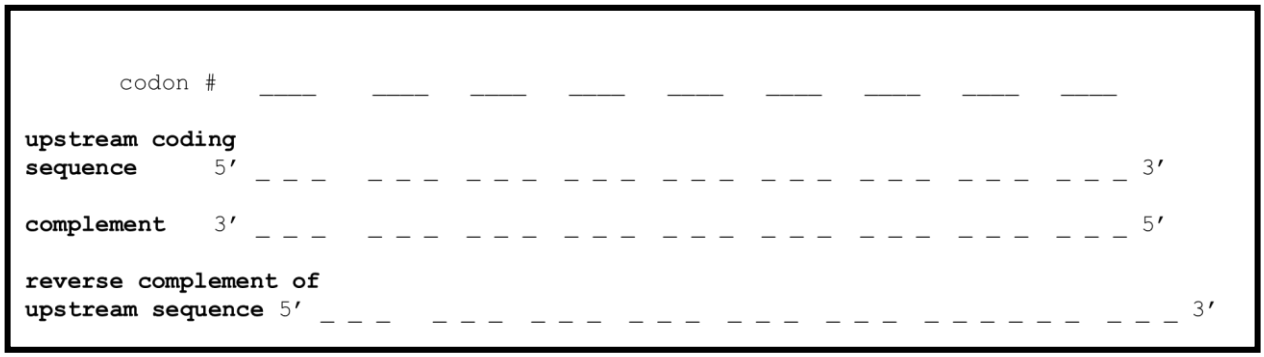
5. Check to see whether the most 3’ nucleotide is a G or C. If not, remove one or more letters from the 3’ end until you reach a G or C. Having a G or C at the 3’ end is called having a GC clamp.

6. Check that the GC content is 40-60%, that there are no extensive repeat sequences, and that your sequence is still > 18 nucleotides long.

7. You have now created your forward primer!

Design the Reverse Primer:

8. Write the nine codon numbers in order that immediately <u>precede</u> your mutated codon in the figure below. For example, if you are mutating E185, the last (9th) codon is going to be D184.



9. Fill the corresponding wild type DNA codons.

10. Fill in the complementary DNA strand.

11. Write the reverse complement (i.e. copy the complement in reverse order, maintaining the 5’ and 3’ orientation.)

12. Check to see whether the most 3’ nucleotide is a G or C. If not, remove one or more letters from the 3’ end until you reach a G or C.

13. Check that the GC content is 40-60%, that there are no extensive repeat sequences, and that your sequence is still > 18 nucleotides long.

14. You have now created your reverse primer!

DAY 1

Day 1 Post-Lab Notebook Tips & Reminders

- Did you finish making all the solutions?
- Did you deviate from the printed protocols?
- Which amino acid residue of ClpX will you mutate and what is its codon?
- What is the replacement amino acid and codon? Why did you choose this replacement amino acid? What is your hypothesis to the ATPase activity?
- Did you design your own primers or select primers that the lab already has?
    - If you designed your own primers, include your two primer sequences (label them as the Forward and Reverse primers appropriately and indicate the 5’ and 3’ ends of each primer)
    - If you selected primers, what are their names? Do they look as expected from the primer design exercise in the step 3b?

Day 1 ILQs

Work on these questions alone or with your partner during your downtime in lab. Your TA will discuss them as a group. You need to write out solutions to receive credit.

Question 1
RT-qPCR is the gold standard for COVID-19 diagnostic test. What does PCR stand for? What is PCR used for? What are the necessary PCR reagents? How is RT-qPCR different than PCR?

Question 2
Primer design:

A. Why do you think we want the mutation on the 5' end of our primer vs the 3' end?

B. How do G-C basepairs differ from A-T basepairs? What kinds of forces are holding the double helix in DNA strands? Why do you think we want a GC clamp on the end of our primers? Do all of the primers in the appendix have the GC clamp?

C. Why would we not want very high or very low GC content in our primers?

Day 1 ILQs

Question 3
Some venomous snakes produce a deadly toxin called fasciculin. Fasciculin binds to and inhibits mammalian acetylcholinesterase, a protein important for the processing of neurotransmitters. When acetylcholinesterase is inhibited, neuromuscular junctions are disrupted and muscle paralysis shortly follows. Mongeese, the fearless snake-fighting mammals, are not affected by fasciculin.

Shown below is DNA **coding** sequence for mouse and mongoose acetylcholinesterase around the region of the active site. The sequences are given in frame and the first codon would code for the 185th amino acid.

```
          185 186 187 188 189 190 191 192 193 194 195 196 197
Mouse:    AAG CAC TGG GTG TTC TAC TCC TGC TGC CCC ACC ACT CCC
Mongoose: AAG CAC AAT GTG ACC TAC GCC TGC TGC CTC ACC ACC CAC
```

Indicate the amino acid changes that might play a role in the “immunity” of the mongoose. For example, if amino acid 184 was ‘His’ in mouse and ‘Ala’ in mongoose, you would write **His184Ala.** This is the 3-letter scientific nomenclature for amino acid substitution. Also discuss how changing the properties of amino acid residues may affect the structure of acetylcholinesterase. *Reminder: the Appendix of the Lab Manual contains a codon table.*

DAY 2

Lab Objective 1 (continued): Reagent preparation

Part 1.3: Buffer and gel preparation

Overview

Today you will also prepare some of the buffers that will be used for generating and purifying your mutant ClpX protein later in the semester. Buffers help create an ideal environment for biomolecules that require a stable pH and salt concentration to work properly. Physiological pH is around pH 7, and concentrations of various salts are often 10-100mM in living organisms.

Today you will prepare an agarose gel and the following solutions:

- PBS
- TAE (TAE will be used to pour an agarose gel at the end of lab)
- SDS-PAGE running buffer

Materials

- (1) 1-L glass bottle for SDS-PAGE running buffer
- (1) 500-mL glass bottle for TAE
- (1) 100-mL/125-mL glass bottle for PBS
- (1) 1-L graduated cylinder
- (2) 50-mL graduated cylinders
- (1) 250-mL flask for mixing agarose gel
- 50X TAE stock (G Bioscience)
- 10X SDS-PAGE Running Buffer stock (G Bioscience)
- 10X PBS stock (G Bioscience)
- Agarose (powder)
- 1000X GelRed dye (diluted in TAE)
- Gel apparatus with tray
- Gel comb
- Regular dH_2O (white tap)
- Ultrapure dH_2O (from Day 1)
- Weigh station with balance
- (1) Ziploc bag

Hazardous Chemicals Used

None; GelRed dye is considered non-toxic, but as a precaution, always wear gloves when handling gels.

DAY 2

Procedure
Special note: Use glass pipets for ultrapure dH_2O and all stock solutions.
Use graduated cylinders for tap DI water.
Always fold the end of a lab tape. Easy to find on a tape. Easy to remove from glassware.

PBS
PBS, or phosphate-buffered saline, is a common buffer used for a variety of biological and biochemical techniques such as storing purified proteins, washing protein blots, and washing cells.

1) Obtain a 125-mL bottle and use lab tape to label it with “1X PBS”, your lab bench number, and the date. Remember to fold a small piece on the end of tape.

2) You will make 125 mL of PBS from a 10X stock of PBS. Calculate the volume of PBS and water you will need.

3) Add the correct volumes of 10X PBS and ultrapure dH_2O to the labeled bottle. (Don’t worry if you are given a 100 mL bottle, it can accommodate 125 mL)

4) Cap the bottle and swirl to mix. Store at room temperature in your cupboard.

TAE
TAE is a buffer used for agarose gel electrophoresis, a procedure that allows one to check the presence and sizes of DNA.

1) Obtain a 500-mL glass bottle, and use lab tape to label it with “1X TAE”, your lab bench number, and the date.

2) We buy 50X stocks of TAE from G Bioscience. Calculate the amount of 50X TAE and water you should combine to make 500 mL of 1X TAE.

3) Add the right amount of 50X TAE and regular dH_2O the bottle. Cap, and swirl to mix.

4) Set aside the 1X TAE; you will use this later today to pour a gel. Afterwards you will store the 1X TAE at room temperature in your cupboard.

SDS-PAGE running buffer
SDS-PAGE is a procedure that is used to separate proteins, usually by size. Today you will make the buffer used in this procedure.

1) Obtain a 1-L glass bottle, and use lab tape to label it with “1X SDS-PAGE Running Buffer”, your lab bench number, and the date.

DAY 2

2) We buy 10X stocks of SDS-PAGE running buffer from G-Bioscience. Calculate the amount of 10X Running Buffer and water you should combine to make 1 L of 1X Running Buffer.

3) Add regular dH_2O and then running buffer into the 1-L bottle, cap, and swirl to mix. SDS is not easily rinsed off by water. Do not use the graduated cylinder for SDS-PAGE running buffer.

4) Store the 1X Running Buffer at room temperature in your cupboard.

Agarose gel
Next week, you will analyze your PCR reaction using agarose gel electrophoresis. Today you will pour the gel.

1) Set up the agarose gel casting tray. You should turn the plastic casting tray in the gel apparatus so that the open ends are sealed by the sides of the gel apparatus. Make sure that the orange rubber seals are properly placed in the grooves of the tray and do not buckle out.

2) Pour some tap dH_2O into the tray to make sure that the casting tray will not leak. Adjust the tray if needed. Leave the tray in place, then pour out the water.

3) Place a comb near the top of the casting tray to create the wells. The ends of the comb should fit into the slots on the sides of the gel casting tray.

4) Measure 50 mL of 1X TAE (using a clean graduated cylinder) and pour it into a 250-mL flask.

5) You will make a 0.8% agarose gel. Calculate the mass of agarose powder that you will need, weigh it out on the balance, and add it to the 250-mL flask. Note, a 1% gel would contain 1 g of agarose in 100 mL of TAE.

6) Microwave the solution in 30 second intervals until the agarose is fully dissolved (note, stuff a single layer of kimwipe over the flask & **NO FOIL** in the microwave). Watch the flask carefully during each interval to make sure that the flask does not overflow. Between intervals, use heat-resistant gloves to gently swirl the flask. Hold the flask up to the light to check whether the solution is uniform and that there are no undissolved pieces of agarose present. If not completely dissolved, continue microwaving in 30-second intervals.

7) Calculate the appropriate amount of 1000X GelRed dye to add to your 50 mL molten agarose solution. Pipet the correct volume of GelRed to the molten agarose, and swirl to thoroughly mix. Work quickly to make sure that the agarose does not solidify.

8) Allow the molten agarose to cool on your bench top until just cool enough to touch for 1-2 seconds.

DAY 2

9) Carefully pour the molten agar into the gel casting tray, trying to avoid introducing bubbles. If you do get bubbles you can use a pipette tip to try to pop the bubbles or move them to the edge of the gel.

10) IMMEDIATELY rinse out the EMPTY flask in the sink with a lot of water so that the remaining gel solution does not solidify in the flask.

11) Allow the gel to solidify on your benchtop (~ 30 minutes – you may move on to Part 2.1, your PCR, while you wait). The gel will turn an opaque white color when solidified. Be careful not to move the gel while it is solidifying.

12) Carefully pull the comb out of the gel. Remove the gel casting tray from the gel apparatus, and slide the gel off of the tray and into a Ziploc bag. Add 200uL of 1X TAE (with a pipette) so that your gel will stay moist over the next week. Zip the bag tight.

13) On a piece of lab tape, write “0.8% agarose gel”, your bench number, and the date. Place the tape over the Ziploc bag, and store the gel in the tray inside the cold room until next week.

*** Note: Day 2 continues on the next page ***

DAY 2

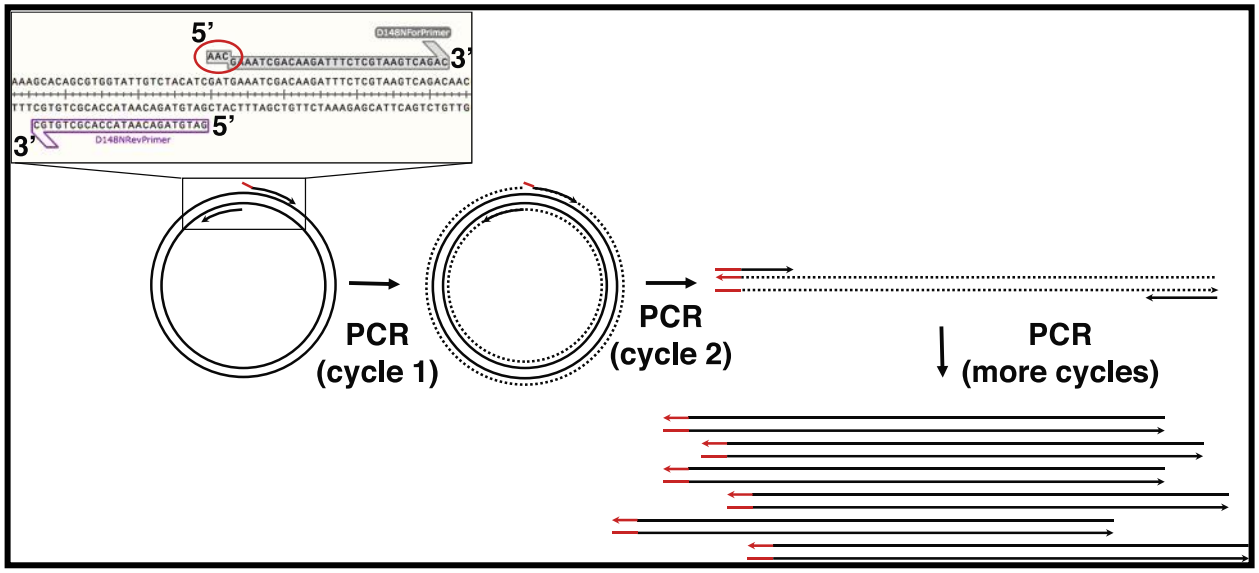
Lab Objective 2: Mutagenesis of the *clpX* gene

Part 2.1: Preparation of "Round-the-horn" PCR mutagenesis reactions

Overview

To better understand how the ClpX ATPase protein functions, you will mutate a particular residue in the ClpX protein and assay the effect this alteration has on enzyme activity. Last week you designed primers that included your mutation of interest. Today, you will use these primers in a PCR to actually produce many copies of mutant *clpX* containing DNA.

Flow Diagram of PCR. *The reaction you set up today will make many linear copies of the plasmid, hopefully containing the desired mutation (shown in red).*



Materials

- pET-ClpX plasmid DNA template (~5.5 kb, 33 ng/μL, see plasmid map in the Appendix)
- 2X Phusion High-Fidelity Master Mix (from New England Biolabs)
- Forward mutagenic primer (100 μM)
- Reverse mutagenic primer (100 μM)
- Ultrapure dH_2O (from Day 1)
- (3) 0.5-mL PCR tubes (not regular 0.5-mL tubes)

Hazardous chemicals used

None

DAY 2

Procedure

Keep all reagents and tubes on ice at all times. Make sure you use a new tip for every solution and between tubes.

1) It is recommended that you transfer a small (~500 µL) aliquot of ultrapure water to a 1.5-mL Eppendorf tube from your larger stock in order to keep the stock clean. Use the water from this smaller aliquot for your PCR reaction.

2) The stock tubes of primers are too concentrated, so you will need to dilute them before you can add them to your PCR samples. Label a 1.5-mL Eppendorf tube as "Primers" along with your bench number. Add the following reagents to this tube:

   3 µL of the forward mutagenic primer
   3 µL of the reverse mutagenic primer
   24 µL of ultrapure water

3) Mix the contents of the tube by gently pipetting up and down several times. Quick-spin the tube for ~5 seconds and leave it on ice. When you set up your PCR samples, make sure you use this diluted "Primers" Eppendorf tube when adding the primers (and not the original stock primer tubes).

4) For each group, obtain three sterile 0.5-mL PCR tubes from your TA. Label the tubes "#1," "#2," and "#3," along with your initials and bench number.

5) Set up the three Round-the-horn PCR reactions as follows:

	Tube #1 (Template + Primers)	**Tube #2 (Template-only)**	**Tube #3 (Primers-only)**
	12.5 µL 2X Phusion Master Mix	12.5 µL 2X Phusion Master Mix	12.5 µL 2X Phusion Master Mix
	2.5 µL primers (from "Primers" tube)	-	2.5 µL primers (from "Primers" tube)
	1.5 µL pET-ClpX template DNA	1.5 µL pET-ClpX template DNA	-
	8.5 µL ultrapure dH_2O	11 µL ultrapure dH_2O	10 µL ultrapure dH_2O
Total Volume	25 µL	25 µL	25 µL

6) Mix the reagents in each tube by gently pipetting up and down several times (try not to introduce any bubbles). Quick-spin all the tubes for ~15 second.

DAY 2

7) Make sure your tubes are tightly capped. Check that they should have the same volumes. Then give them to your TA. They will place your samples in the thermocycler to run the following PCR program:

    1) 98 °C for 30 seconds
    2) 98 °C for 10 seconds
    3) 58 °C for 20 seconds
    4) 72 °C for 90 seconds
    5) Repeat Steps (2) through (4) 29 more times
    6) 72 °C for 4 minutes
    7) 4 °C indefinitely (until the next day when samples will be moved to -20 °C freezer)

Day 2 Post-Lab Notebook Tips & Reminders:

- Did you successfully set up the PCRs?
- Did you finish making all of the solutions?
- Did you deviate from the printed protocols?

Day 2 ILQs

Work on these questions alone or with your partner during your downtime in lab and then discuss them with your TA. You need to write out solutions to receive credit.

Question 1
Today you started your first experiment towards determining if your amino acid residue of interest is important for ATP hydrolysis by ClpX. What are the FOUR lab objectives and sequential orders throughout the semester? (Note the difference between lab objectives and lab experiments)

Question 2
The table below compares PCR with *in vivo* DNA replication. Fill in the boxes for PCR.

	***In vivo* DNA Replication**	**PCR**
What is the end result?	The entire genome is copied.	
Steps of the Process		
1) Double stranded DNA is separated	The enzyme helicase is recruited to the origin of replication and separates the DNA strands.	
2) DNA replication is primed	Millions of RNA primers are synthesized against the single-stranded DNA by the enzyme primase.	
3) Primers are extended, leading to double-stranded DNA	DNA polymerase adds dNTPS to the growing DNA strand.	
Repeat cycles?	No	

Day 2 ILQs

Question 3

You want to amplify Gene X with standard PCR from the linear template shown below. Draw a schematic diagram showing the expected products in your PCR reaction after three rounds of PCR. In your answer, please show the original strands and ALL of the expected products (complete and incomplete) after each round of amplification. How many completed strains are resulted after three rounds of PCR? Come up with an equation to represent the number of completed PCR products after N cycles of PCR. How many completed products are there after 30 cycles? Primers are indicated by the arrows, with the arrows pointing 5' to 3'.

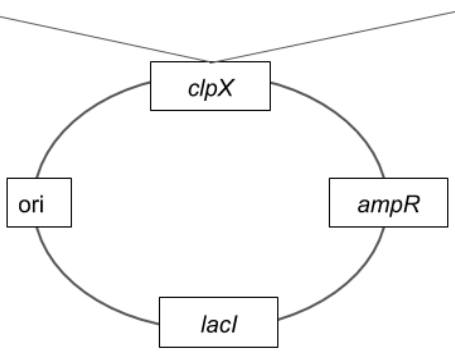
gene X (solid lines) and flanking DNA sequence (dotted lines)

Day 2 ILQs

Question 4

In an attempt to increase scientific aptitude in the Kingdom of Wakanda, Queen Ramonda has instituted a mandatory 7.002 class for all citizens. You volunteer to be a TA! A region of interest from the *clpX* gene on the pET plasmid is shown below:

```
5′ CAG AAG CTG TTG CAG AAA TGC TAC GAT GTC CAG AAA GCA CAG CGT GGT 3′
3′ GTC TTC GAC AAC GTC TTT ACG ATG CTA CAG GTC TTT CGT GTC GCA CCA 5′
    Q   K   L   L   Q   K   C   Y   D   V   Q   K   A   Q   R   G
  163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178
```



Your four students design the following primers:

Shuri	5′ CAG AAG CTG TTG CAG AAA TGC TAC GAT GTC CAG AAA GCA CAG CGT GGT 3′ 3′ GTC TTC GAC AAC GTC TTT ACG ATG CTA CAG GTC TTT CGT GTC GCA CCA 5′ Q K L L Q K C Y D V Q K A Q R G 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 F primer:5′-GTA AAA GCA CAG CGT GGT-3′ R primer:5′-GAC ATC GTA GCA TTT CTG-3′
Nakia	5′ CAG AAG CTG TTG CAG AAA TGC TAC GAT GTC CAG AAA GCA CAG CGT GGT 3′ 3′ GTC TTC GAC AAC GTC TTT ACG ATG CTA CAG GTC TTT CGT GTC GCA CCA 5′ Q K L L Q K C Y D V Q K A Q R G 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 F primer:5′-GTC CAG AAA GCA CAG CGT-3′ R primer:5′-GTA GCA TTT CTG CAA CAG-3′
Riri	5′ CAG AAG CTG TTG CAG AAA TGC TAC GAT GTC CAG AAA GCA CAG CGT GGT 3′ 3′ GTC TTC GAC AAC GTC TTT ACG ATG CTA CAG GTC TTT CGT GTC GCA CCA 5′ Q K L L Q K C Y D V Q K A Q R G 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 F primer:5′-GAT GTC CAG AAA GCA CAG-3′ R primer:5′-ATC GTA GCA TTT CTG CAA-3′
Okoye	5′ CAG AAG CTG TTG CAG AAA TGC TAC GAT GTC CAG AAA GCA CAG CGT GGT 3′ 3′ GTC TTC GAC AAC GTC TTT ACG ATG CTA CAG GTC TTT CGT GTC GCA CCA 5′ Q K L L Q K C Y D V Q K A Q R G 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 F primer:5′-GAT GTC CAG AAA GCA CTT-3′ R primer:5′-GTA GCA TTT CTG CAA CAG-3′

Day 2 ILQs

A. You are concerned that one primer pair will not amplify the plasmid DNA and you help that student redesign their primers to make their desired mutation. Which student needed new primers and why?

B. Indicate the specific amino acid sequence change each student will make if their PCR is successful. *Reminder: the Appendix of the Lab Manual contains a codon table*
   - Shuri:
   - Nakia:
   - Riri:
   - Okoye:

DAY 3

Lab Objective 1 (continued): Reagent preparation

Part 1.4: LB agar plates preparation

Overview

Today, you will prepare LB agar plates in petri dishes. Similar to making sterilized LB media, it is important that the LB agar is sterilized in autoclave before pouring onto the plates. Ampicillin can be destroyed by heat and should be added when LB+Agar is cool enough.

Materials

- (1) 1-L flask for mixing and autoclaving LB+Agar
- (10) sterile petri dishes
- (1) 500-mL graduated cylinder
- (1) Stir bar
- Tryptone (powder)
- Yeast extract (powder)
- Sodium chloride (crystals)
- Bacto-Agar (powder)
- 100X ampicillin stock (liquid; in ethanol)
- Brown sharpie
- Weigh station with balance
- 55°C water bath

Hazardous Chemicals Used:

The ampicillin is made up in EtOH, so we will treat that as hazardous.

Procedure

LB+Amp Agar Plates
Growing *E. coli* on agar plates allows us to select bacterial clones, or individual colonies of bacteria that are genetically identical, because they arose from one initial cell. You will also want to select only for bacteria that contain the gene encoding the mutant ClpX protein. In order to do this, we will add the antibiotic drug ampicillin to the plates. The gene for mutant ClpX will be on the same piece of DNA (called a plasmid) that also contains a gene for ampicillin resistance. Any bacteria with the antibiotic resistance gene, and hence the mutant *clpX* gene, will survive and grow on the agar plate.

1) Obtain a 1-L flask, and label it with a piece of tape that includes “LB Agar”, your bench number (e.g. D2, F4, H2, etc), and the date. Save the foil.

DAY 3

2) You will make 300mL of LB+Agar. The standard recipe for making 1 L of LB+Agar is given below:

    10 g tryptone (partially hydrolyzed proteins)
    5 g yeast extract (yeast cells that have been broken open)
    10 g sodium chloride (a salt)
    15 g bacto-agar
    1 L dH_2O

    Calculate the amounts of each ingredient for 300mL of LB+Agar.

3) For each solid ingredient, measure the appropriate amount using the balance.
    - Place a clean weigh boat on the balance, and press the "zero" or "tare" button to tare the scale. This step ensures that the weight of the container is not counted in the measurement.

