Biochemistry 551 (Online Version) Lab Manual

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Biochemistry 551: Biochemical Methods, Online Version

Course Description: Biochemistry 551 is an integrated lecture, lab and seminar course that covers biochemistry-centered theory and techniques. The course is designed for upper-level undergraduate students majoring in Biochemistry. Students learn how to apply a broad range of biochemical, genetic, and physical techniques to modern biochemical research. Students also learn how to analyze and interpret the primary scientific literature, develop an understanding of the communication of data, and connect biochemical techniques to basic research.

Lectures introduce concepts and theory that are subsequently explored in detail in experiments. The virtual labs are designed to provide experience with techniques that are used in modern biochemical research through interactive online activities. The curriculum incorporates a research project beginning with the PCR amplification and cloning of the *HCAII* gene, which codes for the enzyme human carbonic anhydrase II (HCAII). As the semester progresses, students explore how to overexpress, purify and assay wild type and mutant HCAII protein. Experiments covered include PCR, spectrophotometry, gel electrophoresis, protein overexpression and purification, enzyme assays and fluorescence spectroscopy. Several times during the semester, at-home lab experiments are incorporated to provide hands-on experience to supplement student understanding of the virtual labs.

This lab manual contains detailed descriptions of each online laboratory exercise in this course. Please see the course Canvas site for other course information, such as: a syllabus, schedule,

assignment guidelines, and lecture and seminar materials.

By the end of Biochemistry 551, students should be able to:

- Explain the theory of several fundamental biochemical techniques
- Form hypotheses based on biochemical principles
- Design and perform experiments to collect sound scientific data
- Critically analyze one's own data as well as data from other sources
- Communicate scientific findings in both oral and written form
- Value the collaborative nature of biochemistry

LAB 1: STRUCTURAL ANALYSIS OF HCAII USING PYMOL

Lab 1: Structural Analysis of HCAII using PyMOL

Lab 1 Objectives

This week you will use the molecular graphics program PyMOL to:

- Explore crystal structures downloaded from the Protein Data Bank (PDB)
- Examine the structure of the HCAII protein
- Propose HCAII mutations that you find scientifically interesting

NOTE: This lab requires you to install PyMOL on your computer.

The full version of PyMOL is available to UW students as a free download from this link (opens in a new tab): Campus Software Library

It is important that you download the full version through the

provided link; DO NOT download the "Education Version" directly from PyMOL, which is available for free to everyone but does not include all the required functionality.

Be sure to download the appropriate version of PyMOL. For Mac users, there is only one option. For Windows users, you want PyMOL, not AxPyMOL, and 32-bit vs 64-bit depends on the version of Windows you have installed on your computer. Info from Windows can be found at this link (opens in a new tab): 32-bit and 64-bit Windows: FAQ

If downloading PyMOL v2.0 or later, you must also download the PyMOL License File (available from the same Campus Software Library website).

BEFORE LAB BEGINS make sure you can open PyMOL on your computer!! If you have trouble downloading or installing the software, contact DoIT directly.

It is helpful to use PyMOL with a mouse (rather than a laptop trackpad) if possible.

Lab 1: BACKGROUND

For background related to the HCAII enzyme, please refer to the HCAII lecture and associated materials on Canvas.

This is a review article that you may find helpful as you are starting to think about the HCAII enzyme, but is not required reading (link opens in a new tab): Link to 2008 HCAII Review

Lab 1: PROCEDURE Part 1

> **NOTE:** Before lab, you are expected to read through the procedure instructions. You should be familiar with what you will be doing before lab starts.

> **NOTE:** The instructions for this lab were written for a Mac. However, PyMOL is virtually identical on a PC, so we don't anticipate any problems.

> **NOTE:** The times in parentheses are approximate suggestions of how much time it should take to complete the tasks. Ideally, this lab can be completed in about three hours.

PART 1: GETTING FAMILIAR WITH PROTEIN STRUCTURE (45 min)

Explore the PDB site (5 min)

- Go to the PDB website (link opens in a new tab): Protein Data Bank
- In the search box at the top of the page you can type the name of any protein you like in the search box, or you can use the

"PDB code", a code of four alphanumeric characters that defines a structure. If you don't know what to type try, for example, carbonic anhydrase.

- You will get a list of results. Click on one of them and you will find its description page, including the Primary Citation (the paper where the crystallography was presented), the Molecular Description (the name of the protein and its classification), and possible ligands.
- Under the picture you'll find the Experimental Details, which include the resolution (<1.5 Å: very high; <2 Å high; <2.5 Å medium), space group (the geometry of the crystal), R-value and R-free (quality control numbers; generally these should be ~10% of the resolution or less).
- The PDB code (e.g. "3IEO") is in the top-right corner.
- Try a number of searches for proteins that you have studied. Note down the PDB code to open them in PyMOL later.

Visualize proteins with PyMOL (15 min)

- Open PyMOL.
- Note the tool bar on top, the menus, and the command text box underneath them (next to the "PyMOL>"). (See figure 1.1). Typing a command in the command box can perform many of the functions.
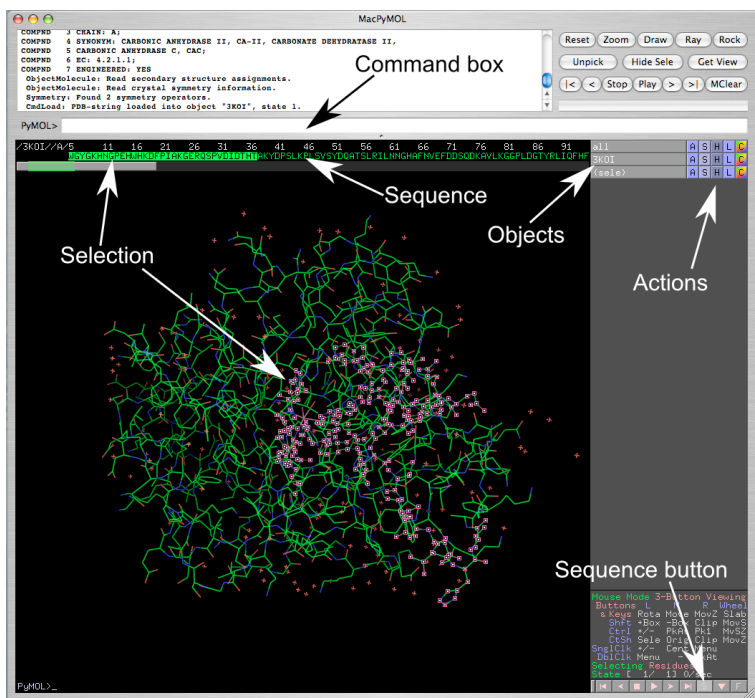
Fig 1.1. PyMOL displaying a protein in 'line' visualization. The sequence of the protein is turned on (by pressing the "S" sequence button on the bottom right). Part of the sequence is selected (highlighted both in the sequence and in the molecule). Actions can be performed on the entire protein (3KOI) or on the selection ('sele').

- Type in the command "fetch <pdb code>" (e.g. "fetch 3IEO"). This will download the structure from the PDB web site and the protein will appear in the main portion of the screen.
- Using the mouse:
  - Clicking on the background and moving the mouse will rotate the molecule around the X and Y axes (clicking at the edge of the screen will rotate around Z axis).
  - Clicking on the molecule will select residues (or chains, or atoms, depending on the current mode).

- Right click will zoom in or out on the molecule (move mouse up and down).
    - The middle button can be either clicked or rolled.
    - Clicking the middle button will allow you to move the structure sideways (translation).
    - Rolling the mouse wheel will slice away Z-planes (you'll see only a slice of the protein), a good way to focus only on some details of the protein.

- Objects: In the right panel you will see two or more objects: *all*, one or more *<pdb code>*(i.e. 3IEO), and possibly some selections '(*sele*)'. You can click on these buttons to display or hide molecules without deleting them (light grey buttons are displayed, dark grey buttons are hidden).
- Next to them there are five buttons: **A**, **S**, **H**, **L**, **C**

    - **A** stands for *action*; you can zoom, center, rename, delete, and duplicate objects from this menu
    - **S** stands for *show*; here you can change the representation of the molecules: e.g. line, cartoon, or stick.
    - **H** stands for *hide*, it performs the same functions as S except that it hides whatever selection you make.
    - **L** stands for *label*
    - **C** stands for *color*: here you change to a specific color, color by chain, by secondary structure, or color by rainbow. Coloring by spectrum/rainbow highlights visually the N- and C-termini of the protein. If you use color by chain/rainbow each chain will have its own rainbow spectrum and it will be easy to see where they start and end.
- Selecting parts of the protein with commands:
    - To select a chain type in the command box 'sele chain A'
    - To select an amino acid name 'resn', type 'sele resn LEU'
    - To select a residue by number ('resi') and chain, type 'sele chain A and resi 74'

- You can also use the 'or' operator for complex logic: 'sele resi 74 or resn LEU' will select residue number 74 and all the Leu in the protein
- Once you enter a selection, the corresponding residues become highlighted and you can perform an action with the **ASHLC** buttons next to '(sele)' in the object box

Play with the visualization modes (10 min)

Experiment with different proteins to familiarize yourself with PyMOL.

- Load the structure, display it in both cartoon and line representations, and color by chains to show the individual subunits.
- Change the PyMOL background color to white (this will make things easier to print): Click on the Display drop-down menu; click Background; choose White.
- Open the sequence display (the little square **S** button in the bottom-right corner, near some controls that look like VCR control buttons, play, stop, etc.). The sequence will show on the top.
- Find a tryptophan in the protein, if there is one: check the sequence for a W, left click on it to select it, and then in the right panel under the 'sele' button use the **A** button to zoom in on this atom. Use the S button to show the side chain in "sticks". If there is no Trp, try Tyr (Y).
- Use the middle button scroll to adjust the visible slab of the molecule (this takes the stuff in the foreground and in the background away and let you focus on the details)
- Repeat for phenylalanine (F). Once zoomed in, let's display the whole structure in lines, (use the show button); now try displaying in sphere representation.
- Hide the sphere representation and display just the phenylalanine side chain in sphere.
- To help see the side chains beneath the spheres, go to settings

in the above tool bar, then transparency, then spheres, then 60%.

- Hide spheres, then select any residue, zoom in, and only for this residue display dots; this is a convenient way to see space filling models of single amino acids.
- Zoom out of the structure, then display the whole molecule as 'surface'; from the **S** button. Another way to view this is show as mesh surface.

Practice following a fold with the structure of a TIM barrel (10 min):

- Remove all the unneeded PDBs from the object box (**A**->delete object).
- Download a TIM barrel, a very common enzyme fold. Fetch 1TRI (an engineered monomeric triose isomerase).
- The TIM barrel (so called because it was first identified in Triose IsoMerase) has an α/β fold, with a central parallel β-barrel (a β-sheet that closes into itself) and α-helices on the outside.
- Display by cartoon, color by rainbow, such as in Fig. 1.2, and follow the fold. You will observe a series of inner β-strands, loops, and α-helices that repeat 8 times, forming an inner β-barrel surrounded by helices.

Fig. 1.2. Top view of the TIM barrel 1TRI. Note the alternating helices and sheets, forming an inner parallel β-barrel surrounded by helices.

- Note that if you display the structure in sphere, you'll see that the center of the β-barrel is not empty.
- The active site is at the "mouth" of the β-barrel.
- Familiarize yourself with the fold.
- Now delete 1TRI and fetch 2X2G. This protein has two molecules in the asymmetric unit (in is not a biological dimer but it crystallizes as a pair).
- Display the protein in cartoon.
- Show the surface of the entire molecule (do not turn off the cartoon).
- Set the surface transparent (menus Setting/Transparency/Surface/60%)
- Now select the ligand: command 'sele resn 3PG'
- Show the selection (the ligand) in spheres

- Zoom into the ligand (**A**ction Zoom next to '(sele)') and examine how the ligand fits into the cavity of the active site.
- Save an image:
  - Selecting the button "Draw/Ray" in the top right corner
  - Select Draw (fast)
  - Select Save Image to File and name the file
  - **NOTE:** Generally, when using images in lab reports, you will be required to ray-trace the image (more on that later). To save images for yourself for future reference, however, this quicker drawing approach is fine.

Lab 1: PROCEDURE Part 2

PART 2: VISUALIZATION OF HUMAN CARBONIC ANHYDRASE II (30min)

- Close and reopen PyMOL to start fresh.
- Change the background to white.
- Download 2VVA. This is a structure of HCAII with CO_2 in the active site.

> **NOTE:** The numbering of HCAII residues is not the logical 1–260 consecutive sequence that one would expect. Because a lot of work was done on HCAI, which has an extra residue, HCAII is numbered with a gap at position 126:
>
> ...Asn 124, Thr 125, Lys 127, Tyr 128...
>
> Thr 125 and Lys 127 are consecutive in the sequence and connected to each other (please verify that in PyMOL). The gap is a numbering convention for convenience in discussion, so that the active site residues in HCAI and HCAII have the same residue number.

- Hide everything and show the protein in cartoon. Color it by spectrum/rainbow.
- Show the sequence.
- First select the catalytic atoms, the Zn^{2+} and the zinc-bound hydroxyl group. Type the command: 'sele resn ZN or resi 2350'. That is, select the residue named ZN or the residue number

2350, which happens to be the number of the water molecule bound to the zinc. (The 'or' ensures that both are selected.)

- Display them in sphere. You may want to rename the selection: press the **A** next to the '(sele)', chose 'rename selection', call it 'Zn-OH', or something else of your choosing.
- Clear the selection by clicking in a white area.
- Find in the sequence the Zn-coordinating histidines (H): H94, H96 and H119 (you could also select them with the following command 'sele resi 94+96+119'), and display them in stick.
- Rename the selection something like 'coorHis'.
- Show the surface of the entire molecule (do not turn off the ribbon).
- Set the surface transparent (menus Setting/Transparency/ Surface/60%)
- Now, play around with the color scheme. If you use the color as it is now, as shown in Fig 1.3, it is very difficult for the viewer to see what is important in the figure. For your lab reports, you want to think about the story you want to tell with this figure, and how to draw the viewer's attention to the important parts.
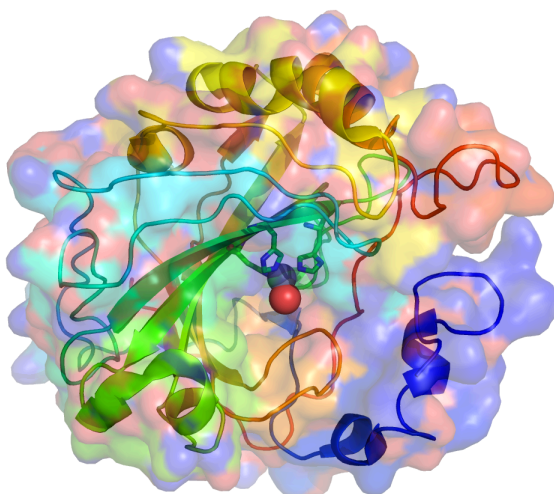
Fig. 1.3. A global view of HCAII from PDB 2ILI. Note how obnoxious the color scheme is.

- Now, play around with the orientation of the model. You should be able to find an orientation such that you can appreciate the location of the active site. There is a clear cavity in the surface, and at the end of it there is the Zn-OH group.

- Take two pictures from different angles and/or at different zooms for your lab notebook.
    - Ray trace the image for quality. Select "Draw/Ray" from the top right corner, then select "Ray (slow)"
    - Then Save Image to File
    - Rotate, and repeat.