    - Use a clean metal spatula from the plastic beaker to transfer the appropriate amount of ingredient to the boat. If you transfer too much on the boat, use the spatula to remove some mass, but be sure to discard the extra in the biohazard waste container. Once a chemical or powder has been removed from its original container it should not be returned, in order to preserve the purity of the original stock.

    - Carefully pick up the boat or paper and transfer the ingredient to the labeled flask. Discard the weigh boats in the regular trash.

    - **Rinse the metal spatulas thoroughly with dH_2O at the sink and dry with paper towels. Using a fresh boat and spatula, repeat the procedure for the next ingredient. Return the cleaned metal spatulas to the plastic beaker.**

4) Using a graduated cylinder, measure 300 mL of dH_2O (white tap at the sink) and add it to the flask. You do not need to mix, as the ingredients will mix in the autoclave.

5) Add a stir bar to the flask.

6) Cover the opening of the flask with the original double-layer of foil.

7) Bring the LB+Agar mix to the instructors at Bench C. Secure the foil with a small piece (~1") of autoclave tape. The instructors will autoclave your LB+Agar with the LB+Agar from other lab members.

8) While the autoclave is running, obtain 10 petri dishes; keep the lids on the dishes until you are ready to use them to maintain sterility. You will indicate that the plates contain LB+Amp by marking a single brown stripe on the side of the plates (hint: it is easy to do this if you stack the plates and drag the marker down the side of the plates).

DAY 3

9) Continue to **Part 2.2** while the autoclave is running. A staff will notify you that the autoclave is finished. Continue onto Step 10.

10) The staff will place your LB+Agar solutions in a 55^{o}C water bath when the autoclave is finished. The LB+Agar should cool in the water bath for at least 15 minutes. The water bath will prevent the LB+Agar from overcooling and solidifying. The staff will also distribute a stock of 100x ampicillin. Calculate the amount of ampicillin you need to add to 300mL of LB+Agar to get a final concentration of 1x ampicillin.

11) When you are ready to pour plates, remove the LB+Agar from the water bath. **PROCEED IMMEDIATELY to Step 12.**

12) Place the flask on a stir plate on low speed. Avoid vortex and bubbles. If the LB+Agar is cool enough to touch, add the ampicillin to the flask. If you cannot comfortably touch the flask, allow it to cool briefly (heat can destroy ampicillin).

13) Keep the flask on a stir plate for about 30 seconds to allow the ampicillin to be distributed evenly. Your TA has a magic wand to remove the stir bar from the flask.

14) Lay your plates in a row near the edge of the bench. Briefly lift the lid of a petri dish and directly pour the molten LB+Amp agar to the dish. Do this carefully until the bottom of the dish is just covered by ~3 mm of agar (or half the height of the dish). Replace the lid quickly, leaving it slightly ajar to speed drying.

15) Repeat for the remaining 9 plates. Try to avoid bubbles. If you do have a few bubbles in your dishes, remove them with the P1000 pipet. If you have a lot of bubbles, let an instructor know so that they can remove them with a Bunsen burner. If you have extra agar mixture in your flask, pour it into a waste beaker. Allow the plates to solidify on your bench, and avoid moving the plates until they are solid.

16) **IMPORTANT**: IMMEDIATELY rinse out the EMPTY agar flask in the sink. **DO NOT POUR ANY AGAR SOLUTION INTO THE SINK**, as it will clog the drain when it solidifies.

17) Acquire a plastic sleeve from your TA. Label a piece of tape with “LB+Amp,” your bench number, and the date. Leave the sleeve next to your drying plates. You may leave your plates to dry overnight and the staff will store them in the cold room to slow the degradation of the ampicillin. Do not pack the plates if there is humidity on the lid. The plates will be stored upside down to prevent moisture from settling on the agar.

DAY 3

Lab Objective 2 (continued): Mutagenesis of the *clpX* gene

Part 2.2: Gel analysis of PCR mutagenesis products

Overview
The Round-the-horn PCR mutagenesis procedure should have created copies of the pET-ClpX DNA template that are identical to the original template except in the region where the desired mutation was introduced. Today we will use gel electrophoresis to confirm if copies of DNA were indeed synthesized.

Before loading your PCR samples into the gel, you will mix them with loading dye. The loading dye contains bromophenol blue, which helps you see your sample as you pipette it into the well and the dye should run between 500 bp and 1000 bp on your 0.8% gel. You cannot see any DNA by eye while your gel is running, but the blue dye front will let you gauge approximately how far your DNA samples have moved through the gel. Another component of loading dye is a dense solution, usually ficoll, which we use, or glycerol. This dense solution helps your DNA fall to the bottom of the well instead of floating out into the buffer.

Materials
- 50 mL agarose gel from Day 2
- 1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA) from Day 2
- Round-the-horn PCR mutagenesis reactions from Day 2
- 6X Loading Dye (0.25% Bromophenol blue, 15% Ficoll)
- 1-kb DNA Ladder (NEB 1/8 dil) in 1X Loading Dye *(see the Appendix for the sizes of the 1-kb DNA ladder fragments)*
- Ultrapure dH_2O from Day 1
- Gel electrophoresis apparatus and power supply

Hazardous chemicals used
None, but for safety, wear gloves when handling gels.

Procedure
Use a new tip for every solution and between tubes.

1) For each group, label three 1.5-mL Eppendorf tubes with the names of your three Round-the-horn PCR reactions (#1 – #3). Remove 3 μL of each PCR reaction and place it in the appropriately labeled Eppendorf tube. Add 3 μL of 6X Loading Dye to each PCR gel sample. Then add ultrapure dH_2O such that each gel sample tube has a final concentration of 1X Loading Dye. *(Save the remainder of each PCR mutagenesis reaction for "Part 2.3: Purification of PCR mutagenesis products.")*

DAY 3

2) Centrifuge the three Eppendorf tubes containing your PCR reactions + Loading Dye for ~15 seconds.

3) Unwrap your agarose gel (from Day 2) and place it in a gel box. Add 1X TAE to the gel box so that it just covers the gel. Make sure the gel is NOT upside down (the wells should be open to you).

4) Load the first lane of the gel with 10 μL of the 1-kb DNA Ladder. Then load 15 μL each of your prepared PCR gel samples into the next three lanes. Your partner may load their samples to the next three lanes. (Note the order of the lanes in your notebook!)

5) Connect the electrodes of the power supply and the gel box in the proper orientation so that the DNA runs towards the **positive (red)** electrode.

6) Turn on the power supply and turn the voltage to ~115 volts. Check that the current is actually running through the gel box (you should see bubbles) and check the running direction of the loading dye to make sure the gel orientation is correct. **While your gel is running, you can proceed to "Part 2.3: Purification of PCR mutagenesis products."**

7) Run the gel at a constant voltage until the dye has migrated about two-thirds of the way down the gel (approximately 45 – 60 minutes). Turn off the power supply.

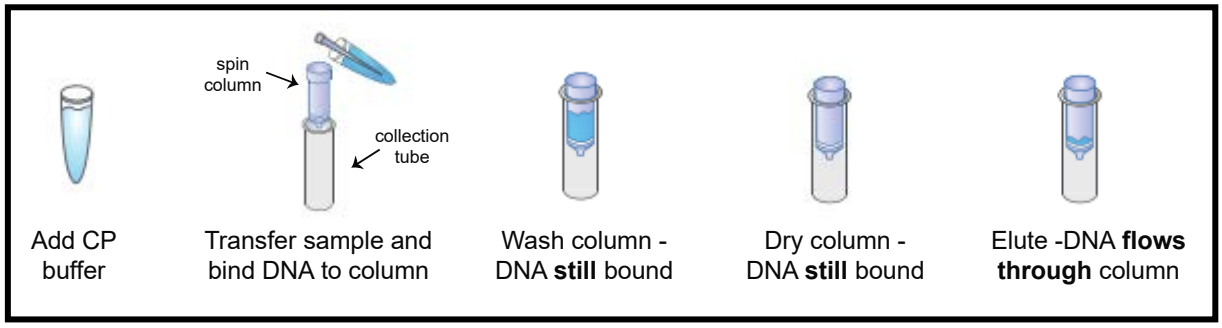
8) Wearing gloves, remove the entire gel tray (with the gel in it) from the gel box and place it in a white plastic box and take it to the gel doc system. The 7.002 staff will take a picture of your gel and upload the image to the 7.002 shared Dropbox folder.

Part 2.3: Purification of PCR mutagenesis products

Overview

Following PCR, the DNA must be purified - separated from the other components of the reaction. You will use the E.Z.N.A. Cycle Pure Kit (Omega) to purify the DNA.

Overview of Spin Column Protocol. *During the first steps, your DNA is on the membrane of the column and the flow-through goes into waste container. During the elution step, you keep the flow-through.*



DAY 3

Materials

- Remainder of PCR mutagenesis reactions #1 and #2 from Part 2.2
- CP Buffer
- DNA Wash Buffer
- Ultrapure dH_2O (from Day 1)
- (2) HiBind DNA spin columns (blue) and collection tubes

Hazardous chemicals used

Guanidine hydrochloride (CP), isopropanol (CP), ethanol (DNA Wash)

Procedure

*This protocol is taken from the E.Z.N.A. Cycle Pure manual (Omega, 2012). Perform this procedure at **room temperature**. Use a new tip for every solution and between tubes. When adding solutions to the spin columns, be careful not to pierce the white silica membrane of the column with your pipette tip.*

1) You will only be purifying the PCR reactions in **Tubes #1 and #2**. You can discard Tube #3. Add five volumes of CP Buffer to each of the remaining PCR reactions in Tubes #1 and #2 (check your calculations with your TA first!). Mix each tube by inverting them five times.

2) Label the two blue HiBind spin columns and their 2-mL collection tubes "#1" and "#2" with your bench number (write on the frosted surfaces). Place the spin columns inside the collection tubes.

3) Transfer all of the PCR/CP Buffer mixture in each tube into the corresponding spin column.

4) Centrifuge the spin columns at 13000 rpm (14000 x g) for 1 minute. Discard the flow-through into a **waste beaker** and place the columns back into the collection tubes.

5) Add 700 μL of DNA Wash Buffer to each column.

6) Centrifuge the spin columns at 13000 rpm (14000 x g) for 1 minute. Discard the flow-through in the waste beaker as before.

7) Centrifuge the spin columns again at 13000 rpm (14000 x g) for 2 minutes.

8) Label two sterile 1.5-mL Eppendorf tubes "#1" and "#2" with your initials and bench number. Remove the spin columns from the collection tubes and place each column into the appropriate labeled Eppendorf tube. The collection tubes can now be discarded.

9) Pipette 30 μL of ultrapure dH_2O directly onto the center of each column membrane; be careful not to touch the membrane with your pipet tip.

DAY 3

10)Let the columns sit undisturbed (with the water in them) for 2 minutes.

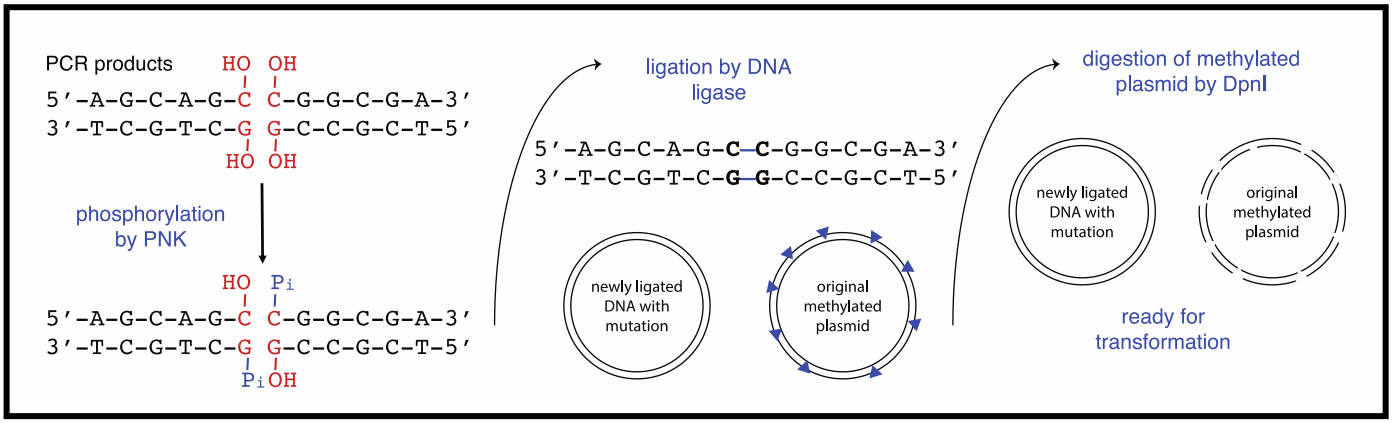
11)Centrifuge the Eppendorf tubes with the columns in them for 1 minute at 13000 rpm (14000 x g). Discard the spin columns and keep the Eppendorf tubes which should now contain ~30 μL of your purified PCR reactions #1 and #2. Place these tubes on ice.

12)Check on your agarose gel from Part 2.2 if you have not done so yet – if it's ready, you can view your gel on the gel doc station and have a picture taken. If your gel is not done, you can proceed to Part 2.4 while you wait for the gel to finish running.

Part 2.4: Digestion, phosphorylation, and ligation of purified PCR products

Overview

You will eventually transform the PCR samples into *E. coli* bacteria to make more copies of the synthesized products for further study, but first you need to prepare the DNA from the PCR to have the best outcome in your transformation. You will prepare **one reaction that will accomplish three important steps**: phosphorylation of PCR products, circularization (ligation), and digestion of wildtype template.



Overview of Three Molecular Cloning Steps.

Materials

- Purified PCR mutagenesis reactions #1 and #2 from Part 2.3
- 10X T4 Ligase Buffer (NEB)
- DpnI restriction enzyme (20 units/μL)
- T4 Polynucleotide Kinase (10 units/μL)
- T4 DNA Ligase (100 units/μL)
- Ultrapure dH_2O from Day 1

DAY 3

Hazardous chemicals used
None

Procedure
Use a new tip for every solution and between tubes. Keep all reagents and tubes on ice.

1) For each group, label two sterile 0.5-mL Eppendorf tubes "Lig #1" and "Lig #2" with your **initials and bench number**.

2) Pipette 10 μL of each purified PCR reaction from Part 2.3 into the appropriately labeled 0.5-mL Eppendorf tube.

3) Label a 1.5-mL Eppendorf tube "Mix." Add the reagents to this tube in the following order:

   12.5 μL of 10X T4 Ligase Buffer
   2.5 μL of DpnI
   2.5 μL of T4 Polynucleotide Kinase
   2.5 μL of T4 DNA Ligase
   80 μL of ultrapure dH_2O

4) Quick-spin the "Mix" tube for ~15 seconds.

5) Pipette 40 μL from the "Mix" tube into each of Tubes #1 and #2. Pipet up and down several times to mix the contents in each tube.

6) Quick-spin Tubes #1 and #2 for ~15 seconds. These tubes now contain the following reagents:

   10 μL of purified PCR reaction DNA (either #1 or #2)
   5 μL of 10X T4 Ligase Buffer
   1 μL of DpnI
   1 μL of T4 Polynucleotide Kinase
   1 μL of T4 DNA Ligase
   32 μL of dH_2O
   Total: 50 μL

7) Give your sample tubes (Lig #1 and Lig #2) to the teaching staff. They will place them in a thermocycler to incubate at 37°C for 16 hours, after which they will be frozen at -20°C until the following lab day. One set of the samples will be returned for Day 4 Transformation. The other set will be saved as your backup. Also give the teaching staff your remaining PCR samples (we will keep them as backup samples for the future).

DAY 3

Day 3 Post-Lab Notebook Tips & Reminders

- Include the gel photo properly labeled such that it's clear **what is loaded in each lane and what the sizes of the ladder bands are**.

- Discuss each lane in your gel in terms of expected vs. observed results and what you can conclude from each lane (similar lanes/samples can be discussed together as a group). If you did not get the expected results, what might be a possible reason to explain the difference?

- Did you deviate from the protocol at any step?

- Did you finish pouring the LB+Amp plates?

Day 3 ILQs

Work on these questions alone or with your partner during your downtime in lab and then discuss them with your TA. You need to write out solutions to receive credit.

Question 1

A. Ginny has performed PCR to amplify a 2.5 kb region of a plasmid that is 5 kb long. She runs her PCR product on a gel and notices that she has bands at 3 kb, 2.5 kb, and 1 kb. Suggest modifications to her PCR protocol that may improve her PCR result.

B. Neville is performing Round-the-Horn PCR on a 7 kb plasmid. He runs a gel to check his PCR, and finds that he only has a very faint smear of bands 5 kb and under. Suggest some potential protocol modifications.

Question 2

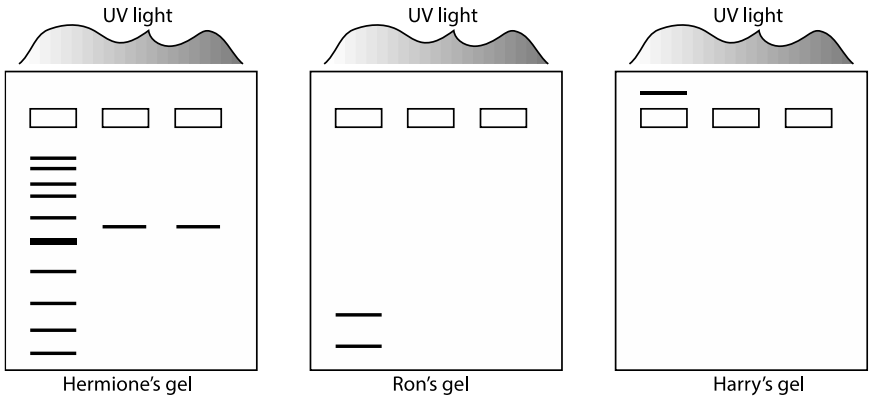
A. Seamus decides to run his first agarose gel. He asks if it is necessary that he use TAE for the gel and buffer. He'd prefer to save some money and use tap water instead. Why is it advisable to use TAE (made from deionized water) instead of tap water?

B. You have convinced Seamus to use TAE, but he now wants to run his gel at 200V to save time. Why is it not advisable to run an agarose at such high voltage?

Day 3 ILQs

Question 3

A. Three biology students each perform PCR on two samples with an expected product of ~3.5 kb. They run their PCRs on agarose gels, with their results illustrated below. Hermione did a brilliant job. What did Ron and Harry do wrong?



B. Hermione goes to purify her PCR products using a silica membrane spin column. She mixes her PCR samples with TE (Tris-EDTA) and is about to load them into the spin column when you stop her with a charm. What is wrong with her purification strategy?

Question 4

The ligation reaction you are performing today will seal together the ends of your PCR products, hopefully circularizing them into plasmids. But it is also possible for two PCR products to ligate together. How do you think we favor the intramolecular ligation (one PCR product circularizing) over the intermolecular ligation (two or more PCR products ligating together)? *Hint: a typical ligation reaction volume is ~10 ul.*

DAY 4

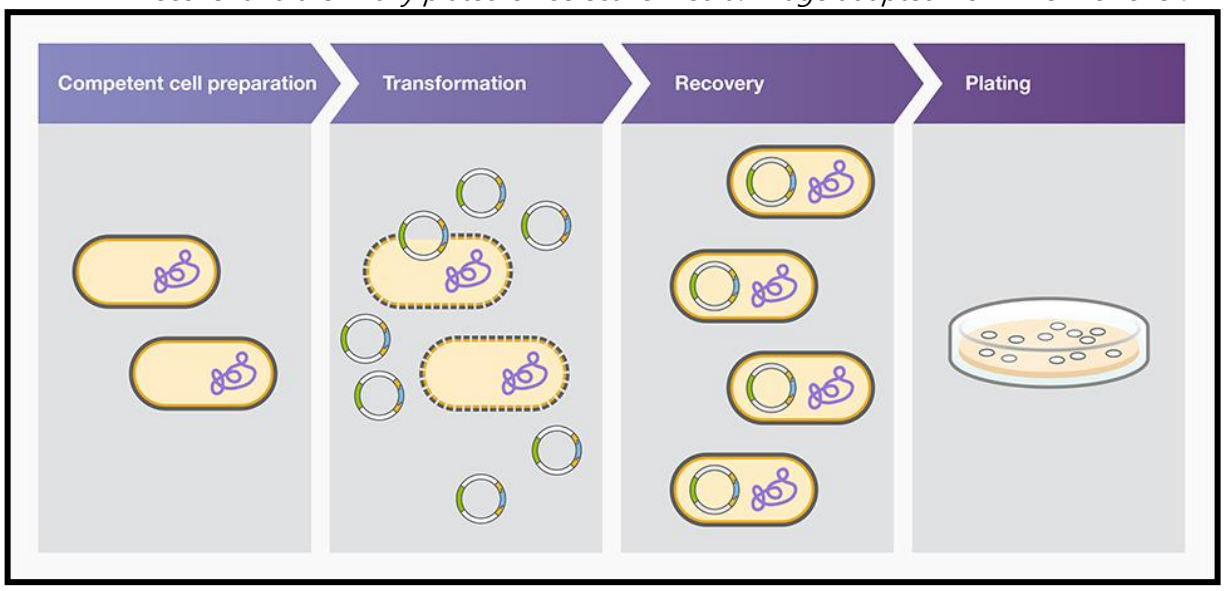
Lab Objective 2 (continued): Mutagenesis of the *clpX* gene

Part 2.5: Transformation of *E. coli* DH5α with PCR ligations

Overview

This week you will transform DH5α bacteria with your PCR mutagenesis digestion/ligation reactions from Day 3 in order to isolate and amplify a mutant ClpX-containing plasmid.

Transformation Workflow. *Cells were pretreated with $CaCl_2$ and frozen at -80. These competent cells are thawed on ice and incubated with DNA. Transformation occurs through heat shock. Cells are allowed to recover and are finally plated on selective media. Image adapted from ThermoFisher.*



Materials

- 200 μL DH5α competent cells (tubes labeled "D")
- PCR mutagenesis digestion/ligation reactions #1 and #2 (from Part 2.4)
- pUC18 plasmid (0.025 ng/μL, control plasmid containing *ampR* gene, see Appendix for plasmid map)
- Ultrapure dH_2O (from Day 1)
- SOC medium
- (4) 14-mL round-bottom Falcon tubes
- (4) LB+Amp agar plates (100 μg/mL ampicillin) (from Day 3)
- (4) 18-mL culture tubes
- LB Broth (from Day 1)

Hazardous chemicals used None

DAY 4

Procedure

Treat competent cells gently – they are very delicate (do not over-pipet them and do NOT vortex them!). Use sterile technique when plating bacteria. Use a new tip for every solution and between tubes.

1) For each group, label four 14-mL round-bottom Falcon tubes with "#1," "#2," "pUC18," and "No DNA," along with your **bench number**. Keep these four tubes on ice.

2) Into the appropriate tube, add 4 μL of each DNA sample (PCR sample #1, sample #2, and pUC18 plasmid). Add 4 μL of ultrapure dH_2O to the "No DNA" tube.

3) Thaw the DH5α competent cells on ice (this should only take a few minutes or so). Add 50 μL of cells to each of the four 14-mL Falcon tubes. Make sure the cells contact the DNA/water at the bottom of the tube (pipette the cells directly onto the DNA). Mix gently by swirling the Falcon tubes (do NOT pipette the cells up and down!).

4) Incubate the cells with the DNA on ice for 30 minutes. During this incubation time, you can prepare LB+Amp tubes while you're waiting (see Step 11).

5) Heat-shock the cells by placing the four Falcon tubes in the rack in the 42°C water bath for **45 seconds**. (Note: Cells should be heat-shocked at exactly this temperature for this exact time to ensure maximum transformation efficiency.)

6) Immediately after the heat-shock step, place the four tubes on ice for 2 minutes.

7) Add 450 μL room-temperature SOC medium to each Falcon tube. Incubate the four tubes on the roller drum in the 37°C warm room for 1 hour to let the cells recover. *(Balance your tubes on the roller drum and make sure to turn the roller drum back **on** after adding or removing your tubes!)*

8) Label four LB+Amp agar plates with the names of your four transformation reactions and your **bench number**.

9) After the 1-hour incubation is complete, plate 100 μL of each transformation mixture onto the appropriately labeled LB+Amp plate, using either sterile plastic spreaders or sterile glass beads in your drawer. Use a new spreader or new beads for each plate!

10) Tape the four plates together (label the tape with your bench number). Incubate the plates upside-down in the 37°C warm room overnight. The 7.002 staff will then store the plates at 4°C until the next lab session.

DAY 4

11) In preparation for next week, you will fill and label tubes of media today. For each group, obtain four glass test tubes from your instructor and label them #1 – #4, along with your bench number (when labeling the tubes, please place the tape on the plastic blue caps, not on the glass tubes).

12) Review sterile technique with your TA or an instructor before starting this step. Using sterile technique, pipette 5 mL of the LB broth that you made on Day 1, into each of the four labeled glass tubes. Leave these tubes on the bench for your instructor to collect. The 7.002 staff will add ampicillin to the media and set up overnight cultures for your samples to be ready for next week. (Ampicillin can degrade over time, which is why we will be adding it right before inoculation).

Day 4 Post-Lab Notebook Tips & Reminders

- Did you complete all steps as documented in the protocol?

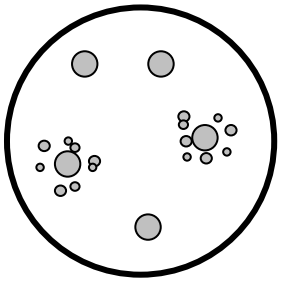
Day 4 ILQs

Work on these questions alone or with your partner during your downtime in lab and then discuss them with your TA. You need to write out solutions to receive credit.

Question 1
Transformation is a very inefficient process. When you attempt to introduce plasmid DNA into bacteria, the vast majority of bacteria do not take up the plasmid. How are you efficiently selecting for bacteria that were successfully transformed?

Question 2
Bumblebee wishes to transform Plasmid K (which contains a kanamycin-resistance Kan^R selectable marker) into DH5α bacteria. He uses a transformation protocol similar to the 7.002 protocol. However, he is impatient, and after heat-shocking the bacteria cells at 42°C, he immediately spread the cells on LB+Kan agar plates, without doing the 1-hour 37°C recovery step. When he checks his transformation plates the next day, there are no colonies on any of the plates. What is the most likely reason why?

Question 3
Some students transformed the pUC18 control plasmid into *E. coli* bacteria and plated on LB+Amp plates. Upon checking their plates the next day, a few students saw some normal-sized colonies surrounded by several smaller colonies, as shown:



Curious about these unexpected small colonies, Jazz picked three of the normal-sized colonies and three of the small colonies and streaked each colony onto a separate fresh LB+Amp plate. She incubated all the plates at 37°C overnight. When she checked the plates the next day, she found that all three of the normal-sized colonies had grown, whereas the small colonies had not.

What is the most likely explanation for the presence of the small colonies on some of the transformation plates?

Day 4 ILQs

Question 4
Convoy performs RTH mutagenesis on pET-ClpX, runs a beautiful gel and successfully purifies his PCR products. He incubates the purified PCR products with T4 DNA Ligase and 1X Ligase Buffer overnight, then directly transforms the ligation products into DH5α *E. coli* bacteria.

From the possible sets of transformation results below (A – F), which set of results would most likely match Convoy's transformation results? Why? Explain your reasoning.

	Results Set A	Results Set B	Results Set C	Results Set D	Results Set E	Results Set F
Reaction #1 (template + primer)	**0 colonies**	**0 colonies**	**100 colonies**	**100 colonies**	**100 colonies**	**300 colonies**
Reaction #2 (template- only)	**0 colonies**	**100 colonies**	**0 colonies**	**100 colonies**	**300 colonies**	**100 colonies**
No DNA	**0 colonies**	**0 colonies**	**0 colonies**	**0 colonies**	**0 colonies**	**0 colonies**
pUC18	**900 colonies**	**900 colonies**	**900 colonies**	**900 colonies**	**900 colonies**	**900 colonies**

.

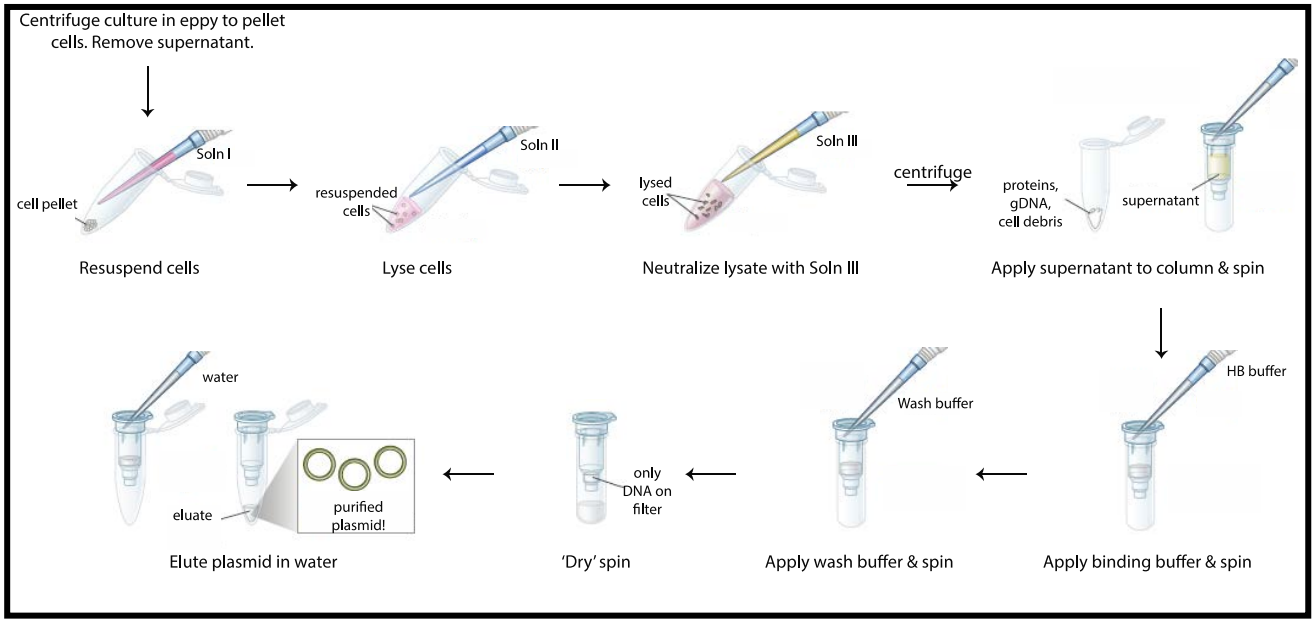
DAY 5

Lab Objective 2 (continued): Mutagenesis of the *clpX* gene

Part 2.6: Miniprep of putative mutant *clpX* plasmid DNA from bacteria

Overview

Today, you will isolate plasmid DNA containing your putative mutated *clpX* gene from the DH5α cells you transformed last week. You will be using the E.Z.N.A. Plasmid DNA Mini Kit (Omega), which is based on the traditional alkaline lysis miniprep procedure.



Miniprep Workflow. *Image adapted from NEB to model the Omega workflow.*

Materials

- (4) 18-mL glass tubes with 5-mL LB+Amp liquid media (100 μg/mL amp)
- Reaction #1 DH5α transformation plate from Day 4
- Solution I Buffer
- Solution II Buffer
- Solution III Buffer
- HB Buffer
- DNA Wash Buffer
- Ultrapure dH_2O from Day 1
- (4) Omega HiBind DNA Mini columns (blue) and collection tubes
- Waste Beaker

Hazardous chemicals used

Acetic acid (Solution III), ethanol (DNA Wash), guanidine hydrochloride (Solution III & HB), isopropanol (HB), and sodium hydroxide (Solution II)

DAY 5

Procedure

This protocol is taken from the E.Z.N.A. Plasmid DNA Mini Kit I manual (Omega, 2013). Use a new tip for every solution and between tubes.

Set up overnight bacteria cultures

(Note: The 7.002 staff has done this part for you already)

1) The day before you perform the miniprep, inoculate each of four tubes of LB+Amp with a different **single** colony from the Reaction #1 transformation plate, using a new stick for each colony. Use 5 mL LB+Amp for inoculation.

2) Incubate the four tubes on the roller drum in the 37°C warm room overnight.

Isolation of plasmid DNA from bacteria cultures *(You start here!)*

3) **You start here!** For each group, label four sterile 1.5-mL Eppendorf tubes with the names of your four overnight bacteria cultures (#1 - #4) and your **bench number**.

4) Transfer 1.5 mL of the bacteria overnight culture from each glass tube into the corresponding Eppendorf tube. Make sure the culture is fully resuspended (e.g. mix by pipetting up and down several times) before you transfer it to ensure the cells are uniformly dispersed.

5) Centrifuge the four Eppendorf tubes for 1 minute at 13000 rpm (14000 x g). Carefully remove the supernatant and discard it in a waste beaker labeled **"Bacteria Waste"** (do not disrupt the cell pellet!).

6) Add 250 μL of Solution I Buffer to each tube. Pipette the mixture up and down in the tube several times to completely resuspend the cell pellet.

7) Add 250 μL of Solution II Buffer to each tube to lyse the cells. Mix well by gently inverting each tube several times. Do NOT vortex the tubes. Continue inverting the tubes gently for at least 2 – 3 minutes, until the solution is of uniform consistency and no cell clumps are present. Do NOT let the lysis step last for more than four minutes though (i.e. once you add Solution II Buffer, you must proceed to Step 8 within four minutes).

8) Add 350 μL of Solution III Buffer to each tube and immediately mix each tube well by gently inverting each tube five times. Do NOT vortex the tubes!

9) Centrifuge the Eppendorf tubes at 13000 rpm (14000 x g) for 10 minutes. During this spin step, label the four HiBind blue spin columns and collection tubes from "#1" to "#4" with your bench number. Place a spin column in each collection tube.

DAY 5

10) After the centrifugation step, use a P1000 to carefully transfer the supernatant from each Eppendorf tube into the corresponding blue spin column, taking care not to touch the silica membrane of the column with your tip. Do NOT transfer any of the cell debris pellet.

11) Centrifuge the four spin columns in the collection tubes for 1 minute at 13000 rpm (14000 x g). Discard the flow-through in the collection tubes into a **new** waste beaker labeled **"Omega Waste."**

12) Add 500 μL of HB Buffer to each spin column and centrifuge for 1 minute at 13000 rpm (14000 x g). Discard the flow-through into your Omega waste beaker.

13) Add 700 μL of DNA Wash Buffer to each spin column and centrifuge for 1 minute at 13000 rpm (14000 x g). Discard the flow-through into your Omega waste beaker.

14) Centrifuge the empty spin columns again for 2 minutes at 13000 rpm (14000 x g). This additional spin step is important for removing any leftover ethanol from the DNA Wash Buffer.

15) Label four sterile 1.5-mL Eppendorf tubes with the names of your four miniprep samples ("MP #1" – "MP #4") and your **bench number**. Remove the spin column from each collection tube and place it in the corresponding "MP" Eppendorf tube. Pipette 30 μL of ultrapure water to the center of each column (but be careful not to touch the membrane with your pipet tip).

16) Let the spin columns sit (with the water in them) for 2 minutes.

17) Centrifuge the Eppendorf tubes with the spin columns in them for 1 minute at 13000 rpm (14000 x g). Save the "MP" Eppendorf tubes (which now should each contain ~30 μL of purified miniprep plasmid DNA) and keep them on ice. The spin columns can now be discarded.

Part 2.7: Preparation of *clpX* miniprep DNA for sequencing

Overview

Before doing anymore experiments with your plasmid, you need to confirm you have successfully mutated the *clpX* gene sequence. You will send your four plasmid samples for Sanger sequencing at Quintara BioSciences.

Quintara will provide the sequencing primer for your reactions, but we need to tell them which primer to use. During your pre-lab, you measured the distance from your mutation to potential primers. Your measurements:

DAY 5

Distance from mutation to T7 promoter primer: _____ bases

Distance from mutation to T7 terminator primer: _____ bases

Which primer should you use? _____

Discuss your answer with your instructor. Your instructor will confirm the correct choice and complete a submission form for all students.

Materials

- (4) pET-ClpX plasmid miniprep DNA samples from Part 2.6
- T7 Promoter Primer (5 μM, 5' – TAATACGACTCACTATAGGG – 3')
- T7 Terminator Primer (5 μM, 5' – GCTAGTTATTGCTCAGCGG – 3')
  (both primers will be provided at Quintara Biosciences)
- Ultrapure dH_2O from Day 1
- 0.2-mL 8-tube strips

Hazardous chemicals used

None

Procedure

Use a new tip for EVERY step when switching between samples and primers.

1) Use the spectrophotometers and the microcuvettes to measure the DNA concentration in each of your miniprep samples (see lab manual Appendix for complete instructions on how to use the specs and microcuvettes). Because you used ultrapure water to elute your plasmid DNA, you should use ultrapure water as your blank.

   Record the final DNA concentration and A_{260}/A_{280} ratio for each of your miniprep samples in your **notebook** (you may also record them here and enter them in your electronic notebook later). Take a photo of the absorption spectrum from the spectrophotometer for each sample as a record in your lab notebook.

   MP #1 [DNA]:_____ A_{260}/A_{280}:_____

   MP #2 [DNA]:_____ A_{260}/A_{280}:_____

   MP #3 [DNA]:_____ A_{260}/A_{280}:_____

   MP #4 [DNA]:_____ A_{260}/A_{280}:_____

DAY 5

2) The Quintara facility requires miniprep plasmid DNA templates be submitted at a final concentration of 80 ng/μL. For each of your four miniprep samples, calculate how much plasmid and how much ultrapure water you need to mix to end up with 12 μL total volume of diluted miniprep DNA at 80 ng/μL final concentration. **Check your calculations with your instructor and include this calculation in your Post-Lab**!

3) Label four 0.5-mL Eppendorf tubes with "dil" (for "dilute") and the names of your four miniprep samples. Dilute each miniprep in sterile water to a final concentration of 80 ng/μL in a final total volume of 12 μL according to your calculations from Step 2. Mix the contents of each tube by gently pipetting up and down several times and quick spin them for ~15 seconds. **When setting up your sequencing samples in Step 7, remember to use these diluted miniprep samples!**

4) The class sequencing samples will be sent to Quintara in small 8-tube strips, numbered sequentially (1, 2, 3, 4, etc). Each lab pair will be assigned four class sample numbers – check with your instructor to find out which four class sample numbers have been assigned to you.

5) Decide which of your four miniprep samples (e.g. MP #1, MP #2, etc) will be associated with each class sample number you were assigned. Record this information in your **notebook** (you will need it for your Post-Lab!). For instance, if you were assigned the four class numbers of 17, 18, 19, and 20, then you load MP #1 to tube 17, MP#2 to tube 18, MP#3 to tube 19, and MP#4 to tube 20. Your sequencing results will return with your group and MP #.

   Temporary record of sample IDs:_____

6) Obtain the appropriate set of 8-tube strips corresponding to your four assigned class sample numbers from your instructor (note that each strip consists of 8 tubes connected together, so you will be sharing the same strip with another group). Do not remove the tube strip from the rack and check to make sure that within the strip there are four tubes correctly labeled with your four assigned class sample numbers.

7) Add 10 μL of each **diluted** miniprep plasmid DNA into its corresponding tube in the strip. Make sure you match each miniprep sample with its appropriate class sample number tube in the strip as in Step 5 (e.g. add MP#1 to Tube 17, add MP#2 to Tube 18, etc). If you accidentally add the wrong sample into the wrong tube, let your instructor know so we can prepare and label a new 8-tube strip for you.

8) When finished, return the 8-tube strips to your TA. These will be collected by the 7.002 staff to be sent off to the sequencing facility.

DAY 5

9) Give the "MP" 1.5-mL Eppendorf tubes containing the remainder of your four (undiluted) DNA minipreps to your TA – these will be stored at -20°C until the next lab day.

Part 2.8: Analysis of *E. coli* DH5α PCR transformations

Overview

We want to know how well our transformations worked for future reference. You will analyze your transformation plates and calculate transformation efficiency of your positive control.

Materials

- (4) DH5α transformation plates from Day 4

Procedure

Use a Sharpie to mark off colonies as you count to prevent counting the same colony twice.

1) Count and record the number of colonies on each transformation plate. Calculate the transformation efficiency for your pUC18 transformation (the only transformation reaction where we know exactly how much DNA was added to the reaction).

$$\text{Transformation efficiency} = \frac{\text{Number of transformants (colonies) on a plate}}{\text{Total } \mu\text{g of DNA used to transform the cells}}$$

The units of transformation efficiency are cfu (colony forming unit) / μg DNA. If you had a lawn on your pUC18 plate (too many colonies to count), assume you had 3000 colonies.

Day 5 Post-Lab Notebook Tips & Reminders

- Be sure to indicate any unique reagents / labels you used that were not specified in the lab manual (e.g. for setting up the samples in Part 2.7).
- Record colony counts on all transformation plates. Remember to comment on each plate in terms of expected vs. observed results and what you can conclude from each. You may include an image of the plates if you would like.
- Remember to include calculations for the transformation efficiency of the pUC18 plate.
- Did you deviate from the protocol?

Day 5 ILQs

Work on these questions alone or with your partner during your downtime in lab and then discuss them with your TA. You need to write out solutions to receive credit.

Question 1
You are explaining your 7.002 project to a new student, but he is having trouble understanding all the different parts of the *clpX* gene. You realize he is not familiar with gene structure in general. Diagram the different elements of a gene. Include a promoter, a ribosome binding site (RBS), start codon, stop codon, and terminator.

Question 2
Four students have DNA samples that they analyze with a spectrophotometer. Their results are shown below. If you wanted to borrow a DNA sample to use for future experiments, whose sample would you borrow? Explain your choice.

	DNA Concentration (ng/ul)	A_{260}/A_{280} ratio
Westley	950	2.01
Vizzini	650	1.82
Fezzick	20	1.79
Inigo	1500	1.62

Question 3
A fifth student, Buttercup, has never heard of Sanger sequencing before. You explain that the Sanger Sequencing reaction is similar to a PCR with two major differences in reaction components. What are those two major differences and what is their purpose?

Day 5 ILQs

Question 4

You explain to Buttercup that you are sequencing your pET-ClpX miniprep sample to see if the desired point mutation modification was made correctly. Buttercup suggests you just use one of your original RTH PCR primers for the Sanger sequencing reaction instead of the T7 promoter or terminator primer. Can you use either one of your original RTH PCR primers for your sequencing reaction to check for the proper *clpX* gene mutation? Explain your reasoning.

DAY 6

Lab Objective 3: Expression and purification of mutant ClpX

Please bring a laptop to lab today

Part 3.1: Analysis of sequencing data

Overview

Today, you will interpret the sequencing results from your four minipreps to see if you successfully introduced your mutation into the *clpX* gene on the pET-ClpX plasmid.

You will use the SnapGene program that you used last week to analyze your four plasmid sequences.

Materials

- (4) sets of sequencing data for your four minipreps (posted in the lab Dropbox folder)

Procedure

1) On the 7.002 Canvas site, you will find the Dropbox link for lab data at the bottom of the page. This link has a folder labeled 'Sequencing Data.' Each bench will have 4 sequence files. Find the files that correspond to your four miniprep samples and download them to your computer.

2) Open your *clp*X plasmid DNA file (either one that you downloaded during pre-labs) in SnapGene. Make sure 'Show Alignments' (the bottom icon in the left toolbar) is selected. 

3) Click on 'Aligned Sequences' dropdown and then 'Align Imported Sequences.' Choose the four miniprep sequences you downloaded in Step 1.

4) Walk through each alignment (it is helpful to look at them one at a time). Note if the miniprep contains the desired mutation and no other mutations. You have space to take notes but remember you to properly record everything in your post-lab notebook entry.

   MP #1:

   MP #2:

   MP #3:

   MP #4:

DAY 6

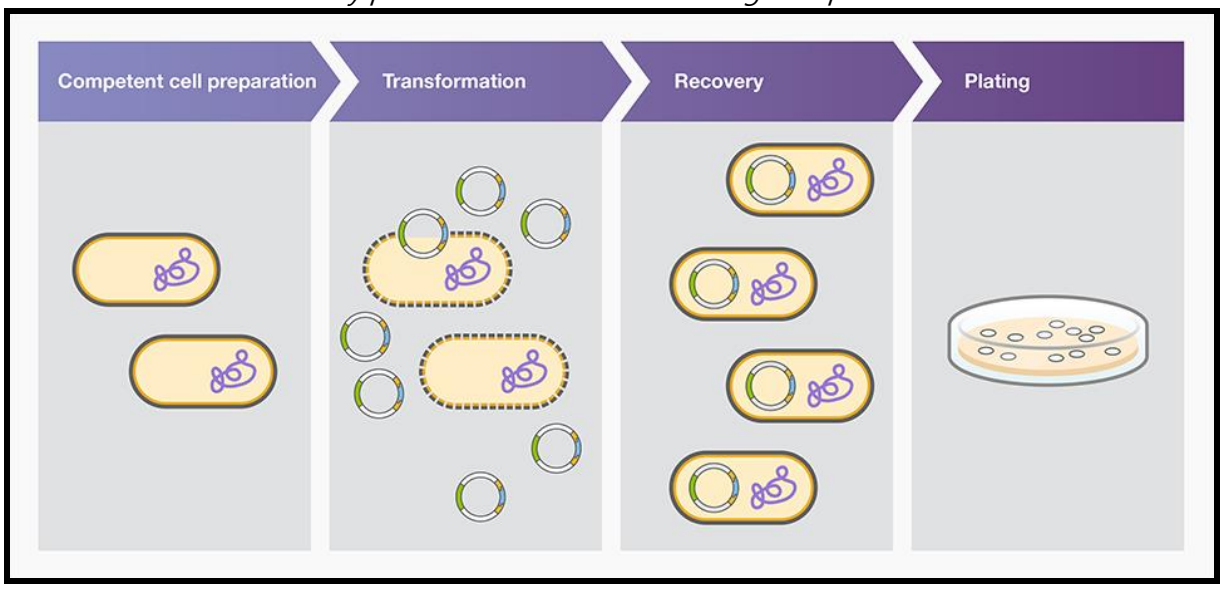
5) Confirm your sequencing results with your TA/instructor. Choose one of your minipreps that has the correct mutation to use for Part 3.2 ("Transformation of *E. coli* BL21(DE3)"). If none of your sequences look good, we will provide you with a backup sample.

Part 3.2: Transformation of *E. coli* LOBSTR BL21(DE3) with mutant *clpX* plasmids

Overview

Your ClpX protein is expressed from your mutated pET-ClpX plasmid, where the *clpX* gene is under the control of the T7*lac* promoter. Expression of *clpX* only occurs under certain conditions, one of which is that T7 RNA polymerase must be present to transcribe the *clpX* gene. To express *clpX*, you will use the *E. coli* strain LOBSTR BL21(DE3), which has been modified so that it contains the T7 RNA polymerase gene in its genomic DNA. Today you will transform *E. coli* LOBSTR BL21(DE3) cells with DNA from the mutated *clpX* miniprep you selected in Part 3.1.

Transformation Workflow Again. *Cells were pretreated with $CaCl_2$ and frozen at -80. These competent cells are thawed on ice and incubated with DNA. Transformation occurs through heat shock. Cells are allowed to recover and are finally plated on selective media. Image adapted from ThermoFisher.*



Materials

- 150 μL of *E. coli* LOBSTR BL21(DE3) competent cells (tubes labeled "L")
- Mutant pET-ClpX miniprep plasmid DNA (from Part 3.1)
- pUC18 plasmid (0.025 ng/μL, see Appendices for plasmid map)
- Ultrapure dH_2O
- SOC medium, pre-warmed to 42°C

DAY 6

- (3) LB+Amp plates (100 μg/mL ampicillin) (from Day 3)
- (3) 14-mL Falcon round-bottom tubes
- (1) 18-mL culture tube
- LB Broth (from Day 1)

Hazardous chemicals used
None

Procedure
Use a new tip for every solution and between tubes and use sterile technique when plating bacteria cells. Treat competent cells gently (do not over-pipette them and do NOT vortex them!).

1) For each group, label three 14-mL Falcon round-bottom tubes as follows: "ClpX," "pUC18," and "No DNA" with your **bench number**. Place these three tubes on ice.

2) Add 2 μL of mutant pET-ClpX miniprep plasmid DNA to the corresponding Falcon tube. For the "pUC18" and "No DNA" tubes, add 5 μL of pUC18 plasmid and 5 μL of ultrapure water, respectively.

3) Collect the competent LOBSTR BL21(DE3) cells from your instructor and place on ice to let them thaw.