> **NOTE**: It is essential that you ray-trace any image to be included in a lab report. It is therefore a best practice to ray-trace any images you plan to save. However, some computers take an excruciatingly long time to ray-trace. If this happens to you, you may save the sessions instead and go back later to ray-trace any images you want for your report (File>Save Session).

- Now select the polar residues of the active site: Tyr (Y) 7, Asn (N) 62 and 67, His (H) 64, Gln (Q) 92, Glu (E) 106, and Thr (T) 199 and 200. First deselect everything by clicking in a black area, then type the following command: 'sele resi 7+62+64+67+92+106+199+200'. Rename the selection 'polar' and display them in sticks.

> **NOTE**: His 64 is displayed in two conformations! This is because in the crystal this side chain spends about 50% of the time toward the active site, and 50% in the other orientation. The crystallographer was able to see electron density for both conformations and fit a side chain in it. This is not unusual for high-resolution structures.

- Now select the hydrophobic residues in the pocket: Ile (I) 91, Val (V) 121 and 143, Phe (F) 131, Leu (L) 141 and 198, and Trp (W) 209: type 'sele resi 91+121+131+141+143+198+209'.
- Now select the CO_2: 'sele resi 1264' and save the selection as

'CO2'. Display it in spheres.

- Now examine the structure and the residues that define the active site. Use cartoon for the backbone of the whole structure. Hide the sticks for the hydrophobic selection, and take a picture with just the polar selection for the lab report. Then, without moving the protein, hide sticks for the polar, show sticks for the hydrophobic residues and take another picture.
- Save what you have gotten so far as a PyMOL session (File>Save Session). Save the file as 2VVA.pse.

Lab 1: PROCEDURE Part 3

PART 3: SURFACE EXPOSURE OF KEY RESIDUES (30mins)

During a future lab, you will use the intrinsic fluorescence properties of tryptophan residues to study the stability of wt and mutant HCAII. The assay relies on the change in fluorescence properties of tryptophan depending on its environment. When a protein is fully folded, most tryptophans are buried; this yields a characteristic spectrum. When a protein is unfolded, all tryptophan residues become exposed, which alters the spectrum. We will use this change in fluorescence upon unfolding to determine whether your mutations affect the stability of HCAII. For now, you will generate some information on the surface exposure of tryptophans. Make sure you save your PyMOL sessions. and figures so you can refer back to them later in the semester.

Calculate surface exposure with an online tool

- Download the 2VVA.pdb file from the PDB website (link opens in a new tab): http://www.rcsb.org/
- Go to the GETAREA website (link opens in a new tab): http://curie.utmb.edu/getarea.html
- Choose the pdb file you just downloaded. Leave the other fields at the default, enter your email address, and hit "Submit for Analysis."
- You should now have a read-out for each residue in HCAII and a determination for whether it is inside or outside on the protein. If you scroll to the bottom, you will also get a count of the total number of surface vs. buried atoms.
- Find each tryptophan residue (TRP) and note whether it is listed as in or out.

Visualize surface exposure in PyMOL

- If you closed PyMOL, reopen and open your saved 2VVA.pse

file.

- Show all residues as spheres and color gray.
- Select all TRP residues by typing 'select resn TRP and not name C+O+N+CA'
- Rename the selection trp.
- Color the tryptophans yellow.
- Look at the relative surface locations of each tryptophan. You may want to make the protein transparent (by choosing Setting, Transparency, Spheres) to see where they all are.
- Make a list of each tryptophan with its residue number and whether you think each is completely buried, minimally exposed, significantly exposed, fully exposed.

    - If you like, you can label the tryptophans for easier identification. With the trp residues selected, go to L -> residues

- Ray trace and save pictures of the trp residues for use in future labs. You do not have to take separate pictures for each residue – just one or two that allow you to see them all.
- Save this session as 2VVAtrp.pse for future use.

Lab 1: PROCEDURE Part 4

PART 4: DESIGNING MUTATIONS IN HCAII (1 hr 15mins)
  Mutagenesis of HCAII active site (30 min)
  Now it is time to model mutating the protein!

- Close PyMOL, reopen it, and load the first session you saved: 2VVA.pse
- To make a mutation, go to the menu: Wizard>Mutagenesis (Fig. 1.4)
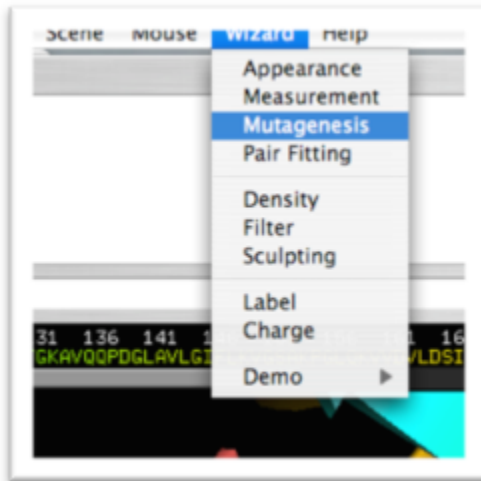

Fig. 1.4. The mutagenesis menu under Wizard.

- On the bottom of the side panel you will see the mutagenesis options. Click on *No Mutation* and select the amino acid type you would like to mutate a side chain to (Fig. 1.5).
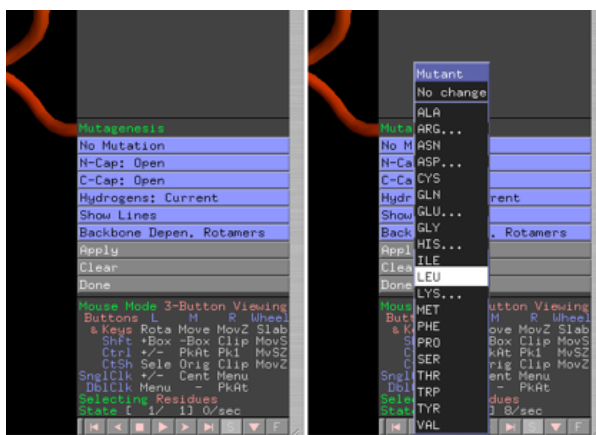
Fig. 1.5. Select the mutated amino acid. Click on No Mutation and pick an amino acid.

- Click on the side chain you would like to mutate, and the new amino acid type will appear in grey on top of the old one (Fig. 1.6). You can change the conformation of the new amino acid using the arrow buttons on the bottom-right corner.
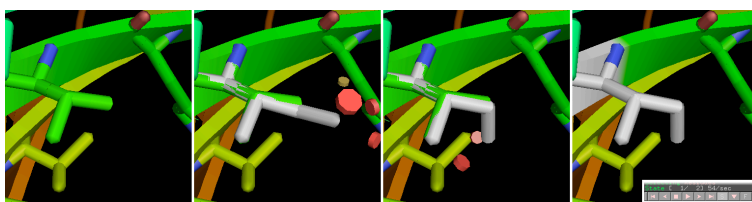
Fig. 1.6. Making a Val-to-Ile mutation: select ILE as the mutated amino acid, then click on the Val side chain. A gray ILE will appear on top. Red disks will show graphically if the new side chain is clashing with other atoms. Use the arrow at the very bottom-right of the PyMOL screen (displayed in the inset) to change conformation.

- You will see some red disks appearing. These are the clashes (atoms going into other atoms). A few small disks mean mild

clashes, which are likely to be relieved by the protein (the backbone and the side chains will have some flexibility that is not present in your rigid model). Big disks (and many of them) means severe clashes.

- It is recommended not to "apply" the mutation, as this causes the original residue and any disks to disappear.

> **NOTE:** PyMOL has a problem mutating amino acids modeled in two conformations, such as His64. The mutation tool will not work for this residue. You can still propose a mutation here, but you won't be able to visualize it.

- Ray trace and save a picture of your mutation. Save the mutation as a separate PyMOL session file (for example, mutation1.pse).
- Reload the 2VVA.pse PyMOL session and work on the next mutation.

You should select three point mutations in the active site and explain the rationale behind each mutation. You may, for example:

- Change the proton-shuttling His64 to a different amino acid. As noted above, you can't visualize mutations to this residue using the mutagenesis tool, but you could still take a look at the residue and think about interesting mutations.
- Change the polarity of the cavity (hydrophobic-to-polar mutations or *vice versa*), while preserving the size
- Change a smaller residue to a larger one in a way that can be accommodated by the structure
- Purposely create clashing mutations that will likely

compromise the structure

- Try to make a mutation that would increase HCAII catalytic activity
- Make mutations that will interfere with the position of the CO_2, or otherwise change the shape of the active site
- Use figure 1.7 as a reference for choosing a mutation. Make sure to comment on how similar (or dissimilar) are the structures of the original residue and the proposed mutation (size/number of atoms, polarity, charge, etc.).
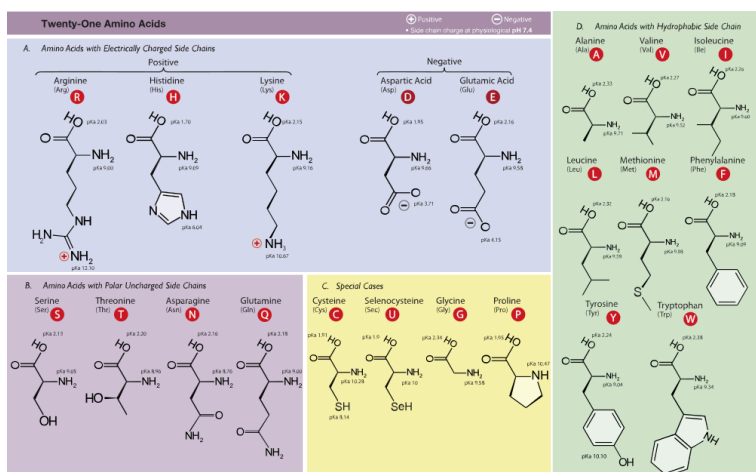
Fig. 1.7. Table of amino acid side chains. The amino acids are grouped by chemical characteristics. Note: histidine can be either positively charged or neutral depending on the pH and the structural context (its pKa is around 6.0). When it coordinates the zinc atom it MUST be neutral because it acts as a Lewis base by donating a lone pair of electrons.
https://commons.wikimedia.org/wiki/File:Amino_Acids-wide.svg

You may choose whatever mutations you want, but you must explain your rationale in your mini lab

Effects of the mutations on DNSA binding (15 min)

- Open a new PyMOL session.
- Fetch PDB 1OKL, of HCAII complexed with dansylamide.
- Select the ligand by name: 'sele resn MNS' and save the selection.
- Consider the mutations you are proposing. Do you expect the binding of the dansylamide to be affected? Are the mutations compatible, or will they clash or generate a void? Be prepared to address these issues in your mini lab report.

Class presents the mutations and votes (30 min)

Once you and your partner have each finished your mutations, decide on your favorite *one* (from your total of six) to present to the class.

- Start by discussing each of your mutations. Once you've decided on your favorite one, go to the class Google slides presentation (your TAs will share the link during lab)
- Look through any slides that other students have already started. If someone else has already chosen your mutation, choose a different one.
- Add a new slide to the presentation. The title should be your mutation, with your names on a second line. Use this slide to present your mutation and your rationale to the class. Be sure to include a clear image of your mutation and where it is relative to the active site. Address why you think this mutation is interesting and what effects you expect to see on the

enzyme.

- Once all the slides are ready, the slide show will be presented. When your slide is up, talk about your mutation for ~1 minute, and answer any questions from the audience.
- After every group has made a suggestion, we will scroll again through the slides and each student will vote for their favorite two mutations. The winners get ultimate bragging rights and will be able to choose their favorite of the mutations actually available to study this semester (most students will have a mutation assigned to them).

Lab 1: LAB ASSIGNMENTS

PRE-LAB QUIZ

Each week, you will take a short Canvas quiz related to preparing for lab. The quiz questions are always listed in the lab manual; please visit Canvas before your lab start time to complete the quiz.

- What will you be doing in lab? Why? (Provide 2-3 sentences).
- Based on what you learned about HCAII during lecture, describe one amino acid you would like to mutate, and to which residue you would change it. Why do you think this mutation would be interesting?

IN-LAB ACTIVITIES

When you arrive on BBCU for lab, you will have a chance to meet your TAs and introduce yourself to them and your classmates. You and a randomly assigned partner will then move to a breakout room. Each student should complete all steps of the procedure individually. If you have questions, you can discuss with your partner. If you still have questions, use the "raise hand" function and a TA will join your breakout room. Your TAs will also be popping into each breakout room periodically to check on your progress. Towards the end of lab, everyone will re-join the main room for presentations of proposed mutations.

Lab 1: MINI LAB REPORT

MINI LAB REPORT 1

Reports must be submitted to Canvas by the start of your next lab.

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Report Guidelines

Results Section

For each of the three mutations you designed during lab, address the following:

- State the residue that you changed and what amino acid you changed it to (e.g. V34K). Include a figure showing the change in the structure. Please make sure that the PyMOL figures you use in the lab report have a white background – this will make them significantly easier to evaluate. Remember that figures need to have informative titles and legends. Your legend should clearly state what is shown in each color and what is depicted as sticks/spheres/etc.
- Justify why you chose to change that amino acid and why you selected the mutant residue that you did. Use your knowledge of the structure from the PyMOL session in Lab 1 and the biochemical properties of the amino acids (Fig. 1.7 or any general biochemistry textbook) to inform your answer. (It is ok to mention the expected phenotype here, if necessary, but that should mainly be discussed in the Discussion Section of the report.)
- Comment on clashes that appear with your mutated residues.
- Study the effect of the mutations you picked on the ligand-binding structure. (The ligand is dansylamide, DNSA). Do they seem compatible, or do the mutations create clashes?

Discussion Section

What effect on HCAII function do you expect to observe for each of the three mutations? Consider the following:

- Different functionality of the new residue (is it a conservative or non-conservative mutation?)
- Effects on structure
- Effects on enzymatic activity
- Effects on ligand-binding

What are the next steps? How would you test the function of the mutants?

LAB 2: PCR AMPLIFICATION OF HCAII AND PETBLUE2

Lab 2: PCR Amplification of HCAII and pETblue2

In Lab 2 we will simulate using the polymerase chain reaction (PCR) to amplify the *HCAII* gene (which encodes the enzyme Human Carbonic Anhydrase II) and the pETblue2 vector, and running the PCR products on an agarose gel. In lab, you will start by working with a partner to design primers for both PCRs. You will then work together to complete a table summarizing each step in the procedure and its purpose. You will then complete virtual lab situations and watch a video of the lab procedures. You will finish by again working with your partner to determine the sizes of the DNA bands you expect to see on the gel. In a future lab, we will simulate using the amplification products in a Gibson assembly reaction in order to clone *HCAII* into pETblue2, an expression vector suitable for protein production in *E. coli*.

Lab 2: BACKGROUND

PCR

As discussed in lecture, <u>PCR</u> can be used to amplify segments of DNA – in this case the *HCAII* gene and pETblue2 vector – using small pieces of ssDNA called primers. PCR is a very powerful and widely used technique in biochemistry and many other fields because it enables specific amplification of a desired segment of DNA. PCR also enables you to introduce mutations and/or additional sequences into the DNA.