4) When the competent cells are completely thawed (it should only take a few minutes), add 50 μL of cells to each of the three 14-mL Falcon tubes. Make sure the cells contact the DNA/water at the bottom of the tube. Mix by GENTLY swirling/tapping the Falcon tubes (do NOT pipette the cells up and down!).

5) Incubate the cells with the DNA on ice for 30 minutes.

6) Heat-shock the cells by placing the three Falcon tubes in the rack in the 42°C water bath for **20 seconds**. (*Note: It is critical that the cells are heat-shocked at exactly this temperature for the exact time stated to ensure maximum transformation efficiency.*)

7) Immediately after the heat-shock step, place the three tubes on ice for 2 minutes.

8) Add 450 μL pre-warmed SOC medium to each Falcon tube.

9) Place the three Falcon tubes on the roller drum in the 37°C warm room and allow the cells to recover at 37°C for 1 hour. *(Balance your tubes and make sure to turn the roller drum back **on** after adding/removing tubes!)*

DAY 6

10) Label the bottom of three LB+Amp agar plates with the names of your three transformation reactions and with your **bench number**.

11) After the 1-hour incubation, plate 50 μL of the "ClpX" transformation mixture onto the appropriately labeled LB+Amp plate. Plate 250 μL each of the "pUC18" and "No DNA" transformation mixtures onto their corresponding LB+Amp plates. Use sterile plastic spreaders or sterile glass beads when plating cells.

12) Tape the three plates together and label the tape with your bench number. Incubate the plates upside-down in the 37°C warm room overnight. The 7.002 staff will then store the plates at 4°C until the next lab day.

13) In preparation for ClpX Day 6.5, you will fill and label tubes of media today. Obtain one glass test tube from your instructor and label it "LB+Amp" with your bench number (when labeling the tubes, please place the tape on the plastic blue caps, not on the glass tubes).

14) Using sterile technique, pipet 2.5 mL of LB broth from Day 1 into the labeled glass tube. Give this tube to your TA. The 7.002 staff will add ampicillin to the media and set up cultures of your samples for Day 6.5.

Part 3.3: Purification buffer preparation

Overview

Today you will prepare buffers that you will use when purifying your ClpX protein. Specifically, you will prepare a pellet buffer (used for as the base of the lysis buffer), a wash buffer to wash your column once your protein is bound, and an elution buffer that will release your mutant protein from the nickel column. You will only need one set of buffers per each group.

Materials

- (3) 15-mL conical tubes
- (1) 1-L graduated cylinder
- 1 M HEPES (liquid)
- 1 M imidazole (liquid)
- 5 M NaCl (liquid)
- 40% Glycerol (liquid)
- Ultrapure dH_2O (from Day 2)

Hazardous Chemicals Used

Imidazole

DAY 6

Procedure

Pellet buffer

Pellet Buffer contains:	The stocks available to you are:
25 mM HEPES pH 7.5	1 M HEPES
500 mM NaCl	5 M NaCl
10 mM imidazole	1 M imidazole
10% glycerol	40% Glycerol

1) Label a 15-mL conical tube with “Pellet Buffer”, your bench number, and date.

2) You will prepare 10 mL of Pellet Buffer. Calculate the amount of each reagent you will need. The remaining volume will be your ultrapure dH_2O.

3) Add each reagent and the dH_2O to the conical tube. Note that the glycerol will be very viscous. Draw up the glycerol slowly, waiting until the glycerol has stopped moving before removing the serological pipette from the glycerol stock. Release the glycerol into the solution in the 50-mL tube, and carefully pipette up and down to remove any residual glycerol from the glass pipette.

4) Cap the 15-mL tube and invert several times to mix. Store your pellet buffer in the rack provided in the 4^{o}C cold room.

Wash buffer

Wash Buffer contains:	The stocks available to you are:
25 mM HEPES pH 7.5	1 M HEPES
150 mM NaCl	5 M NaCl
10 mM imidazole	1 M imidazole
10% glycerol	40% glycerol

1) Label a 15-mL conical tube with “Wash Buffer”, your bench number, and date.

2) You will prepare 10 mL of Wash Buffer. Calculate the amount of each reagent you will need. The remaining volume will be ultrapure dH_2O.

3) Add each reagent and the dH_2O to the conical tube. Note, pipette the glycerol slowly – it is viscous.

4) Cap the 15-mL tube and invert several times to mix. Store your wash buffer in the rack provided in the 4^{o}C cold room.

DAY 6

Elution buffer

Elution Buffer contains:	The stocks available to you are:
25 mM HEPES pH 7.5	1 M HEPES
150 mM NaCl	5 M NaCl
250 mM imidazole	1 M imidazole
10% glycerol	40% glycerol

1) Label a 15-mL conical tube with “Elution Buffer”, your bench, and the date.

2) You will prepare 10 mL of Elution Buffer. Calculate the amount of each reagent you will need. The remaining volume will be ultrapure dH_2O.

3) Add each reagent and the dH_2O to the conical tube.

4) Cap the 15-mL tube and invert several times to mix. Store your elution buffer in the rack provided in the 4°C cold room.

Day 6 Post-Lab Notebook Tips & Reminders

- Record your overall sequencing results for each sample. (Keep in mind that “no sequence obtained” is still a valid result!)
- If a sample had no sequence, suggest why that might have happened.
- Record which miniprep sample you should use or used for the transformation step.
- Indicate if you completed all buffers.
- Indicate if there were any deviations from the protocol.

Day 6 ILQs

Work on these questions alone or with your partner during your downtime in lab and then discuss them with your TA. You need to write out solutions to receive credit.

Question 1
Your UROP advisor gives you a wild-type (WT) *E. coli* strain with an intact *lac* operon and two different mutant *E. coli* strains (Strain A and Strain B) which are defective in regulation of lactose metabolism. She asks you to characterize and identify the specific mutations in Strain A and Strain B. (Note: It is known that Strain A and Strain B each contain only one single mutation.)

You streak the three *E. coli* strains onto LB plates containing X-gal and LB plates containing X-gal and IPTG. IPTG is an allolactose analog that can induce the *lac* operon the same way that lactose can. X-gal is a chemical color indicator that can be cleaved by β-galactosidase (the protein encoded by the *lacZ* gene) to produce a blue pigment. (Note: LB plates have no glucose and no lactose – they use an alternative carbon source instead.)

When you check the color of your cells the next day, you see the following results:

Strain	LB + X-gal	LB + X-gal + IPTG
WT	?	?
Strain A	Blue	Blue
Strain B	Blue	Blue

A. What color would you expect the WT *E. coli* cells to be on the X-gal plates? What color would you expect them to be on the X-gal + IPTG plates? Briefly explain why.

B. From the list below, name two possible locations where either Strain A or Strain B could be mutated. Explain your choices.

lacI coding sequence
CAP binding site
P_{lacZYA} promoter
lacO operator
lacZ coding sequence
lacY coding sequence

Day 6 ILQs

To further characterize the strains, you transform the same three strains with pET-ClpX and plate the transformed cells on XA plates (LB+Amp + X-gal) and IXA plates (LB+Amp + X-gal + IPTG). A map of pET-ClpX plasmid is in the Appendix of your ClpX Lab Manual. When you check your transformants the next day, you see the following results:

Strain	Transformed with pET-ClpX	
	XA plate	IXA plate
WT	?	?
Strain A	Blue	Blue
Strain B	White	Blue

C. What color(s) would you expect the WT *E. coli* strain to be on XA and IXA plates after transformation with pET-ClpX plasmid?

D. From the two possible choices you gave in Part B, state which mutation is in Strain A and which mutation is in Strain B. Explain your reasoning.

Question 2

You are studying ClpX and would like to express ClpX from the pET-ClpX plasmid in *E. coli* bacteria using the 7.002 induction method. However, while you were away from the Spring break, your labmates used up all of the LOBSTR BL21(DE3) cells. Luckily, a neighboring lab offers you three different variations of the expression strain to try instead.

Strain #1: Similar to LOBSTR BL21(DE3), except that all *lacO* sites in the strain's chromosomal genome have been deleted.

Strain #2: Similar to LOBSTR BL21(DE3), except it also contains a plasmid expressing a mutant LacI protein with a mutation in its allolactose-binding domain.

Strain #3: Similar to BL21(DE3)

Day 6 ILQs

A. Which of the strains listed above (#1 - #3) would you not be able to use at all to express ClpX protein from his pET-ClpX plasmid? List all strains that apply. State why the strain(s) would not work for expressing ClpX and explain your reasoning for each.

B. For the strain(s) that you did not pick in Part A, explain why the defect in each strain will not be a problem for your ClpX expression.

DAY 6.5

Part 3.4: Expression of ClpX mutant protein & collection of cell pellet

****Note: This Day 6.5 step will be performed by the 7.002 staff but ALL students should review all the steps for Day 7 Pre-lab.*

Overview
On Day 6, you transformed your mutated pET-ClpX plasmid into LOBSTR BL21(DE3) cells. The 7.002 staff will select colonies from your experimental plate and grow a starter culture in LB+Amp. Once this starter culture has been allowed to grow for several hours, it will be used to inoculate a large-scale culture of Terrific Broth (TB)-Amp autoinduction medium. This large-scale culture will be grown overnight to allow expression of mutant ClpX protein.

The large-scale cultures will be spun down in a floor centrifuge to collect a cell pellet and remove the supernatant. You will start from frozen cell pellet in the following lab day, when you will lyse the cells in the pellet and use affinity chromatography to separate ClpX away from (hopefully) everything else in the cells.

Materials
- pET-ClpX mutant LOBSTR BL21(DE3) transformation plate from Day 6
- (1) 18-mL glass tube containing 2.5 mL LB+Amp medium (100 μg/mL)
- (1) 1-L glass flask with 250 mL TB-Amp medium (100 μg/mL) containing glucose + lactose (TB from Day 2 + added Amp)
- (1) 250-mL plastic centrifuge bottle
- Sterile wooden sticks
- Ultrapure dH_2O

Hazardous chemicals used
None

Procedure
Use sterile technique (e.g. flame tube and flask openings when inoculating cultures, transferring cells, etc.).

Inoculating starter culture and isolating cell pellet
(Note the 7.002 staff have done these steps for you)

1) Using a sterile wooden stick, inoculate 2.5 mL of LB+Amp with a single colony from the pET-ClpX LOBSTR BL21(DE3) transformation plate from Day 6.

2) Grow the LB+Amp starter culture on a roller drum at 37°C until the cells reach mid-logarithmic growth (approximately 3 – 4 hours). The cultures should look cloudy (this will correspond to OD_{600} of ~0.5).

DAY 6.5

3) Transfer the contents of the entire tube of LB+Amp starter culture into a flask containing 250 mL TB-Amp medium. Grow the 250 mL TB-Amp culture for 16 hours at 30^{o}C on a shaker.

4) Transfer the cells to a 250-mL plastic centrifuge bottle and spin the bottle at a high speed. All of the bottles need to be balanced. The weights of the bottles are adjusted by adding ultrapure dH_2O if necessary.

5) Place the bottles in the Sorvall floor centrifuge. Check to make sure the centrifuge is set to spin the cells for 10 minutes at 6000 rpm (5900 x g) at 4^{o}C. Once the centrifuge is done. Gently pull the bottles out (you do not want to disrupt the pellets). Discard the supernatant back into the flask. The cell pellet will be frozen at -80^{o}C until the next lab session.

DAY 7

Lab Objective 3 (continued): Expression and purification of mutant ClpX

Part 3.5: Analysis of *E. coli* LOBSTR BL21(DE3) transformations

Overview
You will analyze the efficiency of your transformation method from last week.

Materials
- (3) LOBSTR BL21(DE3) transformation plates from Day 6

Procedure
1) Count and record the number of colonies on each of your LOBSTR BL21(DE3) transformation plates. Calculate the transformation efficiency for your pUC18 transformation (the only transformation reaction where we know exactly how much DNA was added to the reaction).

$$\text{Transformation efficiency} = \frac{\text{Number of transformants (colonies) on a plate}}{\text{Total } \mu\text{g of DNA used to transform the cells}}$$

The units of transformation efficiency are cfu (colony forming unit) / μg DNA. If you had a lawn on your pUC18 plate (too many colonies to count), assume you had 3000 colonies.

Part 3.6: Purification of mutant ClpX ATPase protein

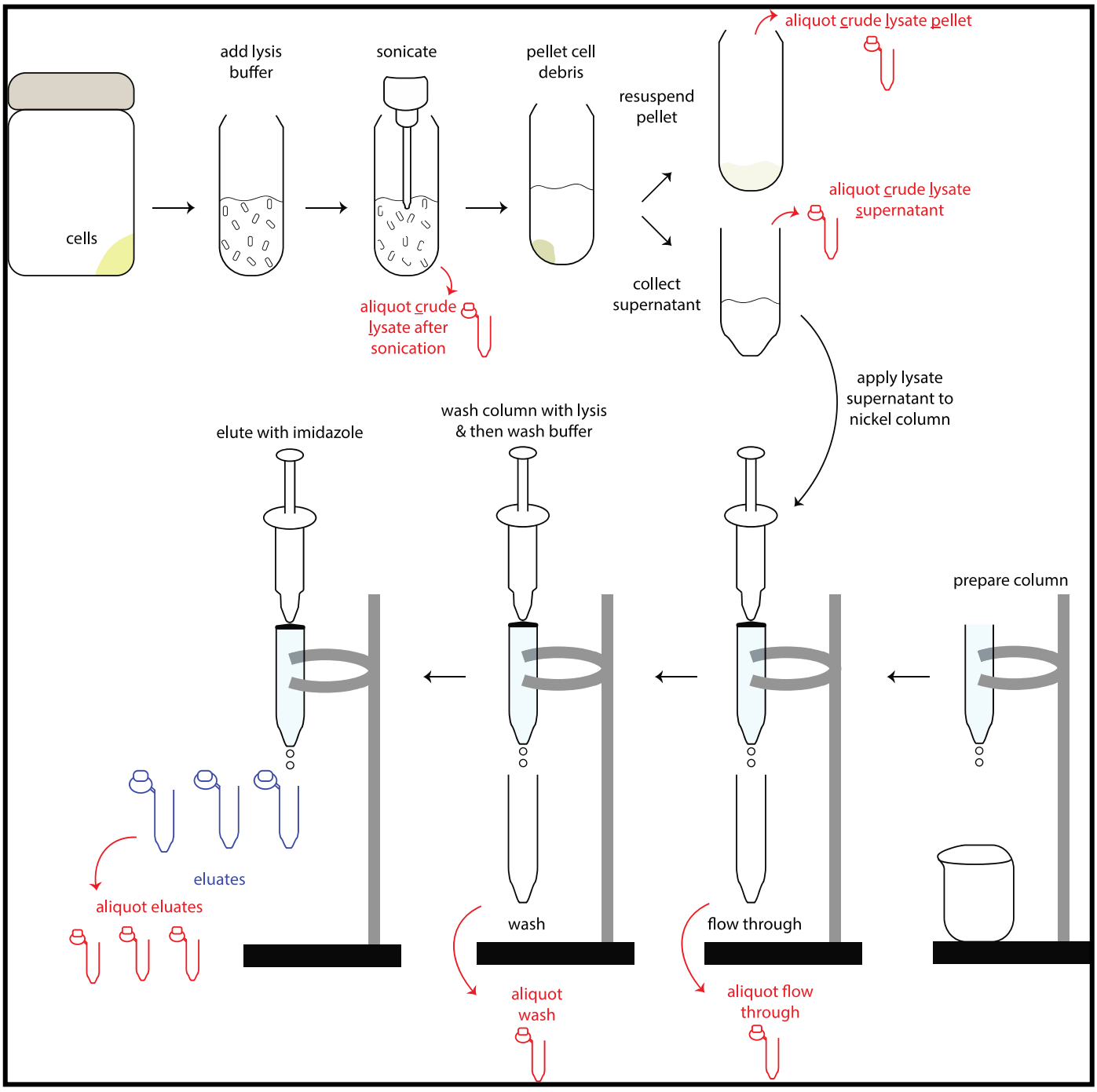
Overview
Today, you will start with a frozen cell pellet. You will lyse those cells both enzymatically and through sonification and load the cell lysate onto a nickel affinity column. Your ClpX protein will bind the nickel column through its N-terminal histidine tag. You will then elute ClpX from the column using imidazole. Along the way you will collect 30-μL aliquots of sample, starting with the cell lysate and ending with the eluates. These 8 aliquots are indicated in red throughout the protocol and will be in 0.5-mL Eppendorf tubes. In a later lab, you will use these aliquots to see how successful each step of your purification process was.

You will also collect three eluate samples in 1.5-mL Eppendorf tubes. They are indicated in blue throughout the protocol. These eluates contain your mutant ClpX protein. Although you will set aside 30-ul aliquots of these for diagnostic assays, you need these samples for the activity assay.

There are a lot of moving parts today. Please see the schematic on the next page for an overview of what you will do.

DAY 7

Workflow of ClpX Purification. *You will collect three eluate protein samples (blue) and eight aliquots (red).*



DAY 7

Materials

- Frozen cell pellet of LOBSTR BL21(DE3) cells expressing mutant ClpX protein, in a 250-mL plastic bottle
- Lysis Buffer (25 mM HEPES pH 7.5, 500 mM NaCl, 10 mM imidazole, 1% protease inhibitor cocktail, 10% glycerol)
- Pellet Buffer (same as Lysis Buffer, but without protease inhibitor cocktail)
- Wash Buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 10 mM imidazole,10% glycerol)
- Elution Buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 250 mM imidazole, 10% glycerol)
- Lysozyme (100 mg/mL, in Lysis Buffer)
- Universal Nuclease (Pierce, 25 U/μL)
- Ultrapure water
- (1) His-Trap Fast-Flow Nickel Sepharose column (1-mL total volume)
- Column adaptor (note: this is NOT disposable!)
- Ringstand and clamps
- (1) 5-mL syringe and small glass transfer breaker
- (1) 30-mL plastic round-bottom centrifuge tube
- (2) 50-mL Falcon tubes
- (2) 15-mL Falcon tubes

Hazardous chemicals used

Dimethyl sulfoxide (DMSO, in the protease inhibitor cocktail); imidazole; and nickel

Procedure

Today, you will be using several different hazardous reagents in relatively large quantities, ***so you should wear safety glasses at all times*** *(in addition to your standard lab coat and gloves). Keep lysate/proteins/reagents on ice whenever possible unless specified otherwise. Make sure you always adhere to the Three Rules of Column Chromatography (listed below) when using the nickel column.*

Recall the Three Rules of Column Chromatography

Rule 1: Never pump air into the column – buffer should drip from the bottom of the syringe before attaching the syringe to the column.

Rule 2: Never let the column run dry - always cap the bottom of the column with the stop-plug when the column is not in use.

Rule 3: Never increase the flow rate through the column above 1 mL/min – buffer should **drip** from the column rather than being a steady stream.

DAY 7

Lysis of cell pellet & preparation of column

1) Let your frozen LOBSTR BL21(DE3) cell pellet (in a 250-mL centrifuge bottle) sit at room temperature until the pellet has thawed (this should only take a few minutes). Once the pellet has sufficiently thawed, add 10 mL of Lysis Buffer to the cells. Completely resuspend the cell pellet by pipetting the solution up and down using a 10-mL glass pipette until no cell clumps are visible. Vortex is ok.

2) Add 200 μL of lysozyme and 10 μL of universal nuclease to the resuspended cell pellet. Make sure the tip is submerged in the cell suspension when pipetting to ensure the reagents are properly added and mixed in. Cap the bottle and gently swirl to mix.

3) Leave the cell suspension on your bench top at room temperature for 20 minutes to incubate (swirl the bottle every few minutes or so during this incubation to mix the contents). During this 20-minute incubation, proceed to Steps 4 – 8 to prepare the nickel column.

4) Place the His-Trap nickel column securely into a clamp on the ringstand. Unscrew and remove the black stop-plug from the top of the column. Pipet ~100 μL of sterile water directly onto the top of the column (this will minimize introduction of air bubbles into the column when you attach the syringe later on).

5) Attach the column adaptor to the 5-mL syringe. Draw 5 mL of ultrapure water into the syringe (you may want to pour the water into a small beaker first and then draw water from the beaker). Remove all air from the bottom of the syringe (gently push the plunger of the syringe until a few drops of water drip out of the adaptor).

6) Attach the syringe (still with the column adaptor on it) to the top of the nickel column. Unscrew and remove the stop-plug from the bottom of the column.

7) Pass 5 mL of water through the column by applying GENTLE pressure to the syringe plunger. Keep the flow rate less than 1 mL/min and collect the flow-through in a waste beaker. ***(Note: A flow rate of 1 mL/min corresponds approximately to one drop exiting the column every two seconds or so.)***

8) After 5 mL of water has passed through the column, cap the bottom of the column with the stop-plug. Remove the syringe (with the column adaptor) from the column.

9) After the 20-minute lysozyme incubation is complete, use a glass pipette to transfer the entire cell suspension from the centrifuge bottle into a round-bottom plastic centrifuge tube. Place a small piece of tape on the side of the tube and label it with your bench number.

DAY 7

10) Bring the round-bottom centrifuge tube containing your cell suspension to Bench B/C and leave it in the labeled ice buckets provided. The 7.002 teaching staff will take your cell suspension and sonicate it for two pulses of 30 seconds each. Afterwards, the teaching staff will return your sonicated cell suspension sample back to your bench.

11) Once you have your sonicated cell suspension back, **transfer 30 μL** of the cell suspension (the "crude lysate") to a 0.5-mL Eppendorf tube. Label this tube "**CL**" with your bench number and keep it on ice.

12) After you've taken the "CL" 30-μL aliquot, give the round-bottom centrifuge tube containing the rest of your sonicated cell suspension to your instructor and then proceed to Steps 13 – 14 to equilibrate the nickel column. While you are equilibrating the column, your cell lysate tube will be spun at 12000 rpm (23400 x g) for 20 minutes at 4°C in the Sorvall floor centrifuge.

13) Draw 5 mL of Lysis Buffer into the syringe (with the adaptor) and remove all air. Attach the syringe (with the adaptor) to the top of the nickel column.

14) Remove the bottom stop-plug from the column and GENTLY pass 5 mL of Lysis Buffer through the column, keeping the flow rate below 1 mL/min. Collect the flow-through in your waste beaker. Cap the bottom of the column with the stop-plug and remove the syringe (with the adaptor) from the column.

15) After the 20-minute centrifugation step is complete, use a 5-mL or 10-mL glass pipette to carefully transfer just the supernatant from the centrifuge tube to a 50-mL Falcon tube labeled "CL-S" (for "Crude Lysate Supernatant") and keep it on ice. **Transfer 30 μL** of the contents of this Falcon tube to a 0.5-mL Eppendorf tube – label this tube as "**CL-S**" with your bench number and keep it on ice.

16) Add 5 mL of Pellet Buffer to the pellet still remaining in the 30-mL centrifuge tube. Resuspend the pellet the best you can and then **transfer 30 μL** of the suspension into a 0.5-mL Eppendorf tube – label this tube as "**CL-P**" (for "Crude Lysate Pellet") with your bench number and keep it on ice. (*Note: It will be very difficult to resuspend the pellet – if necessary, you can vortex the centrifuge tube and/or use a glass pipette to physically mix the cell pellet by hand. If the pellet still does not go completely back into solution, just do your best to transfer ~30 μL to the "CL-P" tube.*)

Purification of ClpX protein from the cell lysate

17) Draw 5 mL of the "CL-S" sample from your 50-mL Falcon tube into the syringe with the adaptor. Remove all air and attach the syringe to the top of the nickel column.

DAY 7

18) Label a 15-mL Falcon tube as “FT” (for flow-through) and place it in a rack under the bottom of the column. Remove the stop-plug from the column bottom and gently pass 5 mL of the “CL-S” sample through the column. Collect the flow-through in the “FT” tube. Cap the bottom of the column with the stop-plug again when finished. (*Note: This is the step when you are most likely to experience back-pressure. Remember, do NOT try to force the sample through the column – apply only gentle pressure on the syringe plunger at all times and keep the flow-rate less than 1 mL/min.*)

19) Repeat Steps 18 – 19 until all of the “CL-S” sample from the 50-mL Falcon tube has been passed through the column. You can use the same “FT” 15-mL Falcon tube to collect the flow-through each time.

20) **Transfer 30 µL** of the contents of the “FT” Falcon tube to a 0.5-mL Eppendorf tube – label this Eppendorf tube as “**FT**” with your bench number and keep it on ice.

21) Draw 5 mL of Lysis Buffer into your syringe with the adaptor. Remove all air and attach the syringe to the top of the nickel column.