<u>Primers</u> are designed to complement the 3′ end of each strand of the DNA template. For Gibson assembly, primers are designed to be complementary to both the vector and insert sequence, with addition of appropriate overlap sequence (see figures 2.1 and 2.2 below). Once the primers have been designed, they can be ordered from a company that chemically synthesizes them.
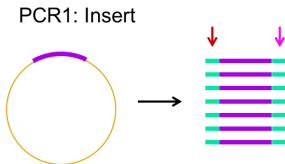
Fig. 2.1. Insert PCR. Arrows indicate the "overlap" sequence, as added by primers. In this case, the overlap consists of vector DNA sequence.
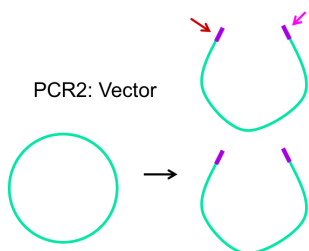
PCR2: Vector

Fig. 2.2. Vector PCR. Arrows indicate
the "overlap" sequence, as added by
primers. In this case, the overlap
consists of insert DNA sequence.

Phusion polymerase is a DNA
polymerase that is resistant to
heat inactivation, making it able
to withstand the temperatures necessary to melt/denature the
DNA template for PCR.

In a typical PCR reaction there are 3 steps in each cycle:

1. Denature the dsDNA (double-stranded DNA) template at 94–96
   ºC. (This temperature will depend on the number and type of
   base pairs in your template).
2. Anneal the primers to the template at 50–65 ºC (This
   temperature will depend on the melting temperature (Tm) of
   your primers).
3. Extend the primers at 72 ºC.

Typically this 3-step cycle is repeated 25-35 times. A final extension
step of ~20 min. at 72 ºC is done to ensure that all of the PCR
products are full synthesized. The thermocycler is then set to hold
at 4 ºC or 10 ºC until the reaction is removed from the machine.

The mechanism of PCR is schematically illustrated in Fig 2.3.
Figure 2.4 shows a diagram of the thermocycler program you will
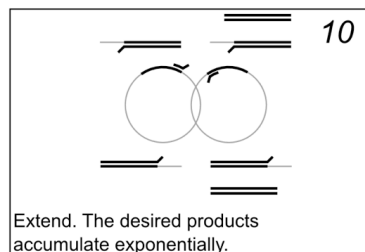use in this lab period.

1 Template DNA. The target region highlighted in black.

2 Cycle 1: denature (94-96º C).

3 Anneal primers (50-65º C). The overhanging regions add the restriction sites.

4 Extend (72º C). The polymerase synthesizes the DNA past the target region.

5 Cycle 2. Denature.

6 Anneal.

7 Extend. The desired products (*) are first produced.

8 Cycle 3. Denature.

9 Anneal. The desired products act as templates, primers anneal with no overhanging regions.

10 Extend. The desired products accumulate exponentially.

Fig. 2.3. Diagram of a PCR.

Fig. 2.4. Diagram of a thermocycler program.

PCR Goal: Amplify HCAII insert and pETblue2 vector using primers designed for Gibson cloning

Run three PCRs:

- Amplify pETblue2 vector
- Amplify HCAII insert

Amplify pETblue2 vector (becomes linear)   Amplify HCAII insert   Negative control
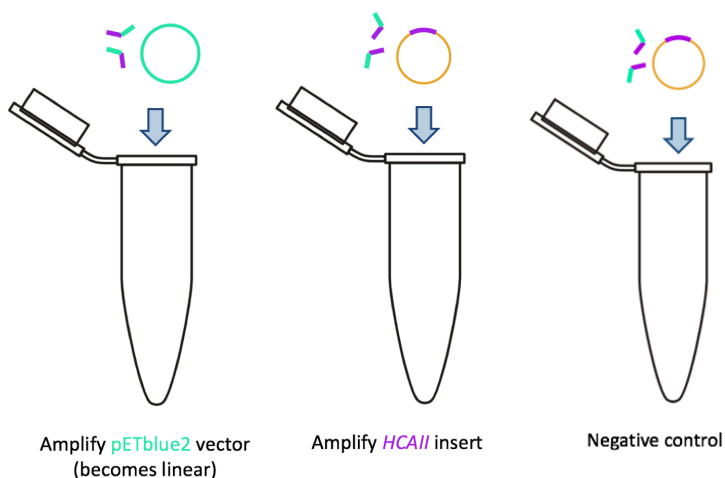
Fig. 2.5. Diagram of three PCRs to be run during Lab 2. Note that the negative control reaction matches the insert reaction in this diagram – the difference is that you will not add enzyme to the negative control. More on that in the next sections.

Agarose Gel Electrophoresis

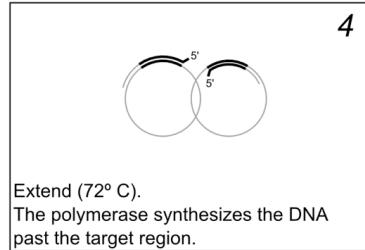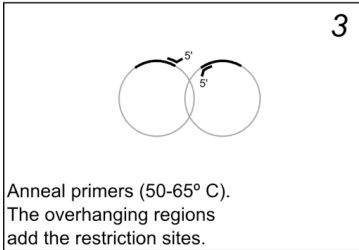Agarose forms a porous gel, as seen in the micrograph image below. You load your DNA in the wells at the top and apply a current, forcing the charged DNA molecules through the gel. This separates the DNA by size, with the smallest DNA fragments able to migrate more quickly through the pores and running farther down the gel.
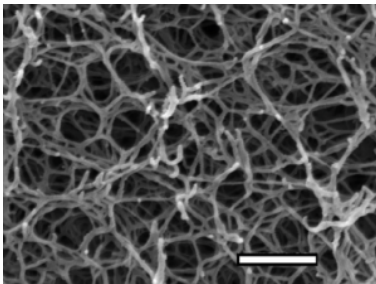
Fig. 2.6. Electron micrograph of a 1.5% agarose gel. White bar = 250 nm
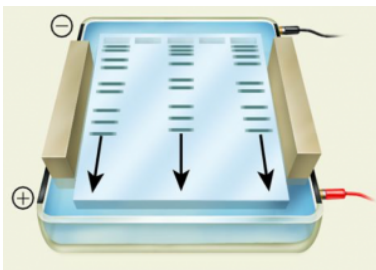
Fig. 2.7. Cartoon image of an agarose gel. The samples are loaded in the wells at the top. An electrical current is applied such that a negative charge is at the top of the gel (black electrode) and a positive charge at the bottom (red electrode).

Different concentrations of agarose are used in gels depending on the relative sizes of the DNA fragments expected or the separation desired; in this experiment you will be running a 1.0% agarose gel. The molecular weight of DNA determines its rate of migration. Typically, the smaller the molecule, the faster it will migrate through a gel. However, DNA pieces of the same size can have different mobilities depending upon whether their shape is linear, circular relaxed, or circular supercoiled. Circular supercoiled DNA migrates faster than either linear or circular relaxed DNA, while linear DNA typically migrates faster than circular relaxed DNA.

Samples containing DNA are mixed with a solution containing bromphenol blue and xylene cyanol as tracking dyes; the bromphenol blue migrates at about the same rate as a 0.3–0.5 kb fragment and the xylene cyanol migrates similarly to a 4–5 kb fragment. Sucrose is also added to make the sample denser than the buffer so the sample will sink to the bottom of the well when it is

loaded. Generally about 2 μL of loading dye is added per 10 μL of sample.

For additional information about agarose gel electrophoresis, please see the appendix: Appendix 3: Additional laboratory details

Preparing an agarose gel

To determine whether our PCR resulted in the desired DNA fragments that will allow us to do Gibson Assembly, we will run a small amount of the PCR product on an agarose gel.

This lab requires 1% w/v agarose gels in TAE buffer.

w/v% = weight of agarose/volume of solution

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https://wisc.pb.unizin.org/biochemistry551online/?p=45

Analyzing a Gel: Ethidium Bromide Staining

Since DNA alone cannot be detected in a gel, ethidium bromide ("EtBr"), a fluorescent dye, is added to the DNA. Ethidium bromide intercalates between the bases of double-stranded DNA or RNA.
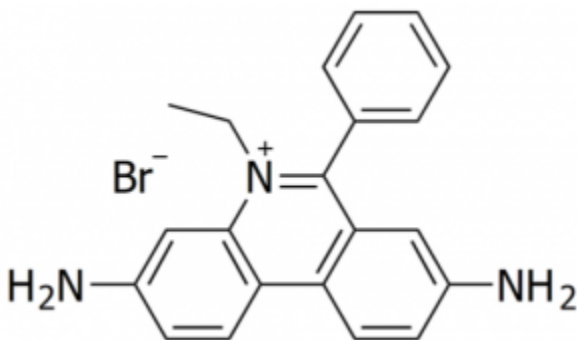
Fig. 3.4. Molecular structure of EtBr.
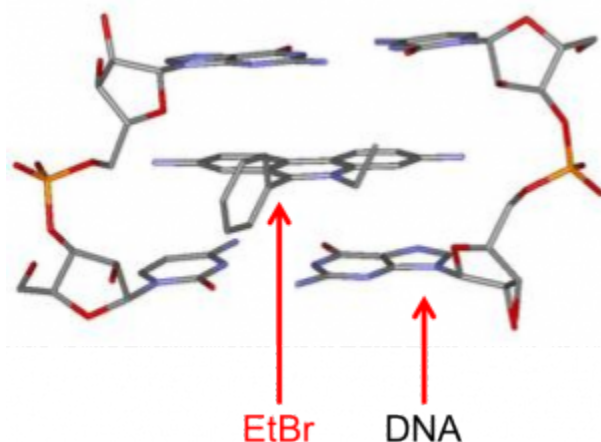
Fig. 3.5. Molecular structure of EtBr intercalated into DNA.

CAUTION: Ethidium bromide is a carcinogen. Make sure you wear gloves when handling it and keep it contained to the designated areas.

EtBr absorbs UV light at 302 nm and produces a reddish-orange fluorescence at 590 nm; the amount of this fluorescence is proportional to the mass of the DNA. Therefore, if you shine UV light on your gel, the EtBr will fluoresce, thus visualizing your DNA.

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https://wisc.pb.unizin.org/biochemistry551online/?p=45

The ladder you load on your gel has bands of known size. This will allow you to estimate the size of your DNA fragments by comparison. A pdf containing information on the ladder we will be using, including the sizes of the bands, is available here (pdf opens in a new tab): NEB quick load DNA ladder

Lab 2: PROCEDURE Part 1

PROCEDURE: PCR

Note: This is the first of several "simulated labs" focused on studying HCAII. The protocols for these labs are written as though you were doing the lab in person, and they include every step that would be required to do so. You are expected to review the protocols before coming to lab, and will be asked about them on the pre-lab quiz. One of your in-lab activities will be to go through the protocol with a partner, and you will have a chance to ask your TAs questions.

Begin by choosing the appropriate primer sets you will need to amplify the *HCAII* gene and pETblue2 vector. Choose from the primers listed below.

- Vector and insert each need to be amplified with overlapping sequence to allow for Gibson assembly (see lecture for more information). If this is done correctly, you will ultimately be able to express his-tagged HCAII protein from your construct. Therefore:
    - Your insert forward primer should contain the *HCAII* start site such that it overlaps with the start site indicated in pETblue2.
    - The stop codon in the *HCAII* gene has to be removed since you want to use the vector's his-tag and stop codon. You need to keep the 3′ end of *HCAII* in frame so that the His-tag in the vector will be expressed.

- ◦ The map of the vector and the *HCAII* gene 5′ and 3′ end sequences are provided as a pdf at this link (opens in a new tab): pETblue2 map
- ◦ Choose which primers will be used for which reaction *before coming to lab*. Do your best. When you arrive to lab, you will discuss with a partner.

Primers available:

Primer A:

5′ GTTTAACTTTAAGAAGGAGATATACCATGGCCCATCACT GGGGGTACGGCAAAC 3′

Primer B:

5′ GAACAGGCAAATCAAAGCTTCCTTCAAACACCACCACCA CCACCACTAATGTTAATTAAG 3′

Primer C:

5′ GTTTGCCGTACCCCCAGTGATGGGCCATGGTATATCTC CTTCTTAAAGTTAAAC 3′

Primer D:

5′ CTTAATTAACATTAGTGGTGGTGGTGGTGGTGTTTGAA GGAAGCTTTGATTTGCCTGTTC 3′

Next, calculate the volume of all of the components for the three different reactions you will be running.

This quick video explains how to do the dilution calculations for PCR, and includes some practice problems:

>
> *An interactive or media element has been excluded from this version of the text. You can view it online here:*
> *https://wisc.pb.unizin.org/biochemistry551online/?p=51*

The table below lists each reagent needed for PCR, the stock solutions that you will be provided, and the desired final reaction conditions:

Reagent	Stock Solutions Provided	Desired Final Reaction Conditions
Buffer	5x	1x
dNTP mix	10 mM (includes 10 mM of each nucleotide)	200 µM
Template	x ng/µL (actual concentration will be provided in lab)	10 ng
Forward primer	10 µM	400 nM
Reverse primer	10 µM	400 nM
Phusion polymerase	1 U/µL (A "U" or "unit" is a standard way of measuring an amount of enzyme in a solution, and relates to its activity)	1 U
Sterile water		To a final volume of 50 µL

Use the information above to create a table that looks like the one below. You should arrive to lab with as much of the table completed as possible. You will finish it with a partner in-lab.

Remember that you will be setting up three separate PCRs:

Reaction #1: *HCAII* template, insert primers

Reaction #2: pETblue2 template, vector primers

Reaction #3: *HCAII* template, insert primers, no Phusion (negative control)

Reagent	Reaction 1: Insert	Reaction 2: Vector	Reaction 3: negative control
5X reaction buffer			
dNTP mix			
Primer A			
Primer B			
Primer C			
Primer D			
DNA template			
H20			
Phusion			
Total Volume	50 µL	50 µL	50 µL

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Finally, set up your reactions! Once you are confident in your calculations, get your aliquots from the stock bench. Keep the aliquots and your PCRs on ice until you put them into the thermocycler. This will help maintain the activity of your *Phusion* enzyme.

Prepare the reactions, per your calculations, into PCR tubes. It is important that you label the PCR tubes clearly and in a way that will allow you to distinguish your reactions from your classmates'. The teaching staff will take the PCRs out of the machines the next morning, and all the tubes will be placed into one large freezer box.

Be sure to add *Phusion* to the reaction last. Use caution when pipetting small volumes! Once all the components have been added to the tubes, mix them with a pipette or by gently flicking the tubes. Do not vortex! Centrifuge briefly in the nanofuge to be sure the samples are all at the bottom of the tubes. You can spin the tiny PCR tubes by placing them inside a 1.5 mL eppendorf tube (people sometimes break the caps off the eppendorf tubes and save them as centrifuge adaptors).

Programming the thermocycler

Once you have finished setting up your PCRs, program the thermocycler to run the following program (also refer back to Figure 2.4):

Step 1: 98 °C for 30 seconds
Step 2: 98 °C for 10 seconds
Step 3: 50 °C for 30 seconds
Step 4: 72 °C for 2 mins
Repeat steps 2–4 for 35 cycles
Step 5: 72 °C for 10 mins
Step 6: 10 °C hold
Keep the reactions on ice while programming the thermocycler.

You need about four people per thermocycler, so don't start the program until at least four people are ready.

NOTE: Stopping the thermocycler during a run can ruin the reactions.

Lab 2: PROCEDURE Part 2

PROCEDURE: GEL ELECTROPHORESIS

Each pair will prepare one batch of 25 mL agarose and use it to pour one gel. They will share that gel to load their samples.

- Calculate the amount of agarose you will need for 25 mL of a 1% gel. Weigh out the agarose and add it to a 250 mL Erlenmeyer flask. Next, add 25 mL 1× TAE buffer to the flask. (TAE stands for "Tris–Acetate–EDTA." A recipe for TAE, and for most buffers used in this course, can be found in the Appendix for future reference: Appendix 4: Buffer formulations)

> **NOTE:** The amount of agarose you will be adding to the TAE will not affect the volume enough to be concerned about. However, if you were using 10 g of agarose, you would need to add the solid first and then add just enough TAE to bring the total volume to 25 mL.

> **NOTE:** 1× TAE buffer will also be used as the running buffer for electrophoresis. Therefore, the gel and the running buffer will have the same ionic strength. Failure to use the running buffer to prepare the gel will result in erratic separation of DNA.

The following video demonstrates the gel pouring process in 551 lab. Note that our pouring systems may be different than those you've used in other labs.
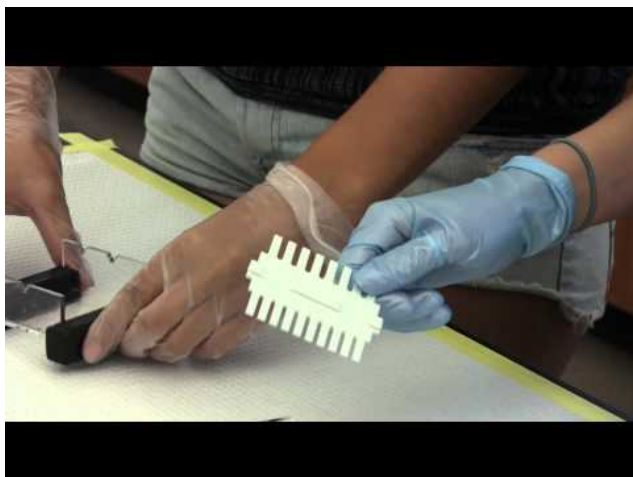
A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/biochemistry551online/?p=278

- Place an inverted 25 mL Erlenmeyer flask or wadded up paper towel in the mouth of the 250 mL flask and heat in a microwave on **medium heat** until all the agarose has completely dissolved. In our microwave, try heating the gel for 45 seconds, swirl to ensure thorough mixing of the agarose, and then heat for additional 15 second intervals until the solution is clear.

> **CAUTION:** Agarose can become superheated and boil over in the microwave or when swirled. Wear a heat-resistant glove when removing the agarose and swirling heated flasks.

- Allow the transparent agarose solution to cool until you can hold the flask in your hand.
- Meanwhile, place the black rubber bumpers onto the ends of the clear plastic gel casting units. The bumpers are a tight fit so they may be difficult to put onto the casting unit. If you have difficulty sliding the bumpers onto the casting unit, try sliding one side on and then the other. Once you have the bumpers on, place the comb into the unit.
- Once the agarose is cool, add 2.5 µL EtBr (10 mg/mL), and gently swirl to mix. Do not remove the EtBr solution from the designated area. Use the designated pipette.
- Fill a casting unit with the agarose.

Lab Safety Notice

EtBr is a carcinogen! When working with it, please observe the following precautions:

- Work with the provided EtBr stock *only* in the designated EtBr area.
- Use the designated EtBr pipette. Dispose of pipette tips in the designated EtBr Tip Waste container.
- Wear gloves and eye protection.
- Dispose of EtBr gels in the designated containers.

- Wait about 5–10 min for the gel to solidify; the gel should appear cloudy, not clear.
- Once the gel has solidified, gently pull the comb straight up out of the gel. Be careful not to destroy the wells when you pull out the comb! Next, remove the bumpers by gently twisting

them off. After removing the comb and the bumpers, place the casting unit into the running box. Two casting units can fit into the same running box, so you can share with another pair.

> **NOTE:** Whether you place your wells on the right or the left will depend on which direction you expect your samples to move and which electrode you use as the cathode or anode. Make sure your gel is facing the correct direction or your DNA will run into the buffer and not the gel!

- Add approximately 350 mL of 1× TAE buffer to the running unit so that the buffer just covers the gel. Make sure that both gels are covered with buffer. If there is used TAE buffer available, please use that.
- For each of your PCRs, place 5 µL H_2O into a new microfuge tube, then add 5 µL of your PCR product. Be sure you pipette the liquid all the way down into the water in the tube. Add 2 µL of 6× loading dye and mix by pipetting up and down.
- Load 5 µL of the 1 kb ladder onto the gel. Load all 12 µL of your PCR+loading dye samples onto the gel in separate wells.

> **NOTE:** Be sure to write down the order in which you load your samples and the volume that you loaded. You will need this information and it will be required for your lab report.

- Once all of the samples have been loaded into both gels, gently place the cover on the running box (be careful not to disrupt

your samples). Attach leads to power supply. Set the voltage at 150 V and press the run button.

> **NOTE:** Check that your anode and cathode are attached appropriately to run your DNA in the desired direction.

> **NOTE:** Give your gel a few minutes to get started and then make sure it is running as expected. There should be small bubbles moving up from each electrode, and you should be able to see your loading dye begin to migrate.

- Continue electrophoresis until the bromphenol blue (the leading, darker dye) has migrated to about 1/2 of the way to the end of the gel.
- Take a picture of your gel using the gel doc, with help from the teaching staff.
- Please pour your TAE buffer into the "Used TAE" carboy so it can be re-used.

Lab 2: LAB ASSIGNMENTS

PRELAB QUIZ

Visit Canvas to take the quiz. The quiz questions are also listed below for your convenience:

Question 1

What will you be simulating in lab? What is the purpose of those experiments? (Provide 2-3 sentences).

Question 2

For your PCRs, you will be provided a 5X reaction buffer stock.

You want your 50 uL reaction to have a final concentration of 1X reaction buffer.

What volume of the 5X stock must you add to each reaction?

Question 3

This lab includes a chemical hazard. Describe the chemical, the potential risks, and steps you would take in a lab to protect yourself and your colleagues.

Question 4

Preparing 1% agarose gels requires adding solid agarose to a buffer and then melting it. If you are going to prepare 25 mL of agarose solution, how much agarose (in grams) will you need?

IN-LAB ACTIVITIES

- This week we will have the first of two TA Talks. One of your TAs will introduce themselves more thoroughly by telling you a bit about how they got interested in science, how their graduate school career is progressing, and what they are currently researching. The talk will last ~15 minutes and then you will have an opportunity to ask questions about their research, graduate school, etc.
- You will then be randomly assigned to a breakout group with a

lab partner, and you will be provided the link to a Google doc. You and your partner will work together to complete it. It will include:

- ◦ Choosing primers.
- ◦ Completing the calculations for setting up the PCRs.
- ◦ A table where you will outline each step in the protocol, state its purpose, and write down any questions you have. Each pair is required to ask a TA at least one question about the protocol.
- ◦ To demonstrate the lab procedures, work through an interactive site, watch two videos, and answer questions.
- ◦ Determine the approximate size of the expected PCR products.
- ◦ Consider several problems that may come up, what might have caused them, and how you could troubleshoot them.
- Sometime during your lab, meet with a TA to ask them at least one question about the protocol, as well as any other questions you might have.
- Complete the lab/partner/self evaluation Google form (link will be provided)
- Get a sharable link to your Google doc and submit it to Canvas (both partners should do this).

Lab 2: MINI LAB REPORT

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Report Guidelines

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NOTE: Please view the Mini Lab Report 1 feedback from your TAs before finalizing your second report. Feedback should be available by 48 hours before your next lab. It is expected that you incorporate suggested changes into future reports. For details on how to view your feedback, see: How to View Mini Lab Report Feedback

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Introduction (draft 1)

This week, you will write a first draft of the Introduction section for your final lab report. As such, think of this as an introduction to the entire 551 HCAII project; not an introduction to doing PCR.

The introduction will provide the context for your lab report by summarizing what is currently known about your topic and stating the hypotheses or questions you are studying. The introduction should briefly describe the rationale of the work and the approach you took. Use the active voice. When presenting published information, use the present tense (i.e. this is what we currently know). Introductions start out with a description of the general background about the problem and focus down to the specific question you are studying.

Your introduction should be 1-2 double-spaced pages long.

Your introduction should address the following:

1.   What is the protein you are working on and why is it

interesting to the general public?

2. Provide relevant molecular details about HCAII, and include a helpful PyMOL figure of the protein.

3. What is the hypothesis or question you are addressing with this project? For this week, choose any mutation you want. Write a detailed, biochemical hypothesis for that specific mutation. The objective is to practice the process of developing a hypothesis; you will work later in the semester to write a hypothesis specific to the mutation you will actually be studying.

4. Give a general description of the experiments you will use to address your question/hypothesis. Since you've only done one lab experiment thus far, don't worry too much about this yet; just look forward in the lab manual and write a sentence or two on what we will be doing this semester.

5. Throughout your introduction, use 2-3 literature sources. Be sure to use in-text citations and include a reference section at the end of your report (scroll to the bottom of Mini Lab Reports for more on references).

A Note on Nomenclature

When referring to a gene, its name should be italicized (*HCAII*), but when referring to a protein, its name should not be italicized (HCAII). Also, some of you may be used to studying bacterial genes, whose names are typically written using lowercase letters. Mammalian genes, by contrast, are written using capitol letters.

Methods

It is difficult to include the right amount of detail in a Methods section. Remember that the goal is to provide enough detail that

someone with a scientific background could repeat your experiment. You do not need to write out detailed protocols.

For your first few mini lab reports, we will tell you exactly which details to include. Gradually, you will be expected to include appropriate information more independently.

For this report, be sure to include:

- A statement on the purpose of the experiment ("In order to amplify the *HCAII* gene and pETblue2 vector, …)
- Cite the lab manual
- Components added to the reactions: for most components, report the final concentrations. DNA and Phusion should be reported as the total amount per reaction (in nanograms and units, respectively).
- Sequences of the primers you used, pointing out which sequences are vector vs. insert
- A description of the negative control
- The %agarose and final concentration of EtBr used in the gel
- How the gel was visualized

Do NOT include:

- Details like "tubes were labeled" or "agarose solution was microwaved." These are standard procedural steps that any scientist would know to do.

Results

You can download the gel image you will use in this section here: Lab 3 Agarose Gel

Start the Results section with a sentence to remind the reader of the overall goal and/or hypothesis for the experiments you are describing – something similar to the purpose statement mentioned above for the Methods section.

Be sure to include:

- A figure of the gel containing the PCR products. Highlight the

expected products with an arrow or box. Label each lane and all of the size markers (for more information on the ladder: NEB quick load DNA ladder). Your figure legend should include:

- A figure number and short title, bolded
- The % agarose
- What is in each lane
- What your box/arrow is highlighting
- DO NOT interpret the gel in the figure legend

- Text description of the PCR products:

  - Which reaction(s) yielded product
  - The apparent size of the product and whether that matches the expected size
  - Whether some reactions yielded more product than others
  - Any other observations you have about the appearance of the product
  - If you observe any unexpected bands, comment on their existence and their apparent size. This is not a Discussion section, so you do not need to discuss what they might be.

LAB 3: LAB-AT-HOME: INTRODUCTION TO BIOTECHNOLOGY METHODS

Lab 3: Lab-at-home: Introduction to Biotechnology Methods

Lab 3 Objectives

- Review the steps of PCR
- Describe how restriction enzymes work and analyze a simulated restriction digest experiment
- Explain the principle behind the separation of biomolecules by gel electrophoresis
- Identify and describe the function of the equipment involved in gel electrophoresis
- Perform an electrophoresis reaction, and analyze the results

This week you will complete the Carolina Distance Learning at-home lab "Introduction to Biotechnology Methods."

The lab assignments will be different than the previous weeks. You do not need to conduct the experiment during your lab time (though you may choose to do so). You do need to log on to BBCU at an assigned time to meet with a TA. There are no pre- or post-lab quizzes this week, and instead of a mini lab report, you will write up answers to questions about the at-home lab.

Lab 3: BACKGROUND AND PROCEDURE

You should have received a hard copy of the background and lab procedure with your lab kit. If not, please refer to the Canvas announcement regarding lab 3, which contains a pdf of the procedure as an attachment.

You may complete the lab experiments on your own at any time this week.

The goal of this lab is for you to get some hands-on experience with electrophoresis, a centrally important biochemical lab technique. The at-home lab is a bit simplistic, but we offer it as a chance to review some of the molecular biology methods we have discussed in the course, and we hope you approach it as a fun change of pace.

You can view a brief introduction video from Carolina Distance Learning about this lab here

Lab 3: LAB ASSIGNMENTS

There is no pre-lab quiz this week.

IN-LAB ACTIVITIES

You are welcome to complete the lab during your assigned lab time, but this is not required. You may complete the lab experiments at any time this week. You ARE required to join BBCU at an assigned time for a 15-minute meeting with one of your TAs (see Canvas Announcement for time assignments). At this meeting, you will discuss:

- Your first mini lab report and feedback
- Your experience during the first two labs (and the third, if you've completed it)
- Any questions or concerns you have about lab

There is no post-lab quiz this week

Lab 3: WRITE UP

There is no mini lab report this week; instead, you need to answer the following questions about your at-home experiments. You do <u>not</u> need to format your responses as you would a lab report; just answer the questions.

Questions from Activity 1: Sickle Cell Anemia Detection and Simulation

- For the wild-type beta-globulin sequence shown in the protocol, how many DNA fragments are present after digestion with *DdeI*?
- For the mutant beta-globulin sequence, how many DNA fragments are present after digestion with *DdeI*? If the number of DNA fragments is different than that of the wild-type, explain why.
- Count the number of base pairs for each fragment, for wild type and mutant. (If a base is not paired because of a sticky end, do not count it.)
  - For the *DdeI*-digested, wild-type beta-globulin sequence, how many base pairs are in each fragment?
  - For the *DdeI*-digested, mutant beta-globulin sequence, how many base pairs are in each fragment?
- On the basis of fragment size, how can the difference between the wild-type sequence and the homozygous mutant sequence be recognized?
- What fragments would be present following *DdeI* digestion of a sample from someone with a heterozygous beta-globulin genotype?
- Insert a photo of your completed gel diagram from protocol Figure 4.
- Based on your gel diagram, what is the genotype of the unknown sample from the newborn? Explain your answer.

Questions from Activity 2: Mini Gel Electrophoresis of Dyes

- Insert a photo of your completed gel
- Which combination of dyes make up each of the two unknown samples?
- What causes different banding patterns for different samples? What do the banding patterns indicate?
- Consider this experiment compared to the simulated *HCAII* experiment we did last week. How was this at-home lab similar to and different from doing gel electrophoresis in a research lab setting? Include two similarities and two differences.
- It is actually quite impressive that you were able to do gel electrophoresis at home for only $22 (batteries not included). Can you imagine any real-world applications for this sort of technology? Describe one idea in detail.