22) Label a 15-mL Falcon tube as “Wash” and place it in a rack under the column. Remove the bottom stop-plug and gently pass 5 mL of Lysis Buffer through the column. Collect this flow-through in the “Wash” tube. Cap the bottom of the column.

23) Draw 5 mL of Wash Buffer into your syringe with the adaptor. Remove all air and attach the syringe to the top of the nickel column.

24) Remove the bottom stop-plug from the column and gently pass 5 mL of Wash Buffer through the column. You can use the same “Wash” 15-mL Falcon tube from before to collect the flow-through. Your column will now have been washed with a total volume of 5 mL of Lysis Buffer followed by 5 mL of Wash Buffer.

25) **Transfer 30 µL** from the “Wash” Falcon tube to a 0.5-mL Eppendorf tube. Label this Eppendorf tube as “**Wash**” with your bench number and keep it on ice.

26) Draw 3 mL of Elution Buffer into the syringe with the adaptor. Remove all air and attach the syringe to the top of the column.

27) Label three 1.5-mL Eppendorf tubes “#1” to “#3.” Place Tube #1 under the bottom of the column, keeping the other two tubes ready close-by.

DAY 7

28) Remove the stop-plug from the column bottom and gently pass 1 mL of Elution Buffer through the column. Collect the 1 mL of eluate in Tube #1. Decrease pressure on the syringe plunger to slow the flow rate. Close Tube #1 and place it on ice. Place Tube #2 under the column bottom. Reapply pressure to the syringe plunger until you have collected 1 mL eluate in Tube #2. Close Tube #2 and place it on ice. Continue in this manner to collect another 1 mL of eluate in Tube #3. Cap the bottom of the column when finished. You should now have three 1.5-mL Eppendorf tubes, each containing around 1-mL fraction of eluate. Keep these tubes on ice.

29) Label three 0.5-mL Eppendorf tubes as "**Elu1**," "**Elu2**," and "**Elu3**" (for Eluate Fractions #1 - #3), along with your bench number. **Transfer 30 μL** of each eluate fraction into its corresponding 0.5-mL Eppendorf tube and keep on ice.

30) Make sure the nickel column bottom is capped with the stop-plug. Remove the syringe/adaptor and cap the top of the column with the other stop-plug – the column should now be plugged at **both** ends with black stop-plugs. **Give the column AND the adaptor back to your instructor – do NOT discard them!!**

31) Throughout the ClpX protein purification procedure, you collected eight 30-μL aliquots in 0.5-mL Eppendorf tubes. Make sure these tubes are labeled with your bench number and keep them on ice. Your TA will collect these samples and stored at -20°C after Part 3.7. You will analyze these samples by SDS-PAGE on the next lab day. Continue onto Part 3.7 with your final samples.

Part 3.7: Determination of purified mutant ClpX protein concentration

Overview

You will next use the spectrophotometer to measure the absorbance of your three eluate samples at 280 nm wavelength of light. With your absorbance values and the equation you derived in your prelab, you will determine the protein concentration of your three eluate samples.

Materials

- Eluate Fractions #1 – #3 from Part 3.6 (1.5-mL Eppendorf tubes containing ClpX fractions #1 – #3)
- Elution Buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 250 mM imidazole, 10% glycerol)
- Cuvettes and spectrophotometer

Hazardous chemicals used

Imidazole

DAY 7

Procedure

Use a new tip for every solution and between tubes.

1) Label four cuvettes as “Blank,” “#1,” “#2,” and “#3” (note: use the specially provided cuvettes from your TA – these are designed for measuring light in the UV wavelength spectrum). Add 200 μL of Elution Buffer to the “Blank” cuvette. Add 200 μL of each ClpX eluate fraction to its corresponding cuvette.

2) Measure and record the absorbance of each eluate fraction at a wavelength of 280 nm using the “Albumin A280” program in the spectrophotometer (see lab manual Appendices for instructions on how to use the spec). Note that the spectrophotometer will calculate the protein concentration based on the extinction coefficient of albumin. Take a photo of the absorption spectrum and record the A280 value for each eluate fraction. Use the Elution Buffer “Blank” cuvette to blank (or “zero”) the spec.

3) A_{280} readings are only accurate up to around ~1.0. If all three of your A_{280} readings are below 1.0, you can continue on to Step 4. If the A_{280} reading for any of your samples is much greater than 1.0, that sample is too concentrated to measure accurately – you will have to dilute that sample first and then remeasure the A_{280}.

    a) Label a clean 1.5-mL Eppendorf tube with the sample fraction number and “1:10.” Add 30 μL of the eluate fraction sample and 270 μL of Elution Buffer to that tube and mix well by pipetting up and down several times.

    b) Label a new cuvette with the sample fraction number and “1:10.” Transfer the 1:10 dilution you made in Step 3a to the corresponding cuvette and remeasure the A_{280}.

4) Transfer 200 μL of each undiluted eluate fraction (#1, #2, and #3) into three clean 0.5-mL Eppendorf tubes. Label the tubes with “Mut C” (for mutant ClpX protein), the eluate fraction number (#1, #2, or #3), and your bench number.

5) Calculate the final concentration (in μM) of protein in each of your eluate fractions #1 – #3 using the equation from your pre-lab:

$$C = \_\_\_\_\_ \ \mu M \times A_{280}$$

    Remember to account for any dilutions you may have made while measuring the A_{280} of each fraction.

6) Write the concentrations on the three “Mut C” tubes and give those three tubes (together with the remaining undiluted samples) to your TA. Your mutant ClpX protein samples will be frozen at -80°C until Day 10 when you will use them in an activity assay. Many proteins are stored at -20°C, especially if they are used frequently, but we are storing the ClpX proteins at -80°C because we will not use them for a month. Return the elution buffer in the 4°C cold room for Day 10. You may dispose of the diluted samples and other buffers.

DAY 7

Day 7 Post-Lab Notebook Tips & Reminders:

- Record colony counts for all plates.
- Comment on each plate in terms of expected vs. observed result and what you can conclude from each plate. Feel free to include photos of the plates.
- Remember to include calculations for the transformation efficiency for the pUC18 plate.
- Record spectrophotometer readings for all samples and include all concentration calculations.
- Attach the adsorption spectrums for all samples and discuss if impurities are observed.
- What do your A_{280} reading results suggest about your overall ClpX purification procedure?
- Indicate if there were any deviations from the protocol.

DAY 7 ILQs

Question 1
Today you are calculating the transformation efficiency of LOBSTR BL21(DE3) cells. How does the efficiency of the LOBSTR cells compare with DH5α (from Day 5)? Does that make sense?

Question 2
Your very nosy labmate is reading through your 7.002 lab manual and notices that the pET-ClpX plasmid used to express your mutant ClpX enzyme has a *LacI* gene sequence on it. They claim it is unnecessary to include a *LacI* gene sequence on the pET plasmid for your recombinant expression procedure.

A. Do you agree with your labmate? Explain why or why not.

Your nosy labmate continues reading through your lab manual and notices that during the TB-Amp induction step (Part 3.3), the *E.coli* bacteria cells were grown at 30^{o}C. They claim that growing the cells at this temperature during the induction step was a mistake.

B. Do you agree with your labmate? Explain why or why not.

DAY 7 ILQs

Question 3
The 7.002 lab has recently acquired some IPTG reagent. IPTG is a molecular mimic of allolactose and can bind LacI in the same way allolactose can. However, IPTG is imported into the cell independently of the LacY permease and is not metabolized in the cell.

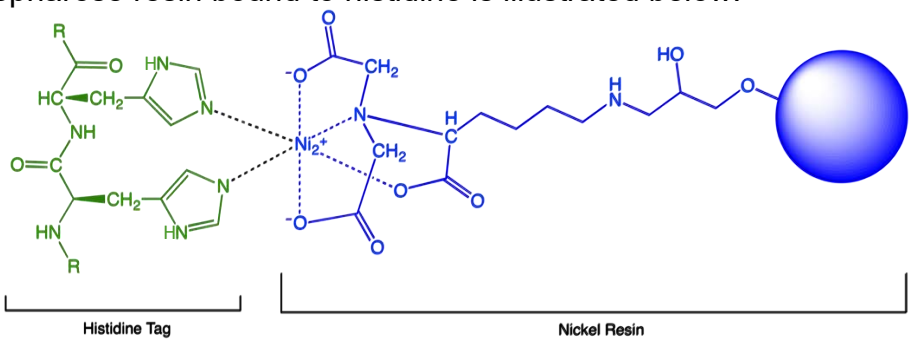
A. Your lab instructor suggests you grow your transformed LOBSTR BL21(DE3) cells in media that contains a finite amount of glucose and IPTG to induce expression of ClpX. Do you agree with your instructor? Explain why or why not.

B. Although the 7.002 lab uses TB + glucose + lactose media to induce recombinant protein expression, most other labs typically use IPTG to induce expression from a lac promoter. List some advantages of each induction method.

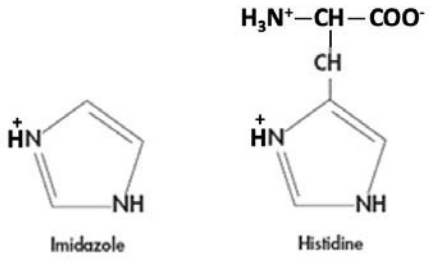
DAY 7 ILQs

Question 4

The nickel – sepharose resin bound to histidine is illustrated below:



And the comparison of histidine to imidazole:



A. In which protonation state (protonated or deprotonated) does histidine bind to Ni^{2+} ion? What type of bond is formed?

B. Why does a high concentration of imidazole elute the protein?

C. Based on your answer to Part A, can you think of another way to elute the protein without using imidazole?

D. Why do you think using imidazole is the preferred elution method?

Day 7 ILQs

Question 5

The nickel column you use is an example of affinity chromatography – where a molecule on the column resin has specific / or somewhat specific affinity for your protein of interest. Brainstorm two other affinity columns (so two other molecules that could be conjugated to resin) that you could use to purify 6xHis-ClpX and indicate how you would elute the 6xHis-ClpX from each column. Some may already exist.

DAY 8

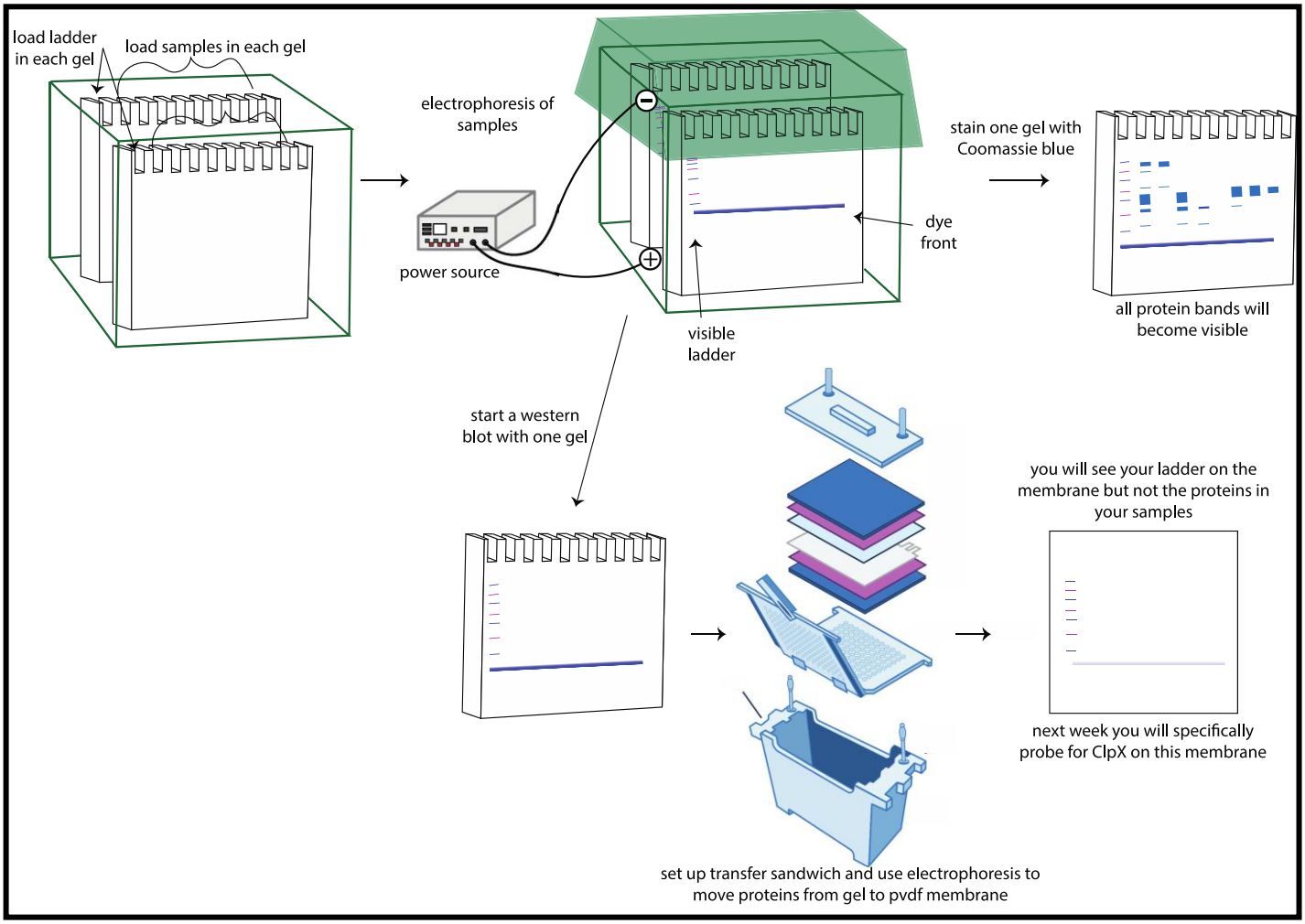
Lab Objective 4: Analysis of mutant ClpX protein purification

Part 4.1: Run two SDS-polyacrylamide gels

Overview

You will use SDS polyacrylamide gel electrophoresis (SDS-PAGE) to separate the proteins in your purification samples by their size. You will load each of your samples into two gels. One gel will then be used for a western blot to verify the presence of ClpX in your samples and the other gel will be stained with Coomassie blue to determine if other proteins are present in your samples.

Workflow of SDS-PAGE & Gel Processing. *You will run two gels and stain one with Coomassie blue and transfer the other to a PVDF membrane. Image adapted from the LI-COR transfer page and LibreTexts:*



Biology.

DAY 8

Materials

- (8) 30-μL protein sample aliquots from the ClpX purification
- 2X Protein Sample Buffer (125 mM Tris/HCl, pH 6.8, 20% glycerol, 4% SDS, 0.04% Bromophenol Blue, 10% β-mercaptoethanol)
- Precision Plus Dual Color Prestained MW Ladder (BioRad, see Appendices for marker sizes)
- (2) 10% SDS-polyacrylamide gel (pre-cast, 10 wells)
- SDS-PAGE Running Buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) from Day 2
- Gel electrophoresis apparatus and power supply

Hazardous chemicals used

Acrylamide, β-mercaptoethanol

Procedure

*Use a new tip for every solution and between tubes. Keep all samples on ice until the heat-shock step. Always **wear gloves** when handling polyacrylamide gels, as there may be residual unpolymerized acrylamide present (which is toxic!).*

Preparing the protein samples

1) Quick spin your eight 30-μL aliquot samples for 15 second. Make sure they are balanced.

2) Add 30 μL of 2X Protein Sample Buffer into each of the eight 0.5-mL tubes containing your protein samples. Mix each tube by gently pipetting up and down a few times (use a fresh tip for each tube!). Quick spin your eight samples **AND** the tube of Precision Plus Prestained MW Ladder for 15 seconds. Make sure they are balanced.

3) Make sure each of the nine tubes (eight samples + MW Ladder) are labeled with your bench number and that the lids are tightly capped shut.

4) Place all nine 0.5-mL tubes (eight samples **AND** the MW Ladder) in a 95°C thermocycler for 2 minutes. After the 2-minute heat step (you'll have to time the 2-minute incubation yourself), remove your nine tubes and quick spin all of them for 15 seconds. Leave your samples and ladder at room temperature until you are ready to load them onto your protein gels.

Preparing the gels

5) Remove the pre-cast polyacrylamide gels from their packaging. The gel (10% polyacrylamide) lies between two plastic plates, one shorter than the other, and has a 10-well comb inserted in the top portion.

DAY 8

6) Carefully pull the green tape off of the bottom of each gel **and tape to your lab bench as proof to yourself (and your instructor) that you did this step**. This exposes the bottom of the gel and allows the electric field to pass completely through the gel.

7) Carefully pull the 10-well comb straight up and out of each gel. *(Note: The comb is wedged in between the plates very tightly and may be difficult to pull out – be very careful when removing them that you don't accidentally damage/disrupt the wells of the gels.)*

8) Disassemble the gel apparatus and place the two gels on the plate holder. The shorter of the two plates for each gel should face the inside of the gel apparatus (creating the "inner chamber"). Lock them into place. Place the entire gel apparatus inside the gel box.

9) Pour SDS-PAGE Running Buffer only into the inner chamber until it is completely filled. Ask the staff if you need extra buffer. Check to make sure that the inner chamber is not leaking too much. If it's good to go, pour Running Buffer into the outer chamber (outside the gels) until it is half way full.

10) Rinse the wells of each gel by gently pipetting some running buffer into each of the 10 wells, making sure that any air bubbles or gel debris floats out of the wells. If you notice any deformities or major rips/tears with the wells or gel, notify a teaching staff. Have an instructor check your gel before you proceed to step 10.

Loading your TWO gels

****Note: Once you start loading a gel, try not to move the gel box too abruptly or else your samples may float out of the well.*

11) Before you load your gels, check that the level of buffer in the inner chamber is above the top of the wells. If not, add some more SDS-PAGE Running Buffer to the inner chamber until the tops of the gels are covered.

12) To load your gels, place your tip containing your sample against the large plate right above the middle of the well you are about to load. Rest the tip between the small plate and large plate – don't force the tip between the plates. **SLOWLY** add your sample to the well, watching the well carefully to make sure that the sample is actually entering and settling in the bottom of the well. Load 10 μL of the Precision Plus Prestained MW Ladder into Lane #1 of each gel. Load 10 μL of each of your samples in the next eight lanes. (Remember to record the lane order in your notebook!!!)

Running and dismantling the gel

13) Once **both** gels have been loaded, attach the green lid to the gel box and plug the leads into the power supply so that the gel runs in the proper orientation (**"red-to-red" and "black-to-black"**). Turn on the power supply and run the gel at ~200 volts. Check that current is running through your gel (*look for bubbles).* If your gel appears to be running improperly or excessively slowly, notify your instructor.

DAY 8

14) Run the gels until the tracking dye from the Sample Buffer is less than 0.5 cm from the bottom of the gel (this will take around 30 minutes). While your gels are running, you can work on Part 4.2.

15) When your gels have finished running, turn off the power supply. Remove the entire gel apparatus (containing both gels) out of the gel box. Unlock the gel apparatus and take out your gel. (Note: It's best to do this near the sink or over a white box – when you unlock the gel apparatus, the buffer in the inner chamber will spill out and make a mess!)

16) We recommend you wear safety glasses for this step. Although rare, the plastic plates can break and broken pieces can fly in unexpected directions. To remove the gel from between the two plates, insert the specially provided metal green tool between the plates at the positions marked with the black arrowheads and gently push up to **carefully** pry the two plates apart. Repeat until you are able to (carefully!) pull apart the plates to expose the gel – the gel will usually stick to one of the plates (it doesn't matter which one).

17) Use a razor blade to **carefully** cut off the wells of the gel (These tend to stick to things and will make your gels more likely to rip or tear if you leave them on. Leave tiny stubs as these can indicate the top of your gel). You may also cut the sides of the gel (where the gel and plate meet but before the first and after the last lanes) because the gel sometimes sticks to the plate at that point. And cut the bottom of the gel, below the blue dye line.

18) Fill a white plastic box with an inch or so of deionized water (from the white taps by the sinks). Take the plate that the gel is still stuck to and submerge the whole thing in the water in the white box. **Gently** wiggle the plate around until the gel slides off the plate, keeping the gel submerged in water the entire time (this will minimize ripping).

19) Repeat Steps 15-17 with your second gel to remove it from the plastic plates as well. One of the gels will now be used for the western transfer, while the other gel will be used for Coomassie-staining.

Part 4.2: Western transfer of SDS-polyacrylamide gel

Overview

Once your proteins have been separated by PAGE, you will use one gel for a western blot. The first step of a western blot is to transfer your proteins to a polyvinylidene fluoride (PVDF) membrane that has a high affinity for **all** proteins. Choose the better of the two gels for Western.

DAY 8

Materials

- Gel from Part 4.1
- Transfer Buffer (20% methanol, 25 mM Tris-base, 192 mM glycine, pH 8.3)
- Container with methanol
- (2) sheets of PVDF membrane (kept between protective sheets)
- (4) sheets of Whatman paper
- (1) transfer cassette with sponges
- (1) storage / Ziploc bag
- Western transfer gel box and power supply

Hazardous chemicals used

Acrylamide, methanol

Procedure

*Always wear gloves when handling the polyacrylamide gels. It is important gloves are **clean** and you try to only touch the very edges/corners when handling the PVDF membrane – this will minimize contaminating the membrane with background protein from your hands/gloves.*

1) Wearing gloves, write your bench number **in pencil** on the bottom right-hand corner of each of the two PVDF membranes. This label will designate the “front-side” and the bottom edge of your membrane after the transfer (the “front” of the membrane will be the side that your gel proteins bind). You can discard the protective sheets that were on your PVDF membranes.

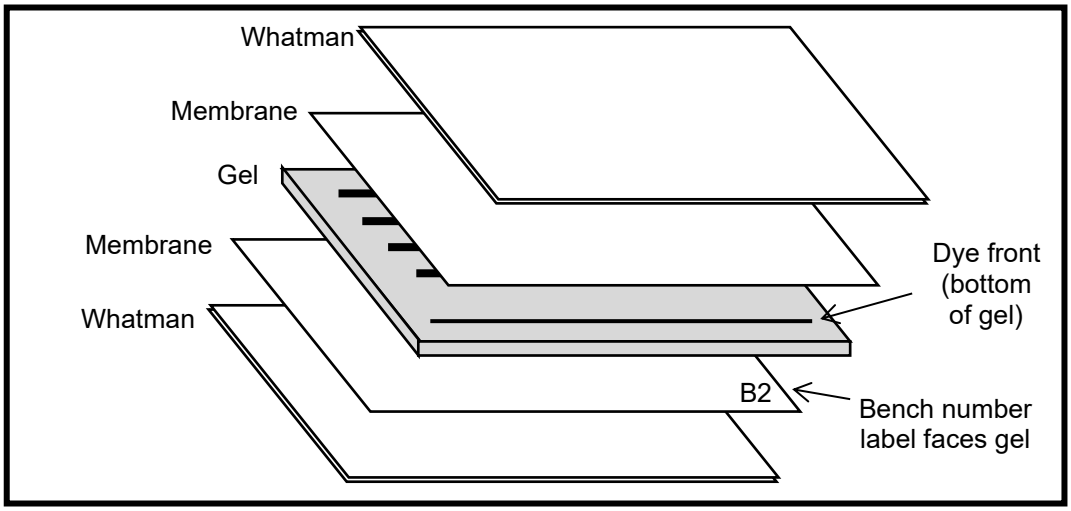
2) Using clean tweezers in your top drawer, submerge your two PVDF membranes in the container of methanol provided by your instructor. Allow the membranes to fully soak for at least 20 seconds. It is very important your membranes be completely soaked in methanol. Note: if this membrane appears to have uneven color, soak in methanol again.

3) Add some (~50 mL) Transfer Buffer in 4°C room to a clean empty tip box. Remove your two PVDF membranes from the methanol and place them in the Transfer Buffer in the tip box.

4) Four sheets of Whatman paper (the thicker rough white paper sheets) are stored in the Ziploc bag. Keep two sheets of Whatman paper dry for storage in step 12. Place **only two** sheets of Whatman paper in the Transfer Buffer in the same tip box as the PVDF membranes. Make sure that both membranes and the Whatman paper are fully submerged and allow them to equilibrate in the Transfer Buffer for at least 5 minutes before setting up your transfer.

DAY 8

5) When you are ready to set up your transfer, assemble the transfer “sandwich” by placing your SDS-polyacrylamide gel between the two PVDF membranes and the two Whatman sheets in the order shown in the diagram below.