LAB 4: GIBSON ASSEMBLY OF HCAII INTO PETBLUE2 VECTOR AND TRANSFORMATION OF E. COLI WITH THE REACTIONS

Lab 4: Gibson assembly of HCAII into pETblue2 vector and transformation of E. coli with the reactions

Lab 4 Objectives

- Prepare DNA for Gibson Assembly
- Use a Gibson Assembly reaction to clone the *HCAII* gene into the pETblue2 expression vector
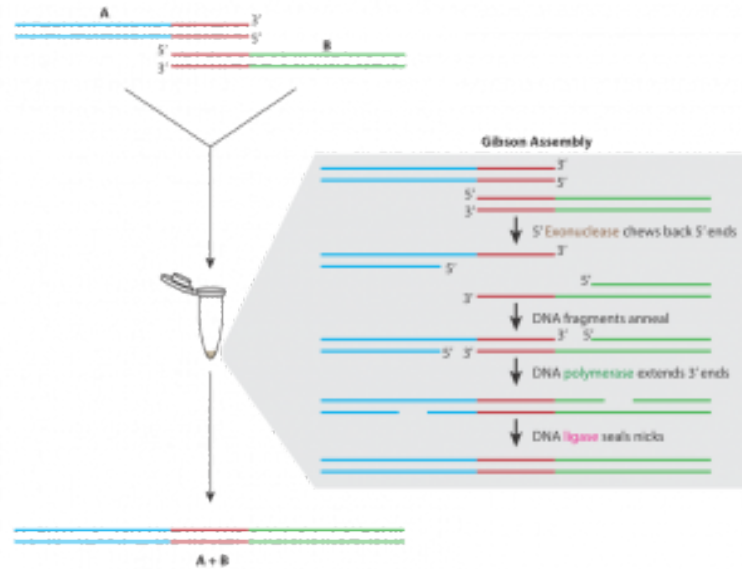- Transform *E. coli* with the cloned DNA using the heat shock method

Fig. 4.1. Outline of the Gibson assembly protocol. Two pieces of DNA have matching sequence (shown in red). Within a Gibson assembly reaction, these DNA pieces undergo several steps: Exonuclease chews back 5′ ends, DNA fragments anneal, DNA polymerase extends 3′ ends, and finally DNA ligase seals the nicks.

Lab 4: BACKGROUND

Preparing for Gibson Assembly

Restriction digest

Restriction enzymes are proteins that cut dsDNA at very specific recognition sequences. The enzyme you will be using (*DpnI*) recognizes methylated DNA. *E. coli* contains enzymes that naturally methylate its DNA, so that any plasmid that originated in a bacterium will be methylated. PCR products, however, will not be methylated and so will not be digested by *DpnI*. This digest then allows us to eliminate the template plasmids, which are not the clones we are trying to obtain, but are still present in the PCR tubes along with our products.

Fig. 4.2. Information on the DpnI enzyme provided by the manufacturer.

The New England Biolabs website is an excellent source of information on restriction enzymes and digestions (link opens in a new tab): www.neb.com

DNA Purification

After digestion, DNA must be purified using a spin column. The

spin columns contain a resin (white) that will bind to DNA, while other components will flow through into the collection tube.
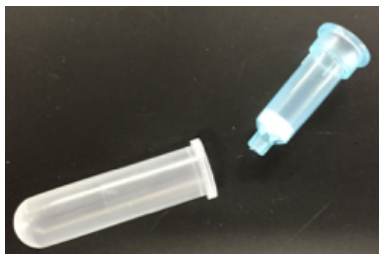

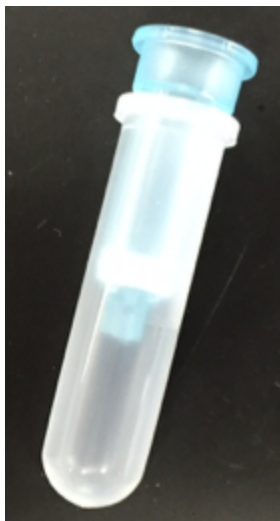Fig. 4.3. Two components of a DNA spin column: the empty collection tube and the column.

Fig. 4.5. An assembled DNA spin column.

For more molecular details on spin columns, please see the lab manual appendix: Appendix 3: Additional laboratory details

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Spectrophotometric Determination of DNA Concentration and Purity

Biological molecules absorb light of different frequencies in a characteristic manner, and we can take advantage of this using spectrophotometry. In general, DNA exhibits maximum absorption of light at 260 nm. In this lab, we will use a spectrophotometer called a NanoDrop to measure the absorbance at 260 nm, or A_{260},

of your purified DNA samples. The NanoDrop reads 2 μL of sample. The inset image below shows the pedestal (within the black circle) where the 2 μL of sample needs to be placed.

Fig. 4.6. Image of a NanoDrop spectrophotometer.

Look at the sample NanoDrop absorbance plot below. The arrow in the center highlights the point of most interest to us: the absorbance at 260 nm. The NanoDrop uses this number to calculate the concentration of DNA (96.2 ng/uL in this example).

The absorbance at 230 nm (left arrow) should be low. If it is high, it may indicate a small molecule contaminant, such as EDTA or carbohydrates. Expected A_{260}/A_{230} values for pure DNA are in the range of 2.0-2.2. If the ratio is appreciably lower, it may indicate contaminants that absorb at 230 nm.

The absorbance at 280 nm (right arrow) should also be low. The expected A_{260}/A_{280} value for pure DNA is ~1.8. If the ratio is appreciably lower, it may indicate the presence of protein in your sample.
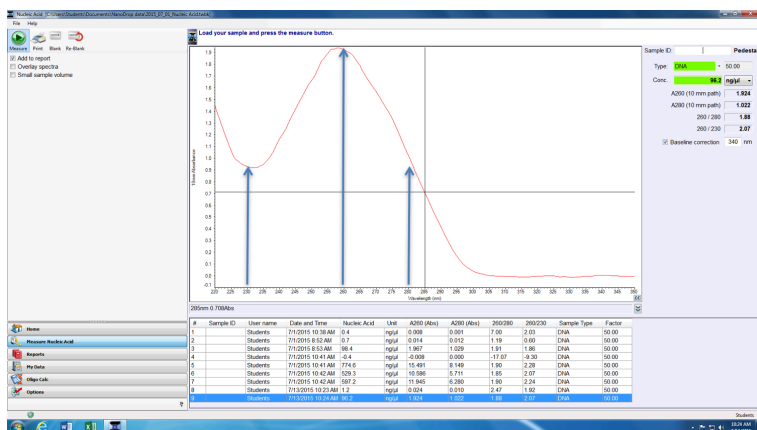
Fig. 4.7. Sample output from a NanoDrop. The arrows indicate, from left to right, the absorbances at 230 nm, 260 nm, and 280 nm.

Gibson Assembly

Developed in 2009, Gibson assembly is a modern approach for joining multiple DNA fragments into a desired clone. In our case, we will be assembling the *HCAII* gene into the protein expression vector pETblue2. However, Gibson assembly can also be used to join up to ten DNA fragments.

If you are interested, here is the original paper describing this method (pdf opens in a new tab): 2009 Gibson cloning reference

Gibson assembly requires that DNA fragments contain ~30 bp of overlapping sequence identity. Typically, this sequence overlap is generated via PCR – insert and vector are amplified via PCR using primers that create sequence overlap. PCR products can then be digested with the enzyme *DpnI* to eliminate template DNA.

Purified DNA fragments with overlapping sequence are then joined in a single, isothermal reaction. The reaction mixture contains three enzymes, in addition to a number of buffer

components to optimize function of each enzyme. Below are the steps in Gibson assembly, with the required enzymes underlined:

- <u>Exonuclease</u> chews back each strand of DNA from the 5' end. This creates single-stranded, sticky overhangs. Overlapping sequences can anneal together.
- <u>DNA polymerase</u> adds nucleotides to fill in any gaps.
- <u>DNA ligase</u> covalently links the annealed DNA fragments, thereby creating a completely double-stranded circular piece of DNA: your clone!

Transformation

When bacterial cells take up DNA they are said to undergo "transformation." Host bacterial cells must be in a state called "competence" which is achieved by treating healthy, mid-log-phase cells with a solution of ice-cold calcium chloride and then freezing them. The cells are then thawed, mixed with the DNA that you wish to transfer, and subjected to a brief pulse of heat, called a "heat shock". This process makes the bacterial cell membrane porous and creates a gradient such that DNA can pass through the membrane and into the cell.

> **NOTE:** The expression "Vector X was transformed into DH5α cells" is used colloquially by biochemists, but is technically incorrect: Vector X was *not* transformed, rather the cells were. Thus the correct phrase should be "DH5α cells were transformed with Vector X."

Competent cells are very fragile and must be treated with care: keep on ice and mix very gently!! The timing and temperature of the heat shock step must be precise; if kept too long at the high temperature,

the cells will die. Furthermore, careful aseptic technique must be used throughout, because any contaminating bacterial cells may grow and compete with desired cells.

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> *https://wisc.pb.unizin.org/biochemistry551online/?p=73*

After the recombinant DNA is transferred to the competent host cells, the cells are incubated in rich medium to allow them to recover from the stress of the heat shock, as well as to begin growing and replicating the newly transferred DNA. Following the recovery period, aliquots of the culture are plated on a selective medium, so that only clones that received the plasmid will grow. The resulting bacterial colonies are then screened to locate those that have the desired characteristics.

For additional information about transformation, please see the appendix: Appendix 3: Additional laboratory details

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> **NOTE:** Remember that there is a difference between "selection" and "screening." Selection allows only the clones with a desired trait to grow. Screening provides a way for us to differentiate among colonies with different traits. Refer to lecture for more details.

Blue/White Screening

Some vectors, such as pETblue2, allow for rapid screening of potential clones using the blue/white method. Screening is necessary because transformed colonies from a cloning reaction may or may not contain the desired clone – screening methods allow you to confirm the presence of your insert.

In the blue/white screening method, the gene of interest (*HCAII* in our case) is inserted into the vector such that it will disrupt the *lac*Z gene. You can see the *lac*Z gene in pETblue2 in the vector map here: pETblue2 vector map

When looking at the vector map, note that inserting *HCAII* into the Multiple Cloning Site will disrupt *lac*Z. The *lac*Z gene encodes for the protein β-galactosidase, which functions to cleave lactose. When plated on LB-agar plates containing X-gal, which is an analog of lactose, cells with in tact β-galactosidase will turn blue. However, in cases where *lac*Z has been disrupted by a cloned insert, the colonies will remain white. Therefore, after plating on LB-Amp-Xgal plates, you will be able to count the number of blue (negative) and white (positive) clones. You will then pick several white colonies to further screen.

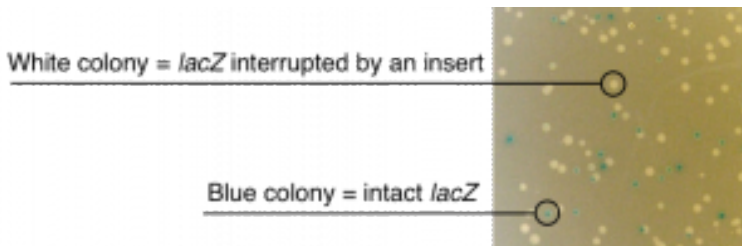Fig. 4.8. Sample plate from a blue/white screening experiment.

White colony = *lacZ* interrupted by an insert

Blue colony = intact *lacZ*

Fig. 4.9. Blue/white screening. Positive clones will appear white because the lacZ gene has been interrupted by an insert. Negative clones will have an intact lacZ gene and appear blue.

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Lab 4: PROCEDURE

> **Note:** This is the next "simulated *HCAII* lab," following directly from Lab 2. The protocol is again written as though you were actually doing the experiments in a physical lab, and includes every step that would be required to do so. You are expected to review the protocols before coming to lab, and will be asked about them on the pre-lab quiz. Similarly to Lab 2, one of your in-lab activities will be to go through the protocol with a partner, and you will have a chance to ask your TAs questions.

Restriction Digest

You will be digesting all three PCR products (the *HCAII* gene and the pETblue2 vector and the negative control) with *DpnI*. Fortunately for us, *DpnI* is functional in the PCR reaction buffer. If the enzyme was not compatible with this buffer, you would have to purify the DNA from the reaction, and then digest.

- Add 1 µL *DpnI* directly to each PCR tube. You should digest all three reactions – including the negative control
- Mix the reactions well by gently flicking the tube with your finger.

> **NOTE:** *DpnI* is an enzyme. Most enzymes are not as stable as HCAII, and you should therefore endeavor to be very gentle with them. Vortexing them may cause

them to denature and lose activity.

- Briefly centrifuge the samples and incubate at 37 °C for 1 hour.

DNA Purification Column

After the *DpnI* digests are complete, you will purify the DNA using small spin columns. Note you are also purifying your negative control. Use one column for each reaction. Be sure to label the spin columns so you don't get them mixed up!

- Add 5× volume of PB buffer to each PCR reaction. (Example: if you have 100 μL of sample, add 500 μL PB). Mix well before moving on to step 3.
- Add the PB buffer + PCR reaction solution (from step 2), to the spin column and spin for 1 min. at 14,000 rpm (>16,000 rcf) in the centrifuge. Be sure to balance the tubes in the centrifuge before you start spinning. Your DNA should now be bound to the column; however, we often recommend that you save the flow-through at this point; if you do not end up with much DNA at the end of the purification, you can determine where you lost it (and whether it can still be recovered).
- Add 750 μL Buffer PE and spin for 1 min. at 14,000 rpm. Again, save the flow-through in the event that you lose your DNA and want to go back and find it later.
- Spin for 1 minute at 14,000 rpm again to dry the column. (Buffer PE contains ethanol, which will interfere with the Gibson assembly reactions if it is not completely removed).
- Place the column into a clean 1.5 mL eppendorf tube labeled with the name of the sample, the date, and your initials.
- Add 30 μL of Elution buffer (EB; 10 mM Tris pH 8.5) to the

center of the column and incubate for 1 minute at room temperature.

- Spin for 1 minute at 14,000 rpm in the centrifuge. The flow-through contains your purified product. In order to get the maximum amount of DNA off of the column, add the flow-through back to the center of the column, incubate for one additional minute and then spin for 1 min. at 14,000 rpm in the centrifuge.
- SAVE THE FLOW-THROUGH CONTAINING YOUR DNA.

Determining the DNA Concentration

Once you have purified your PCR product, you will need to determine the concentration of your product. You will use the NanoDrop spectrophotometer to do this.

The following video demonstrates use of the NanoDrop:

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- Place 2 µL of EB on the platform of the nanodrop and click on "BLANK" (upper left-hand corner) on the computer.
- To check your blank, place another 2 µL of EB on the platform of the NanoDrop and click on "MEASURE." The curve should be close to flat and the DNA concentration should read approximately 0. If not, repeat step 1 and check again. If you are still having trouble blanking the instrument, ask your TA for help.
- Once the blank has been completed, place 2 µL of your purified PCR product onto the platform and click on "MEASURE". Write down the DNA concentration and the 260/280 and 260/230 ratios.

Assembly Reaction

- In lab, you will be provided a video that walks through the following steps:
- Because we want to add equimolar amounts of insert and vector DNA to each reaction, you must start by converting the DNA concentration from ng/µL (as provided by the NanoDrop) into picomoles/µL.
- Determine how much vector DNA you will add to each reaction. For Reactions 1 and 2, try two different amounts of vector DNA. Reaction 3 is a negative control and should contain the lower amount of vector used in reactions 1 or 2 (ideally you would do two negative controls, but to save time and materials we will just do one.)

- If your vector DNA is pretty concentrated (>50 ng/µL), 1 or 2 µL should be plenty.
- If you want to use more than that, note that you may not have the volume of DNA that you think you do. A few microliters are usually lost on the column. You can check the exact volume you have to work with using a pipette.
- Determine how much insert to add to each assembly reaction. For Reactions 1 and 2, use the same number of picomoles of insert as vector. For Reaction 3, use the same volume of the negative control DNA as you did insert in Reaction 1.
- Take care that the total amount of DNA falls between 0.02 – 0.5 pmoles.

Example:

Reaction 1: 0.03 pmol vector, 0.03 pmol insert

Reaction 2: 0.015 pmol vector, 0.015 pmol insert (in 2 µL)

Reaction 3: 0.015 pmol vector, 2 µL negative control DNA

- Set up your reaction as follows, for a final volume of 20 µL:

___ µL Vector DNA

___ µL Insert (or control) DNA

10 µL Gibson Assembly Master Mix

___ µL H_2O

See Appendix for the Gibson Assembly Master Mix recipe (link opens in a new tab): Appendix 4: Buffer formulations

- Incubate the reactions in the thermocycler at 50 °C for 30 minutes.
- Place on ice for a few minutes to cool.

Transformation of *E. coli* with the assembled DNA

NOTE: Proper aseptic technique (also referred to as "sterile technique") is a hallmark of all good biochemical

researchers. In essence, you must take care to be extra aware of your person and any tools that you are working with. If you touch something that is not sterile (be it a fingertip, sleeve, piece of glassware, cough, etc.) to something that is sterile (a jar, or media, or a plate, etc.), then the sterile object *is no longer sterile*! You should thus either throw it away, or sterilize it again before using it. If you have any doubt about an object's sterility, treat it as non-sterile.

- Thaw aliquots of *E. coli* cells <u>on ice</u>.
  - For this lab we are using a strain of *E. coli* called DH5α. While the cells are thawing, chill three empty 1.5 mL eppendorf tubes on ice. Gently pipette 100 µL of DH5α competent cells into each tube – one for each Gibson reaction.

Fig. 4.4. An aliquot of competent cells thawing in an ice bucket.

- Add 10 µL of Gibson assembly reaction to the DH5α cells. <u>Do not pipette up and down</u> to mix! You can mix by gently flicking the tube.
- Incubate for 20 minutes on ice.
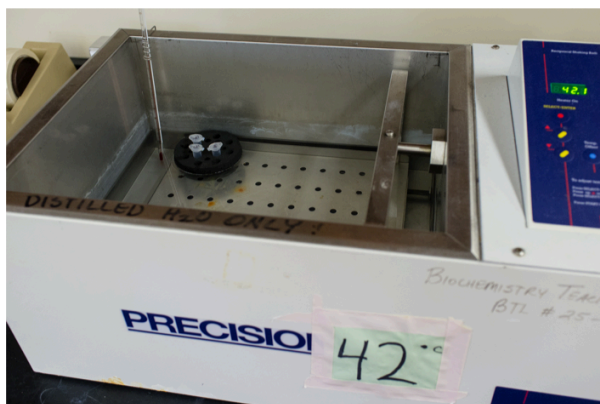- Heat shock for <u>45 seconds</u> at 42°C. Do not exceed 45 seconds.

Fig. 4.5. Heat shock by incubating tubes in a water bath.

- Place on ice for <u>2 minutes</u>. Do not exceed 2 minutes.
- Transfer your tubes from ice to a rack at room temperature. Add 500 µL SOC (a very rich media). Do not put back on ice once you add the SOC.
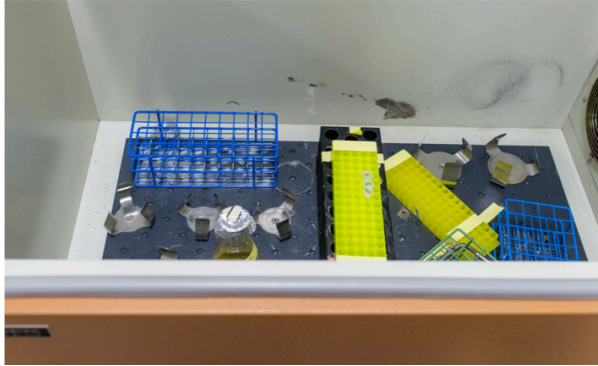- Shake for 45 min. at 37°C in a shaking incubator.

Fig. 4.6. Shaker used to incubate transformation reactions.

- Spin the transformed *E. coli* at 4,000 rpm for 1 min. Remove and discard 300 μL of the supernatant. Gently resuspend the pellet in the remaining supernatant.
- Spread the total volume of each transformed *E. coli* reaction on LB-ampicillin-Xgal plates. Be sure you label your plates with the reaction number, your name and your section. It is best to label the plates around the edge so the writing does not obscure the colonies on the plate.

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- Incubate the plates at 37°C overnight.
- Following the overnight incubation, your plates will be placed at 4°C.

Lab 4: LAB ASSIGNMENTS

PRELAB QUIZ

Visit Canvas to take the quiz. The quiz questions are also listed below for your convenience:

Question 1

What will you be simulating in lab? What is the purpose of those experiments? (Provide 2-3 sentences).

Question 2

In order to set up a Gibson assembly reaction, you will have to calculate DNA molarities. As a first step, calculate the molecular weights of vector (pETblue2) and insert (*HCAII*) in g/mol. To determine the MW of your DNA, assume that one DNA basepair = 650 g/mol. This is an estimate, but it is okay to use this number to calculate the molar amounts of your DNA.

Note: to solve this problem you will need the sizes of the PCR products, as you determined in Lab 2.

Question 3

This lab includes a safety hazard. Describe the hazard and steps you would take in a lab to protect yourself and your colleagues.

IN-LAB ACTIVITIES

- This week we will have the second of two TA Talks. One of your TAs will introduce themselves more thoroughly by telling you a bit about how they got interested in science, how their graduate school career is progressing, and what they are currently researching. The talk will last ~15 minutes and then you will have an opportunity to ask questions about their research, graduate school, etc.
- You will then be randomly assigned to a breakout group with a lab partner, and you will be provided the link to a Google doc.

You and your partner will work together to complete it. It will include:

- ◦ A table where you will outline each step in the protocol, state its purpose, and write down any questions you have. Each pair is required to ask a TA at least one question about the protocol.
- ◦ Completing the calculations for setting up the Gibson Assembly reactions.
- ◦ Complete a virtual bacterial transformation lab.
- ◦ Consider some approaches to troubleshooting common problems.
- Before leaving BBCU, meet with a TA to ask them at least one question about the lab. They will also check your GA reaction calculations.
- Complete the lab/partner/self evaluation Google form (link will be provided).
- Get a sharable link to your Google doc and submit it to Canvas.

Lab 4: MINI LAB REPORT

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Report Guidelines

Methods

Be sure to include:

- Purpose statement/brief summary of the experiment
- Cite the lab manual
- Enzyme used for digestion
- How the DNA was purified (you DO NOT need to include the full protocol for DNA purification) and measured its concentration
- Descriptions of the three Gibson Assembly reactions (using DNA *amounts* instead of volumes wherever possible) and how the reaction was run
- Key information about the transformation: the strain of *E. coli*, the method of transformation (i.e. heat shock), and the antibiotic used in the plate. You do not need to include step-by-step protocol for the transformation.

Results

Include the following information in your Results section:

- The concentrations and purity of DNA. A table might be nice here (note that, like figures, table should have a legend; however, table legends go *above* the table).
- State how many colonies resulted from each of the DNA amounts used, including the negative control. Include separate counts of blue versus white colonies. A table might be nice here.
- Comment briefly on whether the different amounts of DNA resulted in different numbers of colonies.
- Be careful not to add Discussion text here! Your Results should

describe what you observed, not why you think you observed it.

LAB 5: SCREENING FOR PETBLUE2-HCAII CLONES

Lab 5: Screening for pETblue2-HCAII clones

Determine whether the HCAII gene is present in pETblue2 in each of your potential clones; by:

- Using the blue/white screening technique to identify bacteria suspected to contain your clone of interest
- Purifying DNA from these potential clones
- Digesting the purified DNA with restriction enzymes to differentiate positive vs. negative clones (i.e. a restriction digest screen)

Lab 5: BACKGROUND

Minipreps of Plasmids

In order to screen the bacterial colonies for a clone of interest, you need to be able to rapidly isolate small quantities of plasmid DNA from each clone. Small amounts of bacterial cells (2 mLs overnight culture) are used to obtain small amounts (often 1-5 µg) of moderately pure plasmid DNA; these are called minipreps. Miniprep procedures allow you to isolate the plasmid DNA from the more prevalent genomic DNA. The procedure involves pelleting bacterial cells in the microfuge, re-suspending the cells in buffer, precipitating cell debris and protein, and finally separating the plasmid DNA from genomic DNA. Due to time constraints, you will use a commercial miniprep kit. Such kits are widely used in research labs.

Each commercial kit generally includes a resin (specially-treated glass) that is mixed with the plasmid DNA/RNA solution. The plasmid DNA sticks to the resin in a small column. The column is washed with a solution that contains ethanol, so the plasmid remains on the resin. The plasmid DNA is then eluted from the glass resin with water or TE buffer. Sometimes the DNA is stuck to the resin with high salt and eluted with low salt (water or TE). This resin replaces the alcohol precipitation steps done in other procedures and in the purification of genomic DNA.

The steps in a mini prep are outlined below:

Overnight Bacterial Culture

Lyse & Neutralize

Bind & Wash

Elute

Ultra Pure pDNA

→ Nanodrop

Fig. 5.1. Schematic of the miniprep procedure.
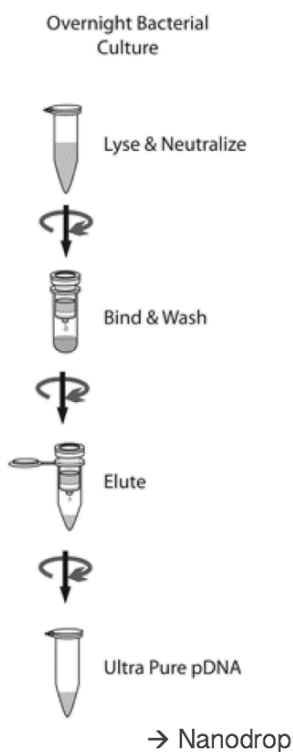
1. Pellet bacterial cells, resuspend in Buffer P1 (resuspension buffer)
2. Lyse for 3 minutes in Buffer P2 (lysis buffer)
3. Add Buffer N3 containing high-salt (neutralization buffer)
4. Pellet cell debris, proteins, and genomic DNA by centrifugation
5. Add supernatant containing plasmid DNA to spin column, spin to bind DNA to resin
6. Wash plasmid DNA bound to column with Buffer PE (wash buffer) to remove salts
7. Spin column while empty to remove any residual Buffer PE
8. Elute DNA from column with Buffer EB (elution buffer, same as lab 3)

For additional information about minipreps, please see the appendix: Appendix 3: Additional laboratory details

Master Mixes

A Master Mix is a convenient tool you can use when running multiple samples that include many of the same components. It works by preparing a large solution that contains all the common components across the reactions. You then pipette the appropriate amount of this master mix into each reaction tube, then add any

components that differ between reactions individually. Using a Master Mix is beneficial because it:

- ◦ reduces pipetting error
- ◦ increases reproducibility
- ◦ saves time

See sample Master Mix below.

Example Master Mix Calculation

The example reaction contains the following components: DNA, buffer, enzyme, and water. My final reaction volume will be 10 µL. Each of my DNA samples has a different concentration and would therefore require a different volume. To make the Master Mix easier, I will start by making dilutions of each of my DNA samples so that they are at the same concentration. In this example I've chosen to make them all 25 ng/ µL. The final volume of these stocks doesn't really matter, I've chosen 20 µL.

DNA Sample	Concentration	Stock volume for dilution	Water volume for dilution
A	25 ng/µL	–	–
B	25 ng/µL	–	–
C	250 ng/µL	2 µL	18 µL
D	100 ng/µL	5 µL	15 µL
E	500 ng/µL	1 µL	19 µL

Next I need to determine the volume of all of the

components for each individual reaction. Water will be used to bring the volume up to 10 µL.

Reagent	Reaction A	Reaction B	Reaction C	Reaction D	Reaction E
DNA	4 µL	4 µL	4 µL	4 µL	4 µL
10x Buffer	1 µL	1 µL	1 µL	1 µL	1 µL
Water	4.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL
Enzyme	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL
Total	10 µL	10 µL	10 µL	10 µL	10 µL

Variable components

In this experiment, I am adding a different DNA sample to each reaction. Therefore, DNA is called the "variable component". Since it varies across reactions, I will leave it out of the Master Mix.

Master Mix with unchanged components:

Single Reaction	# of Reactions	Master Mix
1 µL 10x Buffer	5.5	5.5 µL
4.5 µL H20	5.5	24.75 µL
0.5 µL Enzyme	5.5	2.75 µL
6 µL total volume		

From the Master Mix, I add 6 µL to each of my labeled tubes. Then I add 4 µL of each DNA sample to the appropriate tube.

This video explains how to do a Master Mix calculation, with an example and a practice problem.

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> *https://wisc.pb.unizin.org/biochemistry551online/?p=83*

Lab 5: PROCEDURE

Starting overnight cultures

Note that this needs to be done on the afternoon before the main experiment, so that the *E. coli* cultures can grow overnight.

Take a look at the plates from last lab and identify the one with the most colonies that are potentially positive clones. This is the plate you will use.

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https://wisc.pb.unizin.org/biochemistry551online/?p=88

This video demonstrates the procedure for picking colonies and starting overnight cultures:

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- Label three test tubes with your name
- Add 2 mL LB with 50µg/mL ampicillin to each test tube
- With a sterile toothpick, touch a **single** bacterial colony and drop the toothpick into 2mLs
  - Repeat for a total of three times; i.e. each test tube will contain a single colony
- Incubate at 37°C shaking overnight.

Purification of the DNA

Note that this protocol is similar to the DNA purification done previously, but slightly different. In the previous lab, you started with PCR product, whereas here we are starting with whole cells

(which need to be pelleted and lysed, and leave debris that must be removed).

1. Add 1.4 mL of well-mixed culture to a microfuge tube. If your culture has settled, you can vortex the tube.
2. Spin for 1 minute at 8000 rpm in microfuge to pellet the bacteria. Discard the supernatant into bleach.
3. Resuspend the bacterial pellet in 250μL of Buffer P1 – make sure the pellet is completely resuspended in the buffer either by vortexing or pipetting up and down.
4. Add 250 μL of Buffer P2. Mix by inverting the microfuge tube 4-6 times. Do not vortex or you will shear the genomic DNA. The solution should be viscous and slightly clear. Incubate for 3 minutes at room temperature. Do not exceed 5 minutes.
5. Add 350 μL Buffer N3. Mix by inverting the microfuge tube 4-6 times.
6. Spin the tube for 10 min. at 13,000 rpm. (~ 17,900 x g) in the microfuge. Be nice to your podmates and wait until everyone is ready to start your spin!
7. Obtain a purification column in a collection tube. Apply the supernatant to the column and spin the tube containing the column for 1 min. at maximum speed. Discard the liquid that flows through the column into the tube. You should re-use the clear collection tube.
8. Add 750 μL PE Buffer and spin the column + tube for 1 min. at maximum speed. Discard the liquid that flows through the column. You should re-use the clear collection tube.
9. Spin the column + tube again for 1 min. at maximum speed. Discard the flow through liquid.
10. Transfer the column to a fresh microfuge tube.
11. Add 30 μL EB to the center of the column and let it sit for 1 minute at room temperature.
12. Spin the microfuge tube containing the column (with the added EB) for 1 minute at maximum speed. The liquid that flows through contains your DNA.