- When assembling the sandwich, make sure that the side of the membrane with your bench number label on it is facing the gel and that the bench number label is oriented towards the bottom edge of the gel (where the dye front is).

- Try to avoid air bubbles from being trapped between any of the layers in the sandwich – your proteins won’t transfer through the air.

6) Once assembled, place the sandwich between two sponges inside a transfer cassette. Keep holding the cassette tightly closed when carrying it so that the sandwich won’t come apart accidentally (or if the cassette has a clamp, make sure it is closed).

7) Bring your sandwich/cassette to Bench B/C, where the 7.002 staff will help you insert your cassette into a transfer gel box. Each transfer gel box can hold up to four cassettes – once a box is full, the 7.002 staff will fill it with Transfer Buffer and start the transfer process. The proteins in the gel will be electrophoresed from the gel onto the PVDF membrane at 100 V for 20 minutes.

8) When your Western transfer is complete (~20 minutes), the 7.002 staff will return your transfer sandwich/cassette to your bench. Disassemble your sandwich and examine the two PVDF membranes. The proteins from your gel will have been transferred to only one of the two membranes – determine which of your two membranes contains your protein and verify your selection with your instructor.

9) Rinse out an empty tip box using deionized water (white taps at the sink). Add a layer of dH2O into the tip box. Using clean tweezers, move your selected membrane in the tip box and rinse the membrane briefly.

DAY 8

10) Using clean tweezers, submerge your PVDF membrane in the container of methanol provided by your instructor for 10 seconds.

11) Place your membrane on a piece of Whatman paper to dry (may take ~30 min, will be completely opaque). Once it is dry, cover with another piece of Whatman paper and slide the membrane + Whatman papers into a Ziploc storage bag (labeled with your bench and the date) and leave in the cold room until the next lab session. *Note, if your membrane is not dry by the end of lab, leave it drying on your bench, label your storage bag, and let an instructor know.

Part 4.3: Coomassie-stain SDS-polyacrylamide gel

Overview
Your second SDS-polyacrylamide gel will be stained with Coomassie dye to check the purity of your fractions. Coomassie binds proteins non-specifically and you will be able to tell if there are proteins other than ClpX in your samples.

Materials
- SDS-polyacrylamide gel of ClpX purification samples from Part 4.1
- Bio-Safe Coomassie stain (BioRad)

Hazardous chemicals used
None

Procedure
Be careful with the gel when pouring on/off solutions as they can rip easily.

1) Your protein gel from Part 4.1 should be in a white plastic box. Before staining the gel, you must first wash the gel with water several times to remove any residual SDS, which may interfere with the Coomassie staining.

2) Discard the water from the box containing your gel (the water can just be carefully poured down the sink – make sure the gel doesn't fall out though!). Add enough regular deionized water (from the white taps at the sinks) to the gel so that the gel is fully covered.

3) Cover your box with a lid (you may need to use tape to help secure the lid onto the box) and label the box with a piece of tape with **your bench number**. Place the box with your gel in it (in water) on a tilt table for 5 minutes at room temperature.

4) Repeat Steps 2 – 3 two more times. In total, you will have performed three 5-minute washes.

DAY 8

5) After the third wash is complete, carefully pour off the water. Pour 30 mL of Bio-Safe Coomassie stain into the box (make sure the gel gets fully covered). Put the lid back on the box and incubate the box with your gel in it on the tilt table at room temperature until the next lab day. (**make sure the box is labeled with your bench number!**). Protein bands will reach maximum intensity within 1 hour.

Day 8 Post-Lab Notebook Tips & Reminders

- Record the lane order for your gel.
- Comment on how the gels ran - how did the dye front and ladder bands look?
- Comment on your transfer – does your ladder appear clearly on your membrane?
- Record any deviations from the protocol, including any wells you had trouble loading.

Day 8 ILQs

Work on these questions alone or with your partner during your downtime in lab and then discuss them with your TA. You need to write out solutions to receive credit.

Question 1

In your ClpX purification analysis with Coomassie stain, you observe several bands in each of the eluate fractions. You suspect that the nickel affinity column may retain several other proteins besides the 6xHis-ClpX. Your lab instructor approaches you and says that cobalt affinity column may be a better option to purify the 6xHis-ClpX protein. You learned that cobalt columns have lower capacity but higher specificity to the 6xHis tag than nickel columns.

A. How would you modify the purification protocol if you choose to purify 6xHis-ClpX with cobalt column?

B. Based on your answer to Part A, can you think of another way to obtain higher purity in 6xHis-ClpX protein with nickel columns?

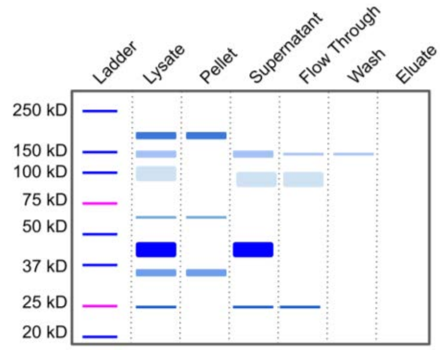
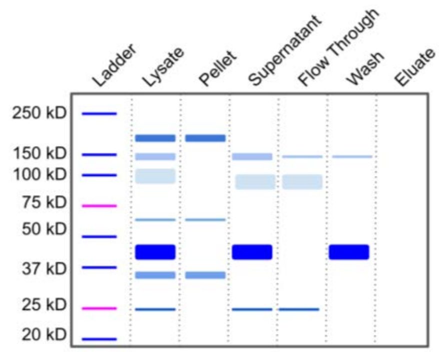
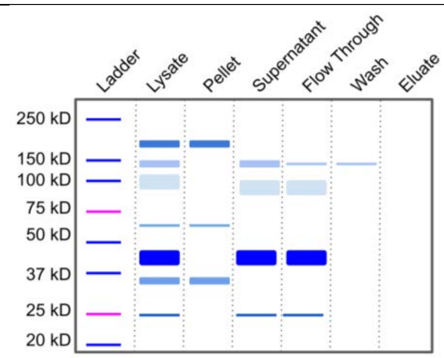
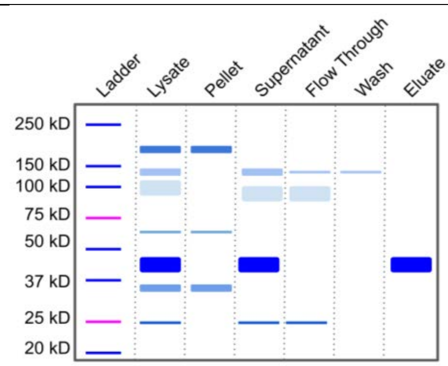
Question 2

A new 7.002 class performs the ClpX purification experiment, but each student alters their protocol:

- Woody adds protease inhibitors with EDTA to his lysis buffer.
- Hamm makes his wash buffer very acidic.
- Bo Peep makes her elution buffer very basic with no imidazole.
- Buzz uses DTT instead of BME in his protein sample buffer.

They perform SDS-PAGE followed by Coomassie staining to analyze their purification process. Unfortunately, the students do not label the boxes containing their gels.
Help Andy, their TA, match each student with their gel results. Explain your answers in the boxes provided on the next page.

Day 8 ILQs

Student name	Commassie-stained gel	Explain how the alteration led to this gel result
		
		
		
		

DAY 9

Lab Objective 4 (continued): Analysis of mutant ClpX protein purification

Part 4.4: Rehydrate western blot, block and probe with primary antibody

Overview
Last week you transferred the proteins in your samples onto a PVDF membrane, dried the membrane, and stored it at 4°C. Before working with your membrane again, you will reactivate it in methanol (similar to what you did before your transfer).

You will then block your membrane with the bovine serum albumin (BSA) protein. Once the PVDF membrane has been coated with either your sample proteins or BSA, you will add an antibody that only recognizes and binds to His-tagged ClpX.

Materials
- Western membrane (from Part 4.2, stored dry in cold room)
- 1X PBS (from Day 2)
- BSA
- Weigh station with balance
- (1) 50-mL conical tube
- (1) hybridization bag
- Primary Antibody Solution (AbCam, mouse monoclonal 6X-His-tag antibody coupled to Alkaline Phosphatase, diluted 1:4000 in 1X PBS-T + 2% BSA)

Hazardous chemicals used None

Procedure
Always use clean gloves and try to touch only the very edges when handling the membrane to avoid background protein contamination.

1) You need to rehydrate your membrane in methanol before you can use it. Using clean tweezers, submerge your PVDF membrane in the container of methanol provided by your instructor. Allow the membrane to fully soak for at least 20 seconds.

2) Rinse your membrane in a tip box with the white tap dH2O at the sink. Put a piece of tape on the tip box and label it with your **bench number**.

3) Wash your membrane with 20 mL of PBS at room temperature for 5 minutes.

4) Make Blocking Buffer: Blocking Buffer contains 1X PBS + 5% BSA (5% means 5 g BSA per 100 mL PBS). Calculate the appropriate amount of BSA to measure for 30 mL of Blocking Buffer. Add the BSA and PBS to a clean 50 mL conical tube.

DAY 9

5) Discard the PBS from the tip box containing your membrane (the PBS can just be poured down the sink – make sure the membrane doesn't fall out though!)

6) Add all 30 mL of Blocking Buffer into the tip box and incubate the box with your membrane in it on the tilt table at room temperature for 45 minutes.

7) When the 45-minute incubation in Blocking Buffer is complete, gently pour the blocking buffer down the sink. Rinse your membrane with dH2O.

8) Place your membrane inside a hybridization bag (if helpful, you can cut the bag so that there is only one sealed edge and then place the membrane in). Once the membrane is within the bag, **seal all but one edge.** (Note: The heat sealer works very quickly – a few seconds is usually more than sufficient to fully seal the edge of the bag. You will hear a little click when it is done.) Feel free to double seal an edge if you are concerned about a seal you made.

9) Carefully pipet 5 mL of Primary Antibody Solution into the bag (make sure your serological pipette does not touch the membrane). Remove as many air bubbles as possible and then seal off your bag with the heat sealer. Check to make sure that there are no leaks along any of the edges of your bag.

10) Write your **bench number** on your sealed bag and place it on the tilt table at room temperature for 1 hr. You may complete Part 4.5 while you wait.

Part 4.5: Destain Coomassie-stained SDS-polyacrylamide gel

Background: After Coomassie-staining, your protein gel will be incubated in water to remove excess Coomassie dye bound weakly to the gel. This will allow the stained protein bands to be more clearly visualized against the gel background.

Materials

- SDS-polyacrylamide gel in Bio-Safe Coomassie stain (from Part 4.3)
- Regular dH_2O (from white tap)

Hazardous chemicals used

None

Procedure

Be careful with the gels when pouring on or off solutions. They are fragile and slippery.

1) Carefully pour off the Coomassie stain solution into the sink.

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2) Add enough deionized water (white taps at the sinks) into the box containing the gel (make sure the gel gets fully covered). Put the lid back on the box and incubate the box with the gel in it on the tilt table at room temperature for at least 30 minutes to destain the gel (it's okay to let this incubation go longer than 30 minutes – the gel is perfectly fine in water for an extended period of time after staining).

3) After the 30-minute incubation, pour off the water from the gel box into the sink. Add fresh deionized water (white taps at the sink) into the box with your gel so that the gel is covered.

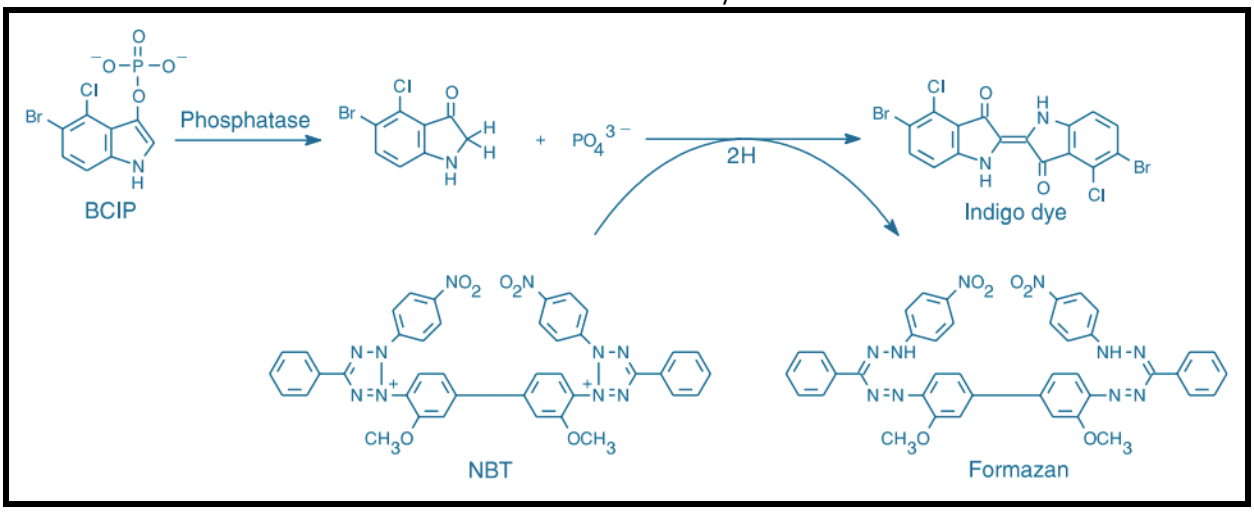
4) Bring your gel to the gel documentation system where an instructor will photograph your gel.

Part 4.6: Washing and detection of Western blot

Overview

The primary antibody you incubated with your membrane is directly bound to AP enzyme. To detect the AP enzyme conjugated to your primary antibody, you will incubate your membrane with 1-Step NBT/BCIP Solution (from Pierce Thermo Scientific). A colorimetric reaction will occur wherever the AP-conjugated primary antibody is on your membrane (presumably where the His-tagged ClpX protein also is).

Colorimetric Reaction. *Alkaline phosphatase (AP) conjugated to the antibody takes a phosphate off BCIP which is then oxidized by NBT yielding formazan and an indigo dye that combine to form a purple precipitate. Image from ThermoFisher Scientific (2002). Multiplexed Proteomics for Detection of Specific Proteins in Gels and on Blots Multiplexed Proteomics.*



DAY 9

Materials

- Western membrane in sealed bag (with primary antibody, from Part 4.4)
- 1X PBS (from Day 2)
- 10% Tween-20 (diluted in PBS)
- 1-Step NBT/BCIP Solution (Pierce)
- Empty P200 tip box (make sure it's clean!)

Hazardous chemicals used

1-Step NBT/BCIP Solution

Procedure

Use clean gloves & tweezers and only touch the very corners/edges when handling the Western membrane.

1) Before you can do the detection step for your western blot, you must first wash your membrane several times in 1X PBS-T to remove any excess or loosely-bound primary antibody. With your remaining PBS, make 1X PBS-T (1X PBS + 0.1% Tween-20). Gauge how much PBS you have left and calculate how much 10% Tween-20 to add. Check your calculations with your instructor!

2) Cut open one edge of the sealed bag containing your membrane and discard the primary antibody solution. Using **clean** tweezers, carefully remove your membrane from the bag and place it inside a **clean** empty tip box such that the side of the membrane containing your proteins is **face up** (i.e. the side of the membrane with your bench number label should be face up if you followed the transfer protocol correctly when making your transfer sandwich).

3) To wash your membrane, add ~15 mL of 1X PBS-T to the tip box containing your membrane. Gently rock the tip box for 10 seconds and then pour off the 1XPBS-T into the sink.

4) Repeat step 3.

5) Add half of the remaining 1X PBS-T to the tip box and incubate it on a tilt table at room temperature for 10 minutes (label your tip box with a piece of tape with your **bench number**!)

6) Pour off the solution in your tip box and repeat Step 5. In the end, you should have performed two rinses followed by two 10-minute washes.

7) Pour off the 1X PBS-T solution in your tip box, leaving just the membrane. Make sure your membrane is face up in the box.

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8) Mix the tube containing the 1-Step NBT/BCIP Solution by gently inverting it several times. Add 5 mL of 1-Step NBT/BCIP Solution to your membrane in the tip box, making sure that the entire surface of the membrane is evenly covered. Note the time.

9) Gently rock the tip box by hand and wait for the appearance of purple bands on your membrane. It may take anywhere from 15 seconds to 5 minutes for the purple bands to fully appear – you will need to keep an eye on the membrane the whole time to follow the progress of the Western detection reaction. Be patient (if you stop the reaction too early, you may fail to detect weaker protein bands), but don't let the reaction continue for too long (if you stop the reaction too late, you may end up with a lot of purple background staining on your membrane).

10) When the most prominent protein bands on your membrane have reached a deep purple color, stop the detection reaction by pouring off the 1-Step NBT/BCIP Solution into a **waste beaker** and then rinsing your membrane in the tip box several times with deionized water (from the white taps at the sinks) – this rinsing step is important to remove residual 1-Step NBT/BCIP Solution that would otherwise continue to cause excess background straining on your membrane. Let the membrane dry on a kimwipe.

11) Once the membrane is dry, you can bring it to the gel documentation system to have the 7.002 staff take a picture.

Day 9 Post-Lab Notebook Tips & Reminders

- Include your western blot image, and properly label each lane and ladder band.
    - **The expected size of ClpX is 42 kDa.** Which band(s) in which lane(s) do you think correspond to ClpX protein?
    - What were the overall expected vs. observed results for each lane of your Western blot? If you did not get the expected results, what might be a possible reason to explain the difference?
    - What can you conclude about the success of your ClpX protein purification procedure?

- Include an image of your Coomasie-stained gel with all lanes and the ladder bands labeled.
    - Describe what you see on each lane of your gel. Did you get the expected result?
    - What conclusion regarding your purification procedure can you draw from your gel?

Day 9 ILQs

Work on these questions alone or with your partner during your downtime in lab and then discuss them with your TA. You need to write out solutions to receive credit.

Question 1
Complete the table below to compare and contrast Coomassie staining and western blotting.

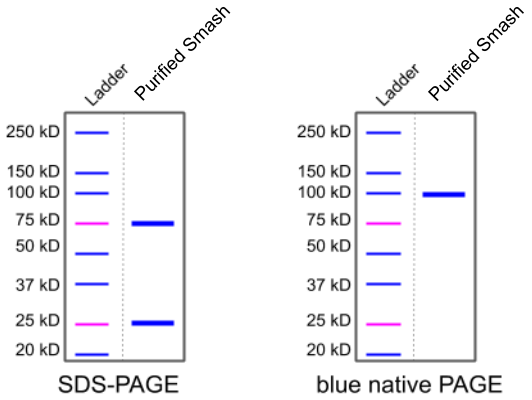
	Coomassie stain	**western blot**
What is similar between the two methods?		
What is visualized?		
What is the detecting agent?		
Detecting medium?		
Information gathered?		

Question 2
You and another lucky UROP, Bruce, are running experiments for Professor Hulk over the summer. Your task is to better understand a protease enzyme called Smash. Prof. Hulk gives you and Bruce purified Smash; you are to perform SDS-PAGE and Bruce blue native PAGE.

A. As you both prepare your samples, Bruce glares at you and asks why the bench smells like rotten eggs. Explain to Bruce why you have beta-mercaptoethanol in your sample buffer and he does not.

Day 9 ILQs

B. The results of your Coomassie stained gels are shown. What can you and Bruce conclude about the protein Smash?



C. You decide to also perform a western blot to confirm the protein you observed was Smash. A helpful postdoc in your lab, Dr. Sterns, approaches you. He says that it would be neat to use an antibody that recognizes a phosphorylated residue on Smash. Would this antibody be a monoclonal or polyclonal antibody? Explain.

D. Inspired by your beautiful western blot, your labmate Betty decides to do a western blot. To save time, she plans to block her PVDF membrane while the gel is running.

- Explain to her how this shortcut would affect her overall results?

- When/how would she first notice the difference?

E. Bruce decides to repeat his experiment but performs native PAGE instead of blue native PAGE. He does a western blot after his electrophoresis and borrows your antibody. He is unable to detect Smash though. Explain why that might be the case.

DAY 10

Lab Objective 5: Analysis of mutant ClpX ATPase activity

Part 5.1: Prepare ATPase reaction buffer

Overview:
Today **each individual student** you will run an assay that detects ATPase activity of ClpX. First you need to make the buffer for the assay. This buffer needs to have the proper pH, salt concentration, and cofactors present so that ClpX can fold and function properly. Students in each group will share the ATPase reaction buffer.

Materials
- (1) 15-mL conical tubes
- 1 M HEPES (liquid)
- 40% Glycerol (liquid)
- 1 M KCl (liquid)
- 1 M $MgCl_2$ (liquid)

Hazardous Chemicals Used
None

Procedure

ATPase assay reaction buffer contains:	The stocks available to you are:
25 mM HEPES pH 7.5	1 M HEPES
100 mM KCl	1 M KCl
5 mM $MgCl_2$	1 M $MgCl_2$
10% glycerol	40% glycerol

1) For each group, label a 15-mL conical tube with “ATPase Assay reaction buffer”, your bench, and the date.

2) You will prepare 5 mL of ATPase buffer. Calculate the amount of each reagent you will need. The remaining volume will be ultrapure dH_2O.

3) Add each reagent and the dH_2O to the conical tube.

4) Cap the 15-mL tube and invert several times to mix.

Part 5.2: ATPase Activity Assay from each student

Overview
You are ready to perform the *in vitro* ATPase assay to measure the intrinsic ATP hydrolysis activity of purified ClpX ATPase enzyme. You will test the mutant ClpX protein you purified, as well as a wildtype ClpX protein that will be given to you.

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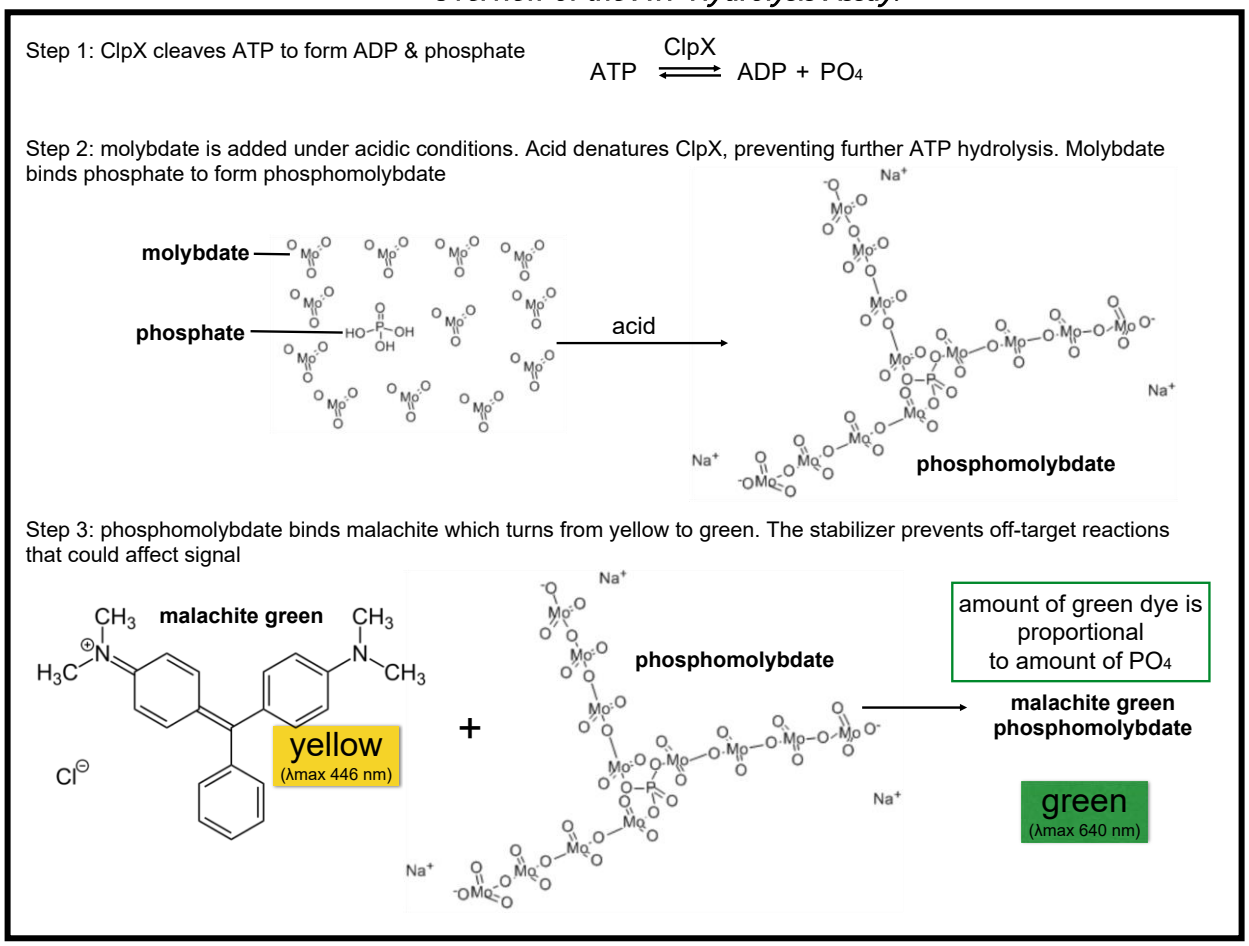
Our assay uses the P_iColorLock Gold reagent containing a malachite green substrate which produces a stable dark green color when bound to free P_i liberated by ATP hydrolysis. The malachite green-phosphate complex absorbs light strongly at 635 nm, so the amount of resulting dark green colorimetric product can be determined with a spectrophotometer. The intensity of the green color produced is proportional to the amount of free phosphate released and therefore is a measure of the enzymatic activity of the purified ATPase protein.