13. In order to get maximum recovery, add the flow-through back onto the column, incubate for 1 min. at room temperature and then spin for 1 min. at maximum speed.
14. Measure DNA concentration of all of your samples using the nanodrop spectrophotometer.

Restriction digest of the purified DNA

We have also seen a restriction digest in lab before. This case is slightly more complicated because rather than simply adding enzyme to DNA, as we did with *DpnI*, this enzyme requires a specific buffer, and we want all the DNA samples to be at about the same concentration.

- Digest ~100 ng of each purified DNA sample. You will also digest negative (pETblue2 vector) and positive (pETblue2 with the *HCAII* insert) control DNA samples. Use a master mix for the digests. If your DNA concentrations are very high, you may need to dilute them. Think about how to do these dilutions such that your Master Mix is easier.

Restriction Digest: Master Mix:
   ___ µL DNA
   ___ µL H20   ___ µL H20
   1 µL 10x Buffer (CutSmart)   ___ µL 10x Buffer
(CutSmart)   (CutSmart)
   0.5 µL *enzyme*   ___ µL *enzyme*
   ———————————————   ———————————————
   10 µL final volume

   Per Digest:
   ___ µL Master Mix
   ___ µL DNA
   ___ µL H20 (if necessary)

$-------------$

10 μL final volume

NOTE: Remember to add the enzyme to your master mix last.

- After setting up all of your digests, mix them well by gently flicking the tubes and then spin them in your nanofuge (the smallest centrifuge) for a couple of seconds to get all of the liquid to the bottom of the tube.
- Incubate your reactions at 37°C for 30 minutes.
- **As soon as you start your digest, pour your gel!** Pay attention to which comb you use when pouring the gel – how many samples will you have to load? Remember that you are sharing your gel with a partner – so you have to count each of your three samples, plus the two digested controls, and one uncut control, plus a ladder. Note that all of the controls are shared between partners.

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https://wisc.pb.unizin.org/biochemistry551online/?p=88

Agarose gel

Run all 10 μL of your digests on a 1% agarose gel to determine whether any of your minipreps contain the desired clone. You will

also run a 10 μL sample containing uncut plasmid (one per gel). Be sure to include a ladder. Refer back to the lab 2 protocol for details on running gels: Lab 2: PROCEDURE Part2

Future Steps

In a research lab, after running your gel you would identify which miniprep(s) appear to contain the clone you want. You would then have the purified DNA sequenced. It is common to sequence more than one potential clone to ensure that at least one has the correct sequence. Once a clone with the correct sequence has been confirmed, it would be transformed into an E. *coli* strain suitable for protein expression. Due to time and financial constraints in the 551 lab, the sequencing of clones and transformation into Tuner(DE3) *E. coli* cells will be done for you.

Lab 5: LAB ASSIGNMENTS

PRELAB QUIZ

Visit Canvas to take the quiz. The quiz questions are also listed below for your convenience:

Question 1

What experiments are being simulated this week? What is the purpose of those experiments? (Provide 2-3 sentences).

Question 2

For starting overnight cultures, how many colonies should be picked, what color should they be, and which plate(s) should they come from?

Question 3

As noted in the procedure, when pouring an agarose gel, you may have different options for what size comb to use (i.e. how many wells for samples will be created in the gel). For this lab, how many samples need to be run on the gel?

IN-LAB ACTIVITIES

- After joining BBCU, you will be randomly assigned a partner.
- Your TAs will provide you a link to a Google doc. You and your partner will work together to complete it, including:
  - Determining the best restriction enzyme to use for a restriction digest screen.
  - Completing the calculations required to use a Master Mix to set up the digest screen.
  - At this point you will check in with your TAs. If everything looks good from parts 1 and 2, they will give you your "Result" from lab – a gel image.
  - You will work with your partner to create a slide that

clearly shows your results as a nice figure.

- You will meet with one TA and about half of your classmates, and each pair will present their slide. There will be time for discussion of each figure and how the results are interpreted.
- Complete the lab/partner/self evaluation Google form.
- Get a sharable link to the Google doc and submit it to Canvas.

Lab 5: MINI LAB REPORT

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Report Guidelines

Methods

Since you have written a couple methods sections, we will now leave it up to you to decide what is important to include. A few general reminders:

- Someone with a scientific background should be able to repeat your experiment by reading your Methods section
- You do not need to include detailed protocols
- Avoid volumes as much as possible; however, note that this may not always be possible

Results

Include the following:

- Gel of the restriction digest screen, appropriately labeled. Remember a good figure legend.
- Analyze the gel by describing all the samples and comparing the lanes to one another (particularly to the controls)
- State how many of the colonies from your cloning were positive and how you know

Discussion:

Note: in this discussion, you will discuss the success of the cloning experiment overall, which covers labs 2, 4, and 5. You may refer to these previous labs in this Discussion.

- Start the discussion by briefly summarizing the goal of the experiment and your results.
- What steps of the cloning procedure may have increased the likelihood of obtaining a positive clone? (Think through the

experimental process from all three labs. Name 2-3 specific steps that were specifically designed to increase the chances of getting a positive clone.)
- If any of your clones were negative or could not be determined based on the gel, state the size of the observed DNA band(s) and explain what you think it could be.
- What would you do to improve the overall cloning strategy, so that you could obtain more positives, and/or a higher percent of positives? (Think of steps that could be further optimized or additional steps that could be added to the protocol. Give 2-3 suggestions for full credit.)

LAB 6: MUTANT EXPLORATION

Lab 6: Mutant Exploration

You and your new official lab partner will work together this week to begin thinking critically about your mutation! After working together, you will present your figure and hypothesis to some classmates and a TA. You will have the chance to discuss each figure and hypothesis, receive feedback on your work, and provide feedback to your classmates.

Lab 6: BACKGROUND

Site-Directed Mutagenesis

By completing labs 2, 4, and 5, we have now cloned the wild type HCAII gene into the pETblue-2 vector. In order to study our mutations of interest, we next need to take that wild type clone and introduce mutations. In 551 we use accomplish this using a protocol called site-directed mutagenesis.

Please review the site-directed mutagenesis section of the PCR & Cloning lectures before lab.

Conservation Analysis: Sequence Alignment

A sequence alignment is a computational tool that arranges sequences to identify regions of similar or even matching sequence. These alignments can be used for a number of purposes, such as identifying evolutionary relationships between different protein sequences. In the case of a mutational analysis, such as we are doing in 551, it can be useful to do a sequence alignment of protein sequences from across different species. This can provide information on whether a given amino acid residue is evolutionarily conserved – i.e., whether the same amino acid is present at the same position across different versions of the protein. If an amino acid is evolutionarily conserved, that suggests that it plays an important role in the protein. If an amino acid is not conserved, it may be less important. Some amino acids are partially conserved, which means that different amino acids are found at that position, but those amino acids all have similar properties – i.e., negative charge.

Below is a sample image of a sequence alignment:

```
gi|62751699|ref|NP_001015729.1|   MAHAWGYGPDNGPATWHHAFPIAHGDHQSPINIVSAEAKYDHHLKPISIKYDPSTAKVIL   60
gi|46048696|ref|NP_990648.1|      MSHHWGYDSHNGPAHWHEHFPIANGERQSPIAISTKAARYDPALKPLSFSYDAGTAKAIV   60
gi|157951596|ref|NP_033931.4|     MSHHWGYSKHNGPENWHKDFPIANGDRQSPVDIDTATAQHDPALQPLLISYDKAASKSIV   60
gi|9506445|ref|NP_062164.1|       MSHHWGYSKSNGPENWHKEFPIANGDRQSPVDIDTGTAQHDPSLQPLLICYDKVASKSIV   60
gi|30466252|ref|NP_848667.1|      MSHHWGYGKHNGPEHWHKDFPIANGERQSPVDIDTKAVVQDPALKPLALVYGEATSRRMV   60
gi|223556019|ref|NP_001138642.1|  MAHHWGYAKHNGPEHWHKDFPIAKGERQSPVDIDTKAAVHDPALKSLCPCYDQAVSQRII   60
gi|109086851|ref|XP_001096360.1|  MSHHWGYGKHNGPEHWHKDFPIAKGQRQSPVDINTHTAKYDPSLKPLSVSYDQATSLRIL   60
gi|4557395|ref|NP_000058.1|       MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYGQATSLRIL   60
gi|302632532|ref|NP_001181853.1|  MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYGQATSLRIL   60
                                  *:* ***   *** **.****:*:;***: * :   *  *: :  *.  .: ::
```

You can see that nine sequences have been aligned, and each amino acid is color-coded according to biochemical property (for example, negatively charged residues are blue). The first amino acid is, of course, methionine, which is completely conserved, as indicated by the "*" at the bottom of the column. The second amino acid is highly conserved, denoted with a ":" because most of the sequences have a serine, but two have an alanine. Resides denoted with a "." are considered mostly conserved. Jumping forward to the eighth position, you can see that this position is not conserved at all, as sequences may contain a glycine, alanine, serine, or aspartic acid here.

How is this useful to studying mutations? If you were proposing to mutate this protein at the eighth amino acid, you might anticipate this having little effect, since the protein seems to tolerate many different amino acids at this position. By contrast, the third amino acid is a completely conserved histidine. If you were proposing to mutate this histidine, you might be more likely to expect some effect. (Of course, sequence conservation is just one piece of information to take into account when forming a hypothesis. The role of the wild type amino acid in the protein, its proximity to the active site, and the biochemical properties of both the wild type and mutated residues are also essential considerations.)

Making an Informative PyMOL Figure

You already have some experience with making nice PyMOL figures, and in this lab you will be applying those principles to creating images that help an audience understand your mutation.

The video linked below includes a number of tips and tricks for creating useful PyMOL figures:

TA Video: How to make a good PyMOL figure

Lab 6: PROCEDURE

Site-Directed Mutagenesis

In a typical semester, the mutagenesis process is carried out by the teaching staff. This semester you will have an opportunity to write a protocol for this process, based on the following technical documentation that outlines the experimental process. You may wish to review it before lab: https://www.agilent.com/cs/library/usermanuals/public/200518.pdf

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Conservation Analysis: Sequence Alignment

- The first step is to download a file containing carbonic anhydrase II sequences from a number of species
  - Go to https://www.ncbi.nlm.nih.gov/homologene
  - Search for "Carbonic anhydrase II"
  - Click on "carbonic anhydrase II"
  - Click "Download" in the top right corner
  - You should see a list of species. They should all be selected by default. Also check that the default "Protein" is selected from the sequences dropdown menu at the top. Click the "Download" button in the top left.
    - A .txt file should have downloaded to your computer.
- The next step is to submit these sequences to a sequence alignment tool
  - Next go to https://www.ebi.ac.uk/Tools/msa/clustalo/
  - Under Step 1, select the button to "Choose file" and choose the homologene.txt file you just downloaded.
  - Press "Submit"
- Find your amino acid residue and note its conservation score (key provided below)

- ◦ * completely conserved
- ◦ : highly conserved
- ◦ . mostly conserved

PyMol Figure(s) of Mutation

Work with your partner to create clear, informative images of your mutation. Refer back to Lab 1 for detailed instructions on how to use PyMOL.

You have two goals:

1. Use PyMOL to study your mutation to help you form a hypothesis regarding its phenotype. Questions to consider include:

   1. Are there significant clashes or voids created? How might these affect the structural stability of the protein?
   2. Where is the mutation relative to the active site? How might a structural and/or biochemical change in this location affect catalysis?
   3. Where is the mutation relative to where ligands bind? How might the mutation affect the ability of the protein to bind ligand? You can examine both CO_2 and the fluorescent ligand DNSA. Note that DNSA is much bulkier, so the two ligands might experience different effects.

2. Create figures of your mutation to include in your lab report. The figures should help the reader understand your mutation and your hypothesis.

- Two useful pdb codes are:
  - 2VVA: this is the wild type protein with CO_2 bound in the active site
  - 1OKL: this is the wild type protein bound to the ligand DNSA
- Use the mutagenesis wizard to model your mutation (refer back to lab 1 if you don't remember how to do this)

Develop a Hypothesis

Goal: develop a hypothesis (to be included in your lab report Introduction) on the effect of your mutation on HCAII.

There will be a number of in-lab activities to get you thinking about what makes a good hypothesis. You will then work with your partner to write your own hypothesis.

Your hypothesis should be clear and succinct, and should be grounded in biochemical principles.

Here are some questions to ask yourself:

- What amino acid residue are you changing? What is the role of that amino acid in the wild type protein? How conserved is

that amino acid?

- What amino acid are you changing to? How do the properties of the new amino acid compare to the wild type in terms of size, shape, charge, polarity, flexibility?
- Where is the amino acid you are changing? If it is in the core of the protein? Near the enzymatic active site? Near the ligand binding site? On the polar or hydrophobic face?

You should consider the three phenotypes that we will be able to test:

- Protein stability
- Kinetic activity
- Ligand binding

Note that your hypothesis does not need to address all three phenotypes – you can focus on one, two, or three – depending on whatever you think will be affected. You MUST hypothesize some change in phenotype.

Presentation of Figure and Hypothesis

Create 1-2 slide(s) in the shared Google doc to present your mutation to your classmates. The slide(s) should contain:

- Whether your amino acid residue of interest is conserved
- What the function of the wild type amino acid residue is
- Your hypothesis
- 1-2 PyMOL figure(s) showing your mutation

You will meet with one TA and half the class, and each pair will present their slide(s). There will be time for discussion. Things to consider during discussion:

- Does the PyMOL figure provide a clear visual explanation of

the mutation?

- Is the hypothesis clear, succinct, and testable?
- If another pair presents on the same mutation you are studying, how similar/different is their hypothesis from yours?

Lab 6: LAB ASSIGNMENTS

PRE-LAB QUIZ

This week's quiz is based on the mutageneic primers needed for the QuikChange PCR. You will be given a mutation number and the primers that were used to generate the mutation. Based on this information, determine the specific amino acid change for your mutation.

IN-LAB ACTIVITIES

You and your partner will meet in a BBCU breakout room to work through the lab procedure together.

- Work through the lab procedure and the provided Google doc, which ask you to:
  - create a protocol for QuikChange mutagenesis
  - complete a sequence alignment
  - generate a PyMOL figure
  - discuss what makes a good hypothesis and critique several sample hypotheses
  - write your own hypothesis
- Put the required information into your slides in the shared class Google slides presentation.
- At the designated time, you will join a TA and several other pairs to present and discuss your slides.
- Complete the lab/partner/self evaluation Google form.
- Get a sharable link to the Google doc and submit it to Canvas.

Lab 6: MINI LAB REPORT

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Report Guidelines

Introduction (draft 2)

This week, you will write a second draft of the Introduction section for your final lab report. Consider the feedback that you and your partner each received on the first draft of the Introduction in Mini Lab Report 2.

Your Introduction should be 2 double-spaced pages long.

The introduction provides the context for your report by summarizing what is currently known about your topic and stating the hypotheses or questions you are studying. The introduction should briefly describe the rationale of the work and the approach you took. Use the active voice. When presenting published information, use the present tense (i.e. this is what we currently know). Introductions start out with a description of the general background about the problem and focus down to the specific problem you are studying.