Overview of the ATP Hydrolysis Assay.



Materials

- Wildtype ClpX protein (tube labeled “C”) (6 μM in Elution Buffer)
- Your mutant ClpX protein samples (three tubes labeled “Mut C #1 – #3”) (from Part 3.5, in Elution Buffer)
- ATPase assay reaction buffer (25 mM HEPES pH 7.5, 100 mM KCl, 5mM $MgCl_2$, 10% glycerol) from Part 5.1
- 20 mM ATP (in Tris, pH 7.0)
- P_iColorLock Gold reagent

DAY 10

- Accelerator reagent
- Stabilizer reagent
- Spectrophotometer and Eppendorf “UVette” plastic cuvettes

Hazardous chemicals used
Ammonium molybdate and hydrochloric acid (in the PiColorLock Gold reagent)

Procedure
Use a new tip for every solution and between tubes. Keep the ClpX proteins and ATP stock tubes on ice until specified otherwise. All other reagents can be kept at room temperature.

1) Based on your ClpX protein concentration calculations (from Day 7 and written on the tubes) and the results of your Coomassie-stained gel & western blot, determine which ClpX eluate fraction (#1, #2, or #3) contains the most/best ClpX protein (check your final decision with an instructor before continuing). You will use the “Mut C” ClpX sample tube from this eluate fraction for the ATPase Assay. For groups with two students, each of you may choose from the same “Mut C” sample or two different “Mut C” samples.

2) For the ATPase Assay, you will want to use the same amount of wildtype or mutant ClpX protein in each assay reaction. You will therefore dilute your mutant ClpX protein and the given wildtype ClpX protein to the same starting concentration (5 μM). **Warning: Know when to use Elution Buffer and ATPase Assay Reaction Buffer!**

    a. Calculate how much mutant ClpX protein and how much Elution Buffer you would have to mix to end up with 60 μL total volume of diluted mutant ClpX protein at 5μM concentration. If the pipetting volume is too small, you can make a larger total volume. **Include this calculation in your Post-Lab!**

    b. Label a 1.5-mL Eppendorf tube with “dilute Mut” on it. Dilute the mutant ClpX protein in Elution Buffer to a final concentration of 5 μM according to your calculations from Step 2a. Mix the contents of the tube by gently pipetting up and down several times. Quick-spin the tube for ~5 seconds and leave it on ice. **When setting up your ATPase Assay reactions, remember to use the diluted mutant ClpX protein where applicable!**

    c. Return your remaining undiluted tubes back to your TA’s ice bucket.

    d. Label another 1.5-mL Eppendorf tube with “dilute WT” on it. Dilute the WT ClpX protein in Elution Buffer to 5 μM in a final total volume of 60 μL. Do not return excess WT ClpX.

3) Label a 1.5-mL Eppendorf tube as “Gold Mix.” Add 450 μL of PiColorLock Gold reagent and 4.5 μL Accelerator reagent to this tube. Mix well by pipetting up and down several times (if needed, briefly centrifuge the “Gold Mix” tube as before). Keep this tube at room temperature on your bench.

DAY 10

4) Label six clean 1.5-mL Eppendorf tubes "#1" – "#6." You will be setting up the assay samples in these six tubes. The final contents of each reaction tube are shown in the table below.

Add ATP LATER!

Tube	Description	WT ClpX	diluted Mut ClpX	ATPase Reaction Buffer	ATP	Total
#1	Buffer only	-	-	200 μL	-	200 μL
#2	Buffer with ATP	-	-	195 μL	5 μL	200 μL
#3	WT ClpX only	20 μL	-	180 μL	-	200 μL
#4	Mut ClpX only	-	20 μL	180 μL	-	200 μL
#5	WT ClpX with ATP	20 μL	-	175 μL	5 μL	200 μL
#6	Mut ClpX with ATP	-	20 μL	175 μL	5 μL	200 μL

5) Record the current time as "T1" in your notes. Then add the ingredients described in the table into each tube **EXCEPT for ATP.** Do NOT add the ATP yet!!!!! Mix each tube well by pipetting up and down several times. When adding WT and mutant ClpX protein, make sure you use the diluted protein you prepared in Step 2 as needed. Keep these six tubes at room temperature on your bench.

6) Record the current time as "T2". Then add the appropriate amount of ATP if needed as shown on the table to each of these six tubes. **Some reactions do not have ATP.** Immediately invert the tubes gently several times to mix them. Record the current time as "T3". Then start a timer for a 10-minute incubation.

7) At the end of the 10-minute incubation, add 50 μL of Gold Mix to each of these six tubes. Immediately invert the tubes gently several times to mix them and start a timer for a 5-minute incubation. Keep these tubes close by – you will eventually be adding stabilizer to each of these tubes.

8) After the 5-minute incubation from Step 7 is done, add 20 μL of Stabilizer reagent to each of Tubes #1 - #6. Invert the tubes gently several times to mix them and leave them on your bench at room temperature to incubate for 30 minutes.

9) After the 30-minute incubation, label two clean 1.5-mL Eppendorf tubes "#5 dilution" and "#6 dilution." Into each tube, pipette 20 μL from tubes "#5" and "#6" and 180 μL ATPase Assay Reaction Buffer. Mix gently up and down several times.

10) Label six Eppendorf UVette cuvettes as "#1" – "#4", "#5 dilution" and "#6 dilution." **Make sure you use the UVette cuvettes provided by your instructor** – these are specially designed for use with small volumes. Transfer 100 μL from each Eppendorf tube "#1" – "#4", "#5 dilution" and "#6 dilution" into the corresponding cuvette.

DAY 10

11) Use the spectrophotometer to measure the absorbance of each sample at 635 nm (see Appendices for instructions on how to use the specs). Use Cuvette #1 as your blank (the Buffer-only sample from Tube #1). Record your results for each sample. You may use the space below if you'd like.

Tube #1:_____ Tube #4:_____

Tube #2:_____ Tube #5 dil:_____

Tube #3:_____ Tube #6 dil:_____

12) Enter your results into the online class spreadsheet. An example of the spreadsheet is uploaded to the supplementary. The spreadsheet will calculate relative absorbance (absorbance with background values subtracted). Make sure you include the absorbance values and relative absorbance values in your post-lab.

13) Once your specific amino acid substitution group has completed their results, discuss what conclusions you can draw about the residue your bay decided to mutate. You may use the space below for notes:

DAY 10

Part 5.3: Generate a standard curve from each student

Overview
Your experimental assay will indicate how active your mutant ClpX is relative to wildtype ClpX. But it is helpful to know specifically how active it is – how much ATP a given amount of ClpX cleaves in a time interval. You can determine this specific activity value by comparing your results to a standard curve. You will record and plot the absorbance values for known amounts of phosphate (P_i) and then use that curve to determine the specific activity for wildtype and mutant ClpX.

Materials
- 175 μL of 50 μM P_i stock tube labeled "50 P_i"
- Sterile ddH_2O
- P_iColorLock Gold reagent
- Accelerator reagent
- Stabilizer reagent
- Spectrophotometer and Eppendorf "UVette" plastic cuvettes

Hazardous chemicals used
Ammonium molybdate and hydrochloric acid (in the P_iColorLock Gold reagent)

Procedure (Note: Each student should complete this part on their own)
Use a new tip for every solution and between tubes. All other reagents can be kept at room temperature.

1) Label a 1.5-mL Eppendorf tube as "Gold Mix." Add 250 μL of P_iColorLock Gold reagent and 2.5 μL Accelerator reagent to this tube. Mix well by pipetting up and down several times (if needed, briefly centrifuge the "Gold Mix" tube as before). Keep this tube at room temperature on your bench.

2) Label four clean 1.5-mL Eppendorf tubes according to the tube labels in the first column You will be setting up the standard samples in these tubes by making serial dilutions. Follow the table below for setting up your serial dilutions.

Tube Label	Std Description	P_i Source	H_2O to add
25 P_i	25 μM	175 μL from 50 P_i stock	175 μL
12.5 P_i	12.5 μM	150 μL from 25 P_i	150 μL
6.25 P_i	6.25 μM	100 μL from 12.5 P_i	100 μL
0 P_i	0 μM	0 μL	200 μL
Note, you should have 200 μL in each tube at the end.			

DAY 10

3) Once you have 200 μL in each standard tubes, add 50 μL of Gold Mix to each. Immediately invert the tubes gently several times to mix them and start a timer for a 5-minute incubation.

4) After the 5-minute incubation is done, add 20 μL of Stabilizer reagent to each tube. Invert the tubes gently several times to mix them and leave them on your bench at room temperature to incubate for 30 minutes.

5) Label five Eppendorf UVette cuvettes for your samples. **Make sure you use the UVette cuvettes provided by your instructor** – these are specially designed for use with small volumes. Transfer 100 μL from each Eppendorf tube into the corresponding cuvette.

6) Use the spectrophotometer to measure the absorbance of each sample at 635 nm. Use the 0 P_i as your blank. Record your results for each P_i concentration.

   You may use the space below to record your results (but still need to include them in the lab notebook).

   25 P_i:_____ 12.5 P_i:_____

   6.25 P_i:_____ 0 P_i:_____

7) Plot a line graph of absorbance vs the amount of P_i (in **nanomoles**). **You will need to do some calculations to determine how many nanomoles are in each standard.** You can check these with your instructor. Include the graph in your post-lab.

8) Using the **relative** absorbance you calculated in Part: 5.2, calculate the specific activity of mutant ClpX and wildtype ClpX. The units of specific activity for an ATPase enzyme are typically written as:

$$\frac{\text{nanomoles (or picomoles) of ATP hydrolyzed per minute}}{\text{Total amount of protein used (in g, mg, μg, etc)}}$$

Note that for our ClpX ATPase assay, the approximate amount of total protein used in each assay reaction was ~4.17 μg protein.

DAY 10

Day 10 Post- Lab Notebook & Reminders:

- Indicate if you have deviated from the protocol.
- Include all calculations from your mutant ClpX enzyme dilution.
- Calculate the time differences between T1-T2 and T2-T3.
- Include all spectrophotometer readings.
- Include the relative level of ATPase activity for the WT ClpX and your mutant ClpX.
- For each sample, comment on the overall expected vs. observed results and what you can conclude from each.
    - How do the WT ClpX and mutant ClpX samples compare with each other? Is that how you expected? Why or why not?
    - If you did not get the expected results, what might be a possible reason to explain the difference?
- Include your standard curve graph, equation, and calculated specific activities.

Day 10 ILQs

Work on these questions alone or with your partner during your downtime in lab and then discuss them with your TA. You need to write out solutions to receive credit.

Question 1

You have constructed an *E. coli* bacteria strain to express your favorite restriction enzyme bound to a histidine tag: 6xHis-BglII and now want to purify the 6xHis-BglII enzyme so that you can sell it to biotech companies and make millions of dollars. You prepare a cell lysate from your cells and collect aliquots of the Crude Lysate (CL) and Crude Lysate Supernatant (CL-S) after spinning the lysate. You load the CL-S onto a nickel column and collect the Flow-Through (FT). You then elute your sample from your column and collect the eluate (Elu).

You analyze your CL, CL-S, FT, and Elu aliquots to determine how much DNA each sample can digest to calculate specific activity for each aliquot (see table below). Unfortunately, you forgot to label your sample tubes!

Sample Tube	Specific Activity	Aliquot (CL, CL-S, FT, or Elu)
A	0 units / mg	
B	100 units / mg	
C	200 units / mg	
D	500 units / mg	

A. In the table above, indicate which tube (A – D) most likely correlates with each of the following aliquots: CL, CL-S, FT, and Elu.

B. Explain your reasoning for Part A for each sample.

Day 10 ILQs

Question 2

You have spent the last few weeks studying ClpX, a protein that uses the hydrolysis of ATP to drive the unfolding of other proteins.

A. List all levels of protein structure that are disrupted in the substrate protein by ClpX?

B. The unfolding activity of ClpX is usually followed by the enzyme activity of ClpP. List all levels of protein structure in the substrate protein that are disrupted by ClpP?

C. Explain how ClpX recognizes protein substrates **and** describe how ClpX will only recognize a protein substrate when it is necessary to unfold that protein?

Appendix: Related Literature and References

Tania A. Baker and Robert T. Sauer. (2012). “ClpXP, an ATP-powered unfolding and protein-degradation machine.” *Biochimica et Biophysica Acta*. **1823:** 15 – 28.

Robert T. Sauer and Tania A. Baker. (2011). “AAA+ Proteases: ATP-Fueled Machines of Protein Destruction.” *Annu. Rev. Biochem.* **80:** 587 – 612.

1-Step NBT/BCIP Solution instructions (Pierce)

E.Z.N.A. Cycle Pure Kit manual (Omega)

E.Z.N.A. Plasmid DNA Mini Kit manual (Omega)

His-Trap FF Ni-Sepharose Column instruction manual (GE Healthcare)

Quintara Biosciences DNA Sequencing website:
https://www.quintarabio.com/service/dna_sequencing

New England Biolabs (NEB) 2013 – 2014 Catalog & Technical References

Overnight Express Autoinduction Systems instruction manual (Novagen)

pET System instruction manual (Novagen)

P_iColorLock Gold Phosphate Detection System instruction manual (Innova Biosciences)

Note: Special thanks to New England Biolabs for their generous donation of restriction enzymes, kinases, ligases, PCR reagents, and DNA ladders for use in this module.

Appendix: 7.002 Lab Safety Guidelines

General Lab Safety Rules

1) No eating or drinking in the lab (this includes water bottles). Food wrappers or containers are NOT allowed in the trash bins in the lab – they must be disposed of outside the lab.

2) Large personal items (bags, coats, etc) may be stored in the lab during class but should be kept out of the way (there is space and hooks under the benches along the side of the lab that you may use to store or hang up such larger items like bookbags and coats). Please keep all aisles and floor space in the lab clear and free of clutter at all times.

3) Wear appropriate clothing:
- No open-toe shoes!!
- Long pants and long sleeves are recommended in the lab
- Tie long hair back

4) Gloves and lab coat should be worn at all times while working in the lab – this protects you and also prevents you from contaminating your experiments. Safety glasses should be worn when appropriate. Remove all protective clothing (gloves, lab coat, and safety glasses) before leaving the lab (including bathroom breaks).

5) Glove rules:
- Remove gloves to handle non-lab equipment (e.g. phones, doors, computers)
- Don't touch your face, hair, etc. with your gloves when working at the bench
- Dispose of all used gloves in the "burn boxes" – do NOT throw gloves in the normal trash!

6) Be aware of your bench space – you are taking a bench that normally share with up to three other people. There should be a lot of distance between you and the next person. Still, take care when using tips and pipettes that you don't accidentally jab someone! Keep your bench space clean and free of clutter – this may have a huge impact on the success of your experiments.

Dealing with accidents/injury

1) All injuries or accidents, no matter how minor, must be reported to an instructor.

2) If you get a hazardous chemical/material in your eyes, immediately wash out your eyes for 15 minutes using the eyewashes located at each sink.

3) If you spill hazardous chemicals/materials on yourself or you catch fire, immediately use one of the lab safety showers (either by the offices near the lab entrance or by Alcove 4.

Appendix: 7.002 Lab Safety Guidelines (continued)

4) If you spill hazardous chemicals/materials on the floor, bench, etc., inform a teaching staff – they will provide instructions on how to properly clean up the spill.

Fire-safety notes

1) When using a Bunsen burner, make sure to keep anything flammable (e.g. paper) away from the burner and keep long hair tied back.

2) Be aware of your surroundings – when reaching for objects or across benchtops, make sure you're not accidentally extending your arm or hand right into a Bunsen burner flame!

3) Always make sure you TURN OFF the gas tap before leaving the lab.

Procedures for handling/disposal of hazardous or toxic chemicals/waste

Handling toxic or hazardous chemicals: Before you come to lab each day you must carefully read over the protocols for the day in your lab manual. In the protocols we have highlighted the harmful chemicals used on certain days. Make sure that you are aware of the hazards of these chemicals before lab, and wear the appropriate protective clothing when using them (gloves, lab coats, and/or safety glasses).

Disposal of toxic or hazardous chemicals: Daily disposal notes describing how to properly dispose of all hazardous material will be posted by the sinks every lab day. Check them each day for specific disposal instructions – if you have any questions, ask a teaching stuff. Certain chemicals cannot be disposed of down the sink (e.g. ethanol). Again, by reading your manual before you come to lab, you will know which chemicals require special disposal procedures. Chemicals that cannot be disposed of down the sink are collected in labelled waste bottles in the fume hoods located in Alcoves 1 - 3. Certain chemicals are incompatible and cannot be collected in the same bottle. *ALWAYS READ THE RED LABEL ON THE WASTE BOTTLE BEFORE ADDING YOUR CHEMICAL.* If in doubt, ask a teaching staff. If you accidentally add the wrong chemical to a waste bottle, immediately inform a teaching staff, as particular combinations of incompatible chemicals can potentially be explosive/reactive.

Procedures for handling/disposal of biohazardous waste

Wear gloves when handling or disposing of biohazardous material (e.g. bacteria, yeast, etc). Solid biohazardous waste (e.g. bacteria plates, yeast plates) and all gloves should be disposed of in the burn boxes. Liquid biohazardous waste (e.g. a liquid bacteria/yeast culture or media) should be treated with 10% bleach and allowed to sit for 20 minutes to ensure all organisms are killed before pouring the liquid waste down the sink (10% bleach is located in the labeled squirt bottles by the sinks). *Note: Do NOT put any liquid in the burn boxes!*

Appendix: 7.002 Lab Safety Guidelines (continued)

Procedures for general disposal/cleaning of lab supplies

1) Gloves: Dispose of in the burn boxes. Do NOT throw out in the normal trash!

2) Plastic pipette tips (200μL or 1mL size): Dispose of in the burn boxes.

3) Plastic Eppendorf tubes, Falcon tubes, and PCR tubes: Dispose of in normal trash unless otherwise instructed. If the tubes contained biohazardous material at any point, they should be treated with bleach for 20 minutes to kill any residual living organisms before being thrown out. Make sure tubes do not contain any hazardous chemicals in them when you throw them out (any hazardous chemicals still remaining should be disposed of in the proper waste container in the hoods).

4) Empty tip boxes or tube boxes: Place on the labeled shelves in Alcove 4.

5) Plastic loops, plastic spreaders, inoculating sticks, and toothpicks: Dispose of in the burn boxes.

6) Glass serological pipettes (5-mL or 10-mL): Place in plastic boxes marked “10% Bleach” located at each bench. (Note: These pipettes get washed and reused – do not throw them out!!) Empty metal pipette cans go in the white bins along the wall.

7) Glass slides/coverslips, any broken glass, razor blades, plastic cuvettes, and syringes: Dispose of in the appropriately labeled red “Sharps” secondary containers located on the benchtops – do **not** put these items directly in the burn boxes.

8) Dirty glass test tubes and plastic centrifuge tubes/bottles: Rinse with water and place in the white bins by the sink or along the walls. All caps/lids should go in the buckets located at each sink. If the tube/bottle contained biohazardous material at any point, they should be treated with bleach for 20 minutes before being rinsed out and placed in the bins. *Please remove ALL tape labels from tubes/bottles before placing them in the bins!!!!*

9) Gel boxes and electrophoresis apparatus: Rinse with water and leave on your benchtop to dry.

Note: NEVER put any liquid in the burn boxes – only dispose of SOLID waste in them!!

Note: All “sharp things” (e.g. anything that could potentially pierce skin, like glass waste, broken glass or a razor blade) should be disposed of in an appropriately labeled red “Sharps” secondary container. Do NOT dispose of sharp objects directly in the burn boxes as they may puncture the bag.

Appendix: 7.002 Lab Safety Guidelines (continued)

Note: Each lab bench station has a benchtop Biohazard Waste Transfer Container for used pipet tips, as well as several small plastic beakers to use as waste containers during the labs. You should label beakers for various waste streams, depending on the protocol for that day (e.g. “Bacteria media waste,” “Omega buffer waste,” etc). During the lab, dispose of all used tips in the provided Transfer Container for convenience – at the end of the day, empty everything out of the Transfer Container into the burn boxes. Collect any media or buffer waste accordingly into the appropriate container as you work. At the end of lab, dispose of the waste properly according to the posted daily disposal notes. Bacteria/media waste should be treated with bleach before being poured down the sink. Hazardous buffer waste should be disposed of in the properly-labeled waste containers in the hoods in Alcoves 1, 2, or 3.

Appendix: Eppendorf BioSpectrometer Instructions

Measuring DNA concentration:

*** You will be using special (read: expensive!) microcuvettes to measure your DNA. Please use them under the supervision of your TA until you are completely familiar with their use. When you are done, wipe them with a Kimwipe and return them to your TA. ***

1) Turn the power on (the switch is in the back left corner of the spec). Wait for the "SELF-TEST" to complete. Press "OK" to go to the Main Menu.

2) On the Main Menu, press the right arrow once to highlight "Favorites." Press the right and down arrows to highlight "dsDNA 1 mm" in the Methods column. ("ds" stands for double stranded DNA, and 1 mm is the path length for the special microcuvette we will be using to measure DNA).

3) Press the white oval "enter" button – this will take you to the "dsDNA 1 mm" parameters page. Press "Next" to proceed (note: do NOT change any parameters!).

4) Now you are ready to measure your blank solution. Take a microcuvette out of its metal box and lift one glass arm to expose the two black circular wells to hold your sample. Pipet 2 µl blank solution into one of the wells and close the arm by snapping gently. Look from the side to make sure that you have indeed generated a column between the two pieces of glass. (If the sample does not form a continuous column between the two wells, lift the arm and snap it closed again carefully.)

5) Lift the blue sample compartment door on the spec and insert the cuvette with your blank into the sample holder. Make sure the cuvette is in the correct orientation in terms of light path! Also, make sure to use the microcuvette each time in the same orientation as you did with your blank (e.g. the side which says Eppendorf facing you or not).

6) Close the sample compartment door. Press the white round "blank" button to zero (or blank) the spec. The display panel should give a concentration reading of 0.

7) Open the sample compartment door and remove the cuvette with your blank. Lift the cuvette arm and use a Kimwipe to carefully wipe off the blank solution from the cuvette.

8) Load 2 µl of your DNA sample into the microcuvette as previously described. Insert the cuvette with your sample and close the compartment door. Press the blue round "sample" button and the spec will display the DNA concentration and the A_{260}/A_{280} ratio for your sample. Repeat for any additional samples you have. Always make sure to wipe off the microcuvette with a clean Kimwipe before/after measuring each sample.

9) At any point, you can press the blue oval "exit" button to return to the Main Menu.

Appendix: Eppendorf BioSpectrometer Instructions

Measuring a single wavelength:

1) Turn the power on (the switch is in the back left corner of the spec).

2) Wait for the “SELF-TEST” to complete. Press “OK” to go to the Main Menu.

3) On the Main Menu, press the down arrow once to highlight “Absorbance.” Press the right arrow twice to highlight “Single λ” in the Methods column.

4) Press the white oval “enter” button – this will take you to the “Single λ” parameters page.

5) If needed, press “Edit” to change the wavelength. Leave the “Cuvette” setting at 10mm (or 1 cm, the standard pathlength of the cuvettes used in most labs). Use the number keypad to change the “Wavelength” setting to your desired wavelength (e.g. 280 nm or 600 nm, etc). Press “Save” to save any changes you made.

6) Once the wavelength setting is correct, press “Next>” to proceed.

7) Lift the blue sample compartment door and insert the cuvette with your blank into the sample holder. Make sure the cuvette is in the correct orientation such that the light path passes directly through the sample!

8) Close the sample compartment door. Press the white round “blank” button to zero (or blank) the spec. The display panel should give an absorbance reading of 0.

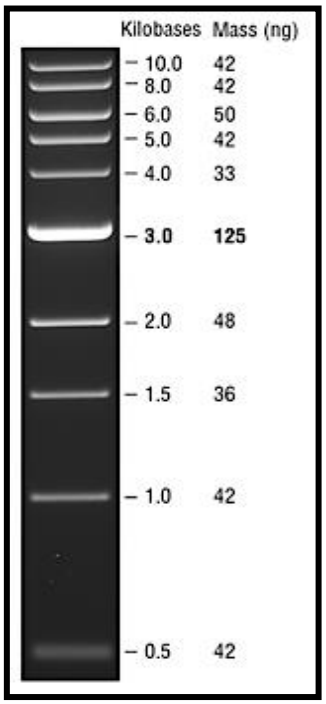
9) Open the sample compartment door and remove the cuvette with your blank.

10) Insert the cuvette with your sample and close the compartment door. Press the blue round “sample” button and the spec will display the measured absorbance for your sample. Repeat for any additional samples you have.

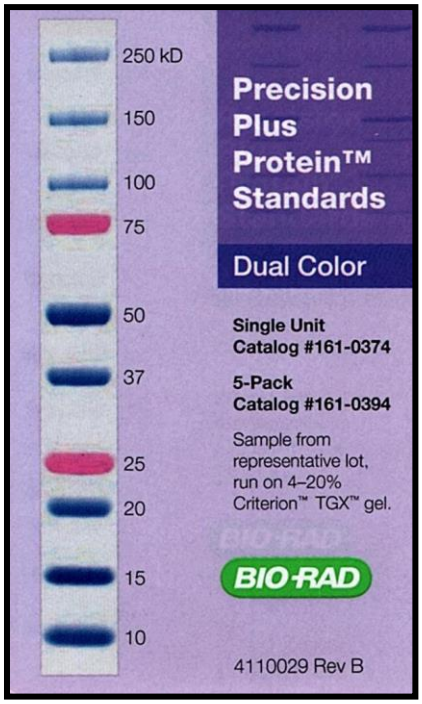
11) At any point, you can press the blue oval “exit” button to return to the Main Menu.

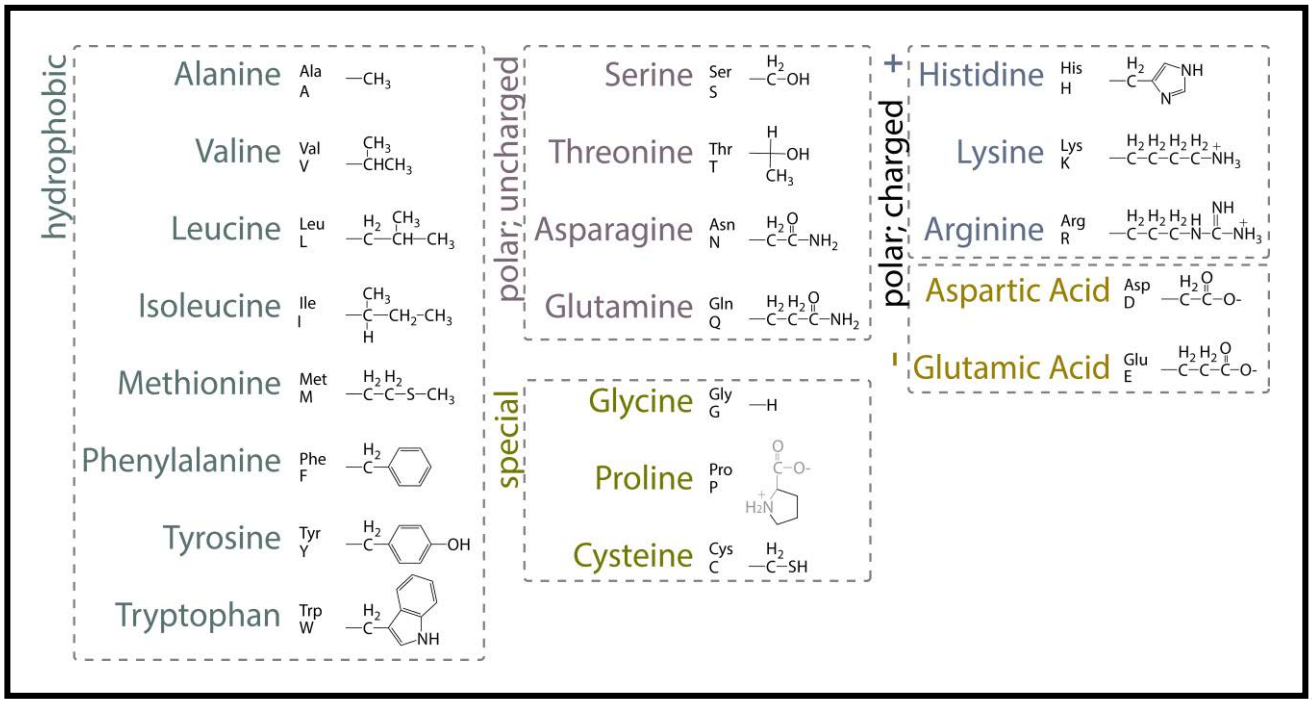
Appendix: Molecular Weight Markers and Amino Acid Structures

1-kb DNA ladder (NEB)



BioRad Precision Plus Dual Color Pre-stained High Range Standards

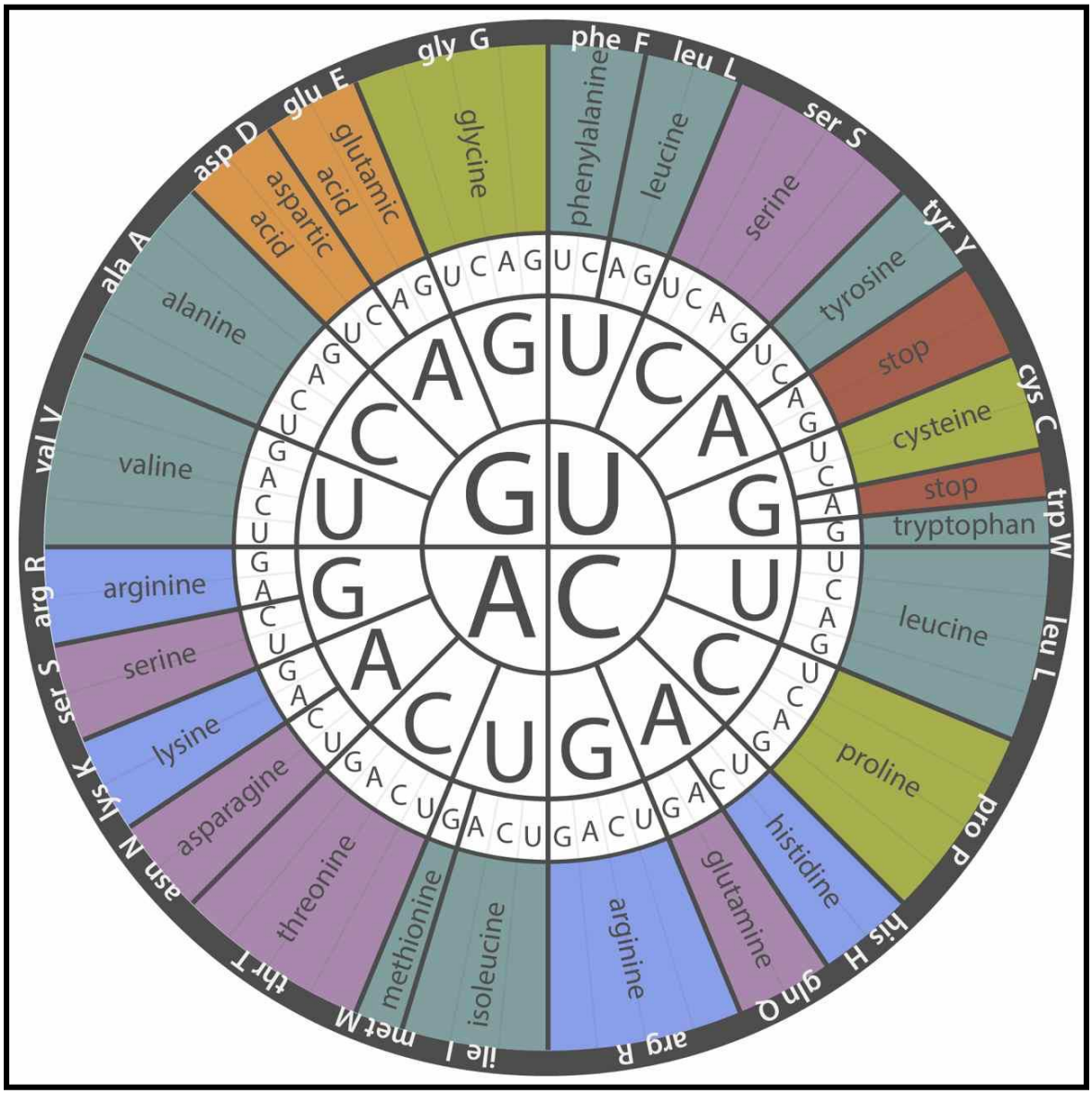




Thornton, © 2013 Broad Institute

Sera

Appendix: Codon Chart



Sera Thornton, © 2013 Broad Institute

Example uses of this chart:

- To determine which amino acid is coded by the codon 5' – AGC – 3', find the big A in the center, followed by the middle-sized G, and the small-sized C, and you will see the amino acid serine.
- To determine a codon for lysine, work backwards from the lysine box to get 3' to 5' sequence and then flip. Codons for lysine would be 5' – AAA – 3' and 5' – AAG – 3'.

Appendix: Primers Available for RTH Mutagenesis

Forward Primers

Primer Name	Primer sequence (5'-->3'). First codon contains mutation.
D184A_F	GCT gaa atc gac aag att tct cgt aag
D184E_F	GAG gaa atc gac aag att tct cgt aag
D184H_F	CAT gaa atc gac aag att tct cgt aag
D184N_F	AAT gaa atc gac aag att tct cgt aag
D184R_F	CGT gaa atc gac aag att tct cgt aag
D184S_F	AGT gaa atc gac aag att tct cgt aag
E185A_F	GCA atc gac aag att tct cg
E185D_F	GAT atc gac aag att tct cgt
E185F_F	TTC atc gac aag att tct cgt
E185G_F	GGA atc gac aag att tct cg
E185K_F	AAA atc gac aag att tct cg
E185L_F	CTA atc gac aag att tct cgt
E185N_F	AAC atc gac aag att tct cgt
E185Q_F	CAA atc gac aag att tct cg
E185R_F	CGA atc gac aag att tct cgt
E185V_F	GTA atc gac aag att tct cg
F298V_F	GTT ggt ctt atc cct gag ttt att gg
K125A_F	GCA acg ctg ctg gct gaa acg
K125E_F	GAA acg ctg ctg gct gaa acg
K125L_F	CTG acg ctg ctg gct gaa acg
K125P_F	CCA acg ctg ctg gct gaa acg
K125Q_F	CAA acg ctg ctg gct gaa acg
K125T_F	ACA acg ctg ctg gct gaa acg
K125W_F	TGG acg ctg ctg gct gaa acg
R370A_F	GCT ggc ctg cgt tcc atc gt
R370D_F	GAT ggc ctg cgt tcc atc gt
R370F_F	TTT ggc ctg cgt tcc atc gt
R370G_F	GGT ggc ctg cgt tcc atc gt
R370L_F	CTT ggc ctg cgt tcc atc gt
R370S_F	AGT ggc ctg cgt tcc atc gt
Y153A_F	GCT gtg ggt gaa gac gtt gaa aac atc a
Y153C_F	TGT gtg ggt gaa gac gtt gaa aac atc a
Y153D_F	GAT gtg ggt gaa gac gtt gaa aac atc a
Y153F_F	TTT gtg ggt gaa gac gtt gaa aac atc a
Y153N_F	AAT gtg ggt gaa gac gtt gaa aac atc a
Y153P_F	CCT gtg ggt gaa gac gtt gaa aac atc a
Y153S_F	TCT gtg ggt gaa gac gtt gaa aac atc a

Reverse Primers

Primer Name	Primer sequence (5'-->3')
E185_R	atc gat gta gac aat acc ac
R370_R	ggc acc ggt ttt acg cgc cat
K125_R	acc gga acc ggt cgg acc gat c
Y153_R	acc ggc ttc ggt cag tgt agt c
D184_R	gat gta gac aat acc acg ctg tgc
F298V_R	ctt gat cag atc ttc cgg ttc aac c
T121_R	cgg acc gat cag cag aat gtt ac
S123_R	acc ggt cgg acc gat cag cag aat g

Appendix: ClpXΔN Coding Sequence

Previous research has shown that wildtype full-length ClpX and wildtype ClpXΔN (missing the first 61 amino acids of the protein) have similar binding properties and ATPase activity. In 7.002, we utilize the ClpXΔN variant in our experiments simply because the ClpXΔN variant is known to be more robustly expressed and purified from *E. coli*.

The sequence given consists of *ClpX* coding sequence (top line) that corresponds to amino acid residues 62 – 424 (bottom line, every fifth amino acid is numbered). The amino acid residues are represented by their one letter abbreviations.

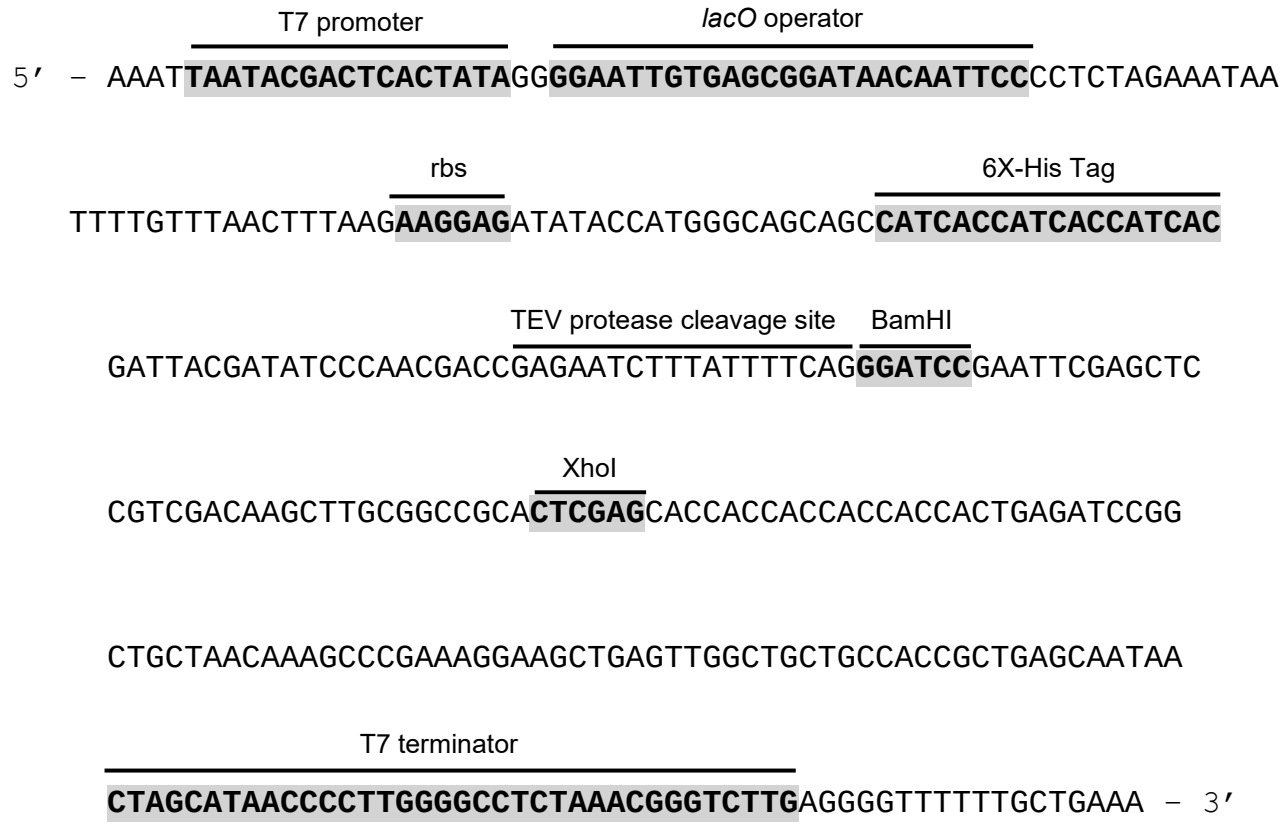
```
agt gcg cta ccg acg ccg cat gaa att cgc aac cac ctg gac gat tac gtt atc
 S   A   L  P65  T   P   H   E  I70  R   N   H   L  D75  D   Y   V   I

ggc cag gaa cag gcg aaa aaa gtg ctg gcg gtc gcg gta tac aac cat tac aaa
G80  Q   E   Q   A  K85  K   V   L   A  V90  A   V   Y   N  H95  Y   K

cgt ctg cgc aac ggc gat acc agc aat ggc gtc gag ttg ggc aaa agt aac att
 R   L  R100 N   G   D   T  S105 N   G   V   E  L110 G   K   S   N  I115

ctg ctg atc ggt ccg acc ggt tcc ggt aaa acg ctg ctg gct gaa acg ctg gcg
 L   L   I   G  P120 T   G   S   G  K125 T   L   L   A  E130 T   L   A

cgc ctg ctg gat gtt ccg ttc acc atg gcc gac gcg act aca ctg acc gaa gcc
 R  L135 L   D   V   P  F140 T   M   A   D  A145 T   T   L   T  E150 A

ggt tat gtg ggt gaa gac gtt gaa aac atc att cag aag ctg ttg cag aaa tgc
 G   Y   V  G155 E   D   V   E  N160 I   I   Q   K  L165 L   Q   K   C

gac tac gat gtc cag aaa gca cag cgt ggt att gtc tac atc gat gaa atc gac
D170 Y   D   V   Q  K175 A   Q   R   G  I180 V   Y   I   D  E185 I   D

aag att tct cgt aag tca gac aac ccg tcc att acc cga gac gtt tcc ggt gaa
 K   I  S190 R   K   S   D  N195 P   S   I   T  R200 D   V   S   G  E205

ggc gta cag cag gca ctg ttg aaa ctg atc gaa ggt acg gta gct gct gtt cca
 G   V   Q   Q  A210 L   L   K   L  I215 E   G   T   V  A220 A   V   P

ccg caa ggt ggg cgt aaa cat ccg cag cag gaa ttc ttg cag gtt gat acc tct
 P  Q225 G   G   R   K  H230 P   Q   Q   E  F235 L   Q   V   D  T240 S

aag atc ctg ttt att tgt ggc ggt gcg ttt gcc ggt ctg gat aaa gtg att tcc
 K   I   L  F245 I   C   G   G  A250 F   A   G   L  D255 K   V   I   S

cac cgt gta gaa acc ggc tcc ggc att ggt ttt ggc gcg acg gta aaa gcg aag
H260 R   V   E   T  G265 S   G   I   G  F270 G   A   T   V  K275 A   K

tcc gac aaa gca agc gaa ggc gag ctg ctg gcg cag gtt gaa ccg gaa gat ctg
 S   D  K280 A   S   E   G  E285 L   L   A   Q  V290 E   P   E   D  L295
```

Appendix: ClpXΔN Coding Sequence (continued)

```
atc aag ttt ggt ctt atc cct gag ttt att ggt cgt ctg ccg gtt gtc gca acg
 I   K   F   G  L300 I   P   E   F  I305 G   R   L   P  V310 V   A   T

ttg aat gaa ctg agc gaa gaa gct ctg att cag atc ctc aaa gag ccg aaa aac
 L  N315 E   L   S   E  E320 A   L   I   Q  I325 L   K   E   P  K330 N

gcc ctg acc aag cag tat cag gcg ctg ttt aat ctg gaa ggc gtg gat ctg gaa
 A   L   T  K335 Q   Y   Q   A  L340 F   N   L   E  G345 V   D   L   E

ttc cgt gac gag gcg ctg gat gct atc gct aag aaa gcg atg gcg cgt aaa acc
F350 R   D   E   A  L355 D   A   I   A  K360 K   A   M   A  R365 K   T

ggt gcc cgt ggc ctg cgt tcc atc gta gaa gcc gca ctg ctc gat acc atg tac
 G   A  R370 G   L   R   S  I375 V   E   A   A  L380 L   D   T   M  Y385

gat ctg ccg tcc atg gaa gac gtc gaa aaa gtg gtt atc gac gag tcg gta att
 D   L   P   S  M390 E   D   V   E  K395 V   V   I   D  E400 S   V   I

gat ggt caa agc gaa ccg ttg ctg att tat ggc aag ccg gaa gcg caa cag gca
 D  G405 Q   S   E   P  L410 L   I   Y   G  K415 P   E   A   Q  Q420 A

tct ggt gaa taa
 S   G   E  STOP
```

Appendix: pET Vector Cloning Site Sequence

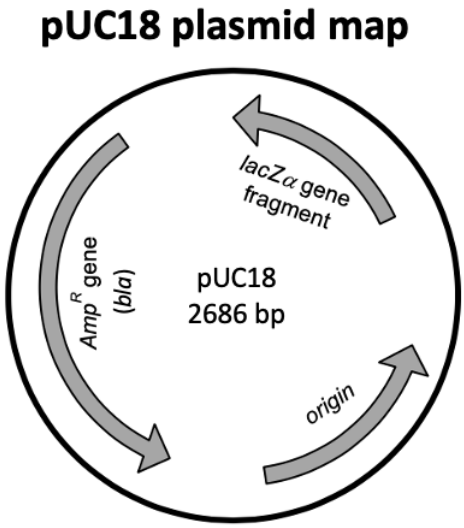
The diagram below shows the cloning/expression region of the pET empty vector used for expressing ClpX in 7.002. Only the coding (sense) strand of the pET empty vector is shown. Unique restriction sites are indicated.

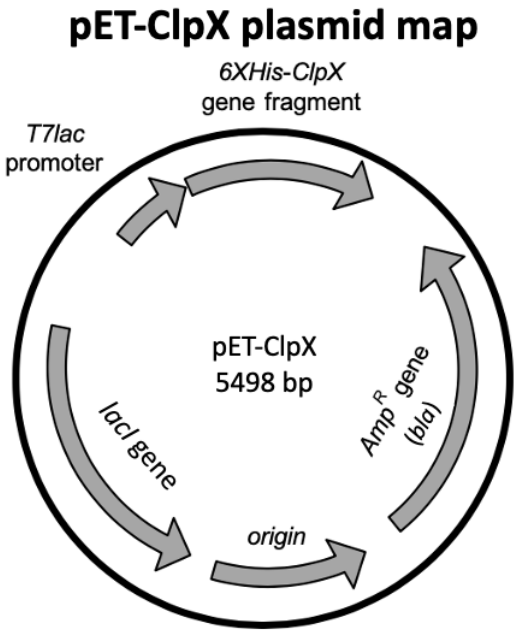


Note: For the pET-ClpX plasmid used in 7.002, the ClpXΔN coding sequence (1,091 bases) was cloned into pET vector using the BamHI and XhoI restriction sites. This means the vector was digested with BamHI and XhoI and then the ClpXΔN coding sequence was ligated into the vector, replacing the sequence between the two cut sites.

Appendix: Plasmid Maps and Strain Info

Commonly Used Plasmids





Commonly Used Strains

Features of DH5α *E. coli*

- High transformation efficiency
- *endA1* mutation: inactivates an intracellular endonuclease that degrades plasmid DNA
- *recA* mutation: eliminates homologous recombination
- *hsdR17* mutation: eliminates EcoKI endonuclease, which recognizes foreign, unmethylated DNA

Features of LOBSTR BL21(DE3) *E. coli*

- High expression of recombinant protein with minimal background of histidine-rich proteins
- DE3: T7 RNA Polymerase is under the lacUV5 promoter (a promoter that is inhibited in the absence of lactose but does not need the CAP activator protein)
- BL21: a cytosolic protease (Lon) and a cell membrane protease (OmpT) are inactivated
- LOBSTR: two His-rich proteins (ArnA and SlyD) are mutated so they will not bind the nickel column during purification

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