Your introduction should address the following:

1. What is the protein you are working on and why is it interesting to the general public?
2. Provide molecular details about HCAII that are relevant to your mutation. Including a helpful figure of wild type HCAII is up to your discretion.
3. Clearly state your hypothesis. The statement of your hypothesis should flow naturally from the information you've provided so far. Be sure to consider the feedback you received during your in-lab presentation. Including information from the sequence alignment here is at your discretion.
4. Give a general description of the experiments you used to address your hypothesis. (You have not done the experiments yet... but pretend that you have, and write in the past tense.)

5. Throughout this section you should use 4–5 literature sources (at least 3 from the primary literature). Be sure to reference all of your sources.

LAB 7: PROTEIN EXPRESSION AND PURIFICATION IN E. COLI

Lab 7: Protein expression and purification in E. coli

Lab 7 Objectives

- Overexpress HCAII protein in *E. coli* (wt and mutant!)
- Purify HCAII from *E. coli*, obtaining as high quantity and purity as possible
- Throughout both these protocols, collect samples for SDS-PAGE

  ◦ This will allow you to analyze how well you overexpressed and purified HCAII

In this lab, you will simulate growing *E. coli* and inducing overexpression of wild type and mutant HCAII. You will then simulate purifying the proteins via nickel column.

If this lab were being conducted in-person, each pair would team up with another pair, with one purifying wild type and the other mutant. For the simulated labs, everyone will work with data for the wild type protein.

Lab 7: BACKGROUND

Overexpression in *E. coli*

The pETblue2 vector you cloned *HCAII* in previous labs contains a part of the *lac* operon that allows regulation of protein expression from this vector. The vector contains sequences encoding the gene of interest (*HCAII* in the case of this lab), the *lac* operator and the repressor that binds to the operator and prevents unwanted transcription of the gene.
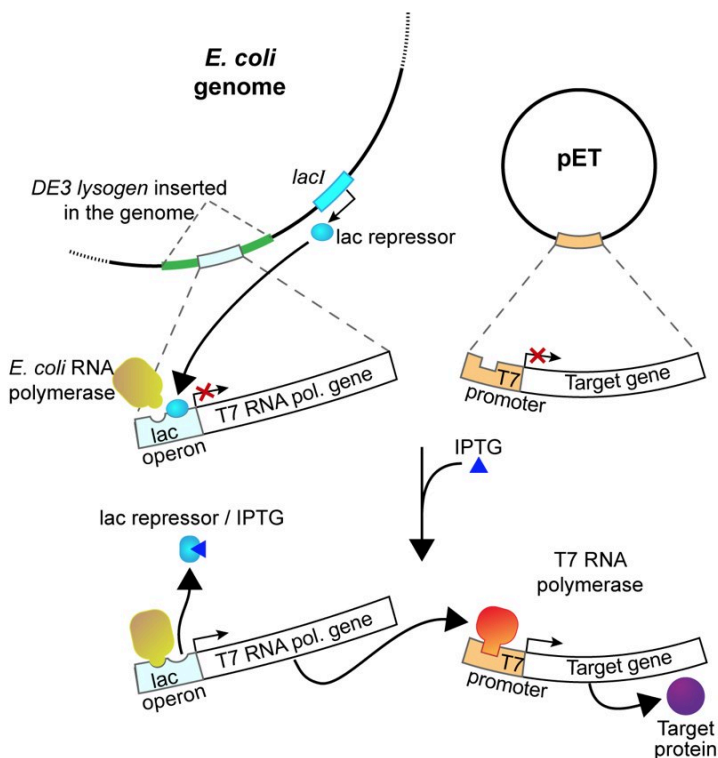
Fig. 7.1. The lac operon. The gene of interest is cloned onto the vector under the control of a specific promoter, called the T7 promoter. Only a specific RNA polymerase (T7 polymerase) can transcribe from this promoter. The T7 polymerase gene itself is under the control of the lac operon, on the genome of the E. coli.

In this experiment, you will use isopropyl-1-thio-β-D-galactopyranoside (IPTG), a commonly used inducer for the *lac* operon, to induce HCAII expression from our clones. Unlike allolactose, the natural inducer of the *lac* operon, IPTG cannot be metabolized and will thus induce the operon to a higher level. IPTG is often used to induce expression of many different recombinant proteins in *E. coli*. In our case, IPTG will be used to induce the

expression of T7 RNA polymerase, which will then transcribe the His-tagged *HCAII* gene you cloned into pET-blue2. These RNAs will then be translated into His-tagged HCAII protein.
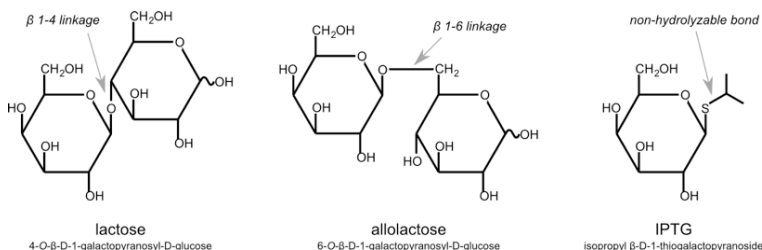
Fig. 7.2. *Chemical structure comparison between lactose, allolactose and IPTG.*

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https://wisc.pb.unizin.org/biochemistry551online/?p=112

Cell Growth

When inducing *E. coli* to overexpress protein, it is important that cells are grown to a relatively high density before the induction. Once the *E. coli* begin to overexpress HCAII, their growth is expected to slow down significantly. This is because the cells are devoting much of their energy to producing protein, rather than cell division.

The following video contains more information on bacterial cell growth, how to measure it, and what it means for protein overexpression:

OD_600 Correlates to Cell Number

- Optical density is not technically the same as absorbance
  - The light bounces off the bacteria so that it does not reach the detector
  - The more bacteria, the more reflection, the less light reaches the detector, the higher OD

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Note that there are additional videos from Christine in the Protocol section, demonstrating several techniques mentioned here.

Purification Step 1: Cell Lysis

Cell lysis is a process for disrupting cells in order to release the contents (like HCAII!) out of the cells

There are a number of methods for cell lysis (see lecture!); in lab we will use the **Bugbuster reagent**

- ◦ Works by using lysozyme and lipases/detergents to dissolve the cell membrane
- ◦ This is the most 'gentle' method of cell lysis, but also the most expensive (only the best for 551 students)

Purification Step 2: Bulk Purification

Bulk purification steps are quick and easy methods to remove a number of contaminants from a sample.

In our case, we will use centrifugation to separate soluble protein from cell debris.
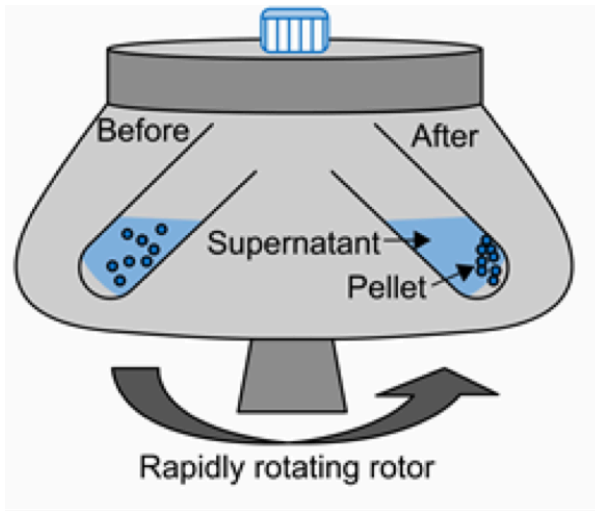
Fig. 7.4 Centrifugation

- Soluble fraction (i.e. supernatant, lysate) contains:
  - Soluble proteins
  - Small molecules
- Insoluble fraction (i.e. pellet) contains:
  - Cell wall/membranes
  - Insoluble proteins
  - Unlysed cells

Purification Step 3: Fine Purification (chromatography)

See lecture for more details on the different types of chromatography methods! Here is a list of the most commonly used types of chromatography for protein purification:

- Size – size exclusion
- Charge – ion exchange
- **Specific interactions – Affinity (our method of choice!)**
  - Remember that we added a histidine-tag to HCAII when we cloned into pETblue2 (see picture below)
  - We will now use this histidine-tag for affinity purification

pETBlue-2 cloning/expression region
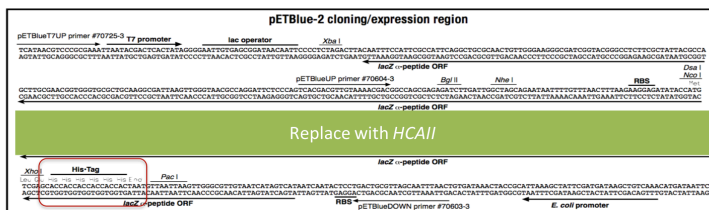
Fig. 7.5. *Schematic of HCAII cloned into the pETblue2 vector.*

Purification Methods: His-Tag

Since you cloned the *HCAII* gene such that your HCAII protein has a His-tag at the C-terminus, you can use nickel resin to purify the protein. The histidines coordinate with nickel ions in the resin, as seen below, effectively gluing your protein to the column. Imidazole, which is chemically similar to histidine, can be used to outcompete protein bound to the nickel resin and thereby specifically elute the desired His-tagged protein. Washing the column with buffer containing a low concentration of imidazole first should remove most of the contaminating protein (i.e. proteins that bind weakly to the nickel resin). The His-tagged protein can then be eluted with a higher concentration of imidazole.
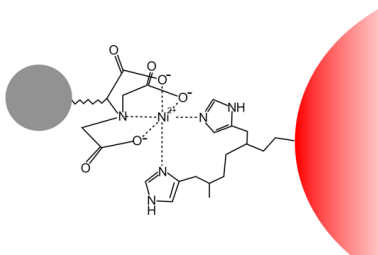
Fig. 7.6. Ni-NTA *(bound to an agarose bead, red, to form a resin) coordinates histidines in a his-tagged protein (grey).*
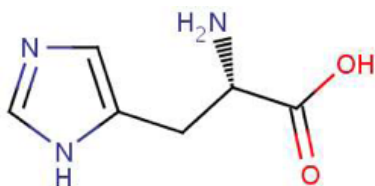
Fig. 7.7. Structure of histidine
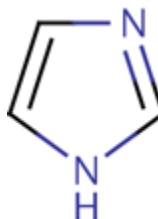
*Fig. 7.8.
Structure of
imidazole*

Purification Step 4: Identifying Purified Protein

After running a column, you are left with a number of elution fractions, only some of which contain the protein of interest. You will test each elution fraction for the presence of protein using the NanoDrop spectrophotometers.

Just like in previous labs, we will use the NanoDrop spectrophotometers to measure absorbance values at 230, 260 and 280 nm. Because we are now interested in protein rather than nucleic acid, we will primarily use the absorbance at 280 nm, or A_{280}. Also be sure to note the 260/280 ratio, as a measure of purity.

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> *https://wisc.pb.unizin.org/biochemistry551online/?p=112*

We can use Beer's Law to determine the protein concentration from the A_{280}. Beer's Law states:

A = elc

Where:

- A is the absorbance
- e is the molar absorbtivity, also known as the extinction coefficient
- l is the pathlength (1 cm in our system), and
- c is the concentration

The extinction coefficient is characteristic for a particular substance. It can be estimated from the sequence of a protein using the equation:

e = (#tryptophans x 5500) + (#tyrosines x 1450)

The experimentally determined extinction coefficient for HCAII is $50,420 \text{ cm}^{-1}\text{M}^{-1}$.

NOTE: This is the extinction coefficient for WILD TYPE HCAII. The extinction co-efficient will be different for certain mutants.

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https://wisc.pb.unizin.org/biochemistry551online/?p=112

Purification Step 5: Dialysis

The dialysis process is commonly used for removing salt or small molecules (like imidazole!) from protein samples. It works by separating molecules according to size through the use of a semi permeable porous membrane. Pores in the membrane allow

solvents, salts, and small metabolites to defuse across but block larger molecules.
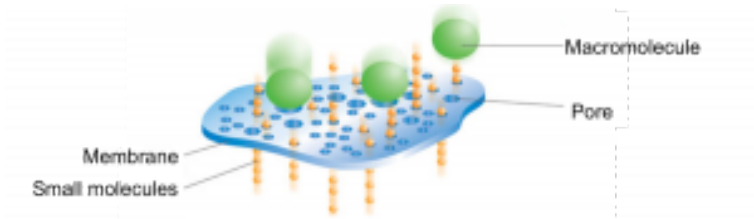
Fig. 7.9. Schematic of the dialysis process

Dialysis requires:

- A large (>100x) excess volume of dialysis buffer compared to the sample
- Time to equilibrate (>4 hours with stirring). Note that this is an *equilibration*. The imidazole concentration in your protein sample after dialysis is *not* exactly 0, but it is very low.
- Multiple changes of dialysis buffer

Lab 7: PROCEDURE

E. coli Growth

- Each pair works with ~200 mL of an E. *coli* culture expressing wild type HCAII and ~100 mL of an E. *coli* culture expressing mutant HCAII, each in mid-log phase (OD_{600} 0.6-0.9).

The following series of videos demonstrates the process of starting a culture and monitoring its growth.

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- Use the provided *E. coli* to set up 3 100 mL cultures in 250 mL flasks:
    - 100 mL IPTG-treated *E. coli* + wild type
    - 100 mL negative control *E. coli* + wild type (i.e. no IPTG)
    - 100 mL IPTG-treated *E. coli* + mutant
  - Use the sterile flasks provided on the back lab bench – DO NOT use the normal flasks in your drawers!
- Remove 0.5 mL from each culture as your 0 time point. Store in labeled 1.5-mL eppendorf tubes, on ice, until you are ready to process them (see below).
- Add IPTG to a final concentration of 500 µM to each culture, and then <u>immediately</u> place each flask in a 37°C water bath.

Collect samples for SDS-PAGE

Throughout this protocol, there are several steps where small samples are taken and processed for future analysis via SDS-PAGE. These steps are always boxed so that you can easily identify them.

- Collect 0.5 mL from each culture at each of the following time points: 45, 90, and 150 minutes
- Spin each sample of cells for 1 min at 4,000 rpm.
- Discard the supernatant into bleach. Do not disturb the pellet.
- Resuspend in 30 μL water. Add 30 μL of denaturing sample buffer (containing tracking dye, SDS, and β-mercaptoethanol).
- Heat the samples to 95ºC for 10 min. in the heat block.

Fig. 7.12. Heat block for preparing SDS-PAGE samples.

- Spin briefly in the microfuge to remove liquid from caps.
- When all of the experimental samples have been prepared, all of the microfuge tubes can be stored in your freezer box at -20°C until the next period when the SDS-PAGE gel will be performed.

Note that, in the box above, samples were collected for SDS-PAGE at several time points. If desired, you could measure the OD_{600} of these samples before preparing them for SDS-PAGE. The OD_{600} provides an estimate of cell number, so that measuring the OD_{600} over time would allow you to create a growth curve for your different cultures.

Prepare the remaining culture for purification.

- Once you have collected all of your samples, transfer your remaining wild type and mutant cultures to two separate 250 mL centrifuge bottles – DO NOT combine the wild type and mutant cultures.
- Another group will add their culture to these bottles as well, so that there will be 200 mL of wild type and 200 mL of mutant to spin.
- Be sure your bottles are well-labeled.
- Balance the two bottles against each other on a scale, using water to adjust if necessary.
- Pellet the cultures in a large floor centrifuge by spinning at 6,000 x g for 15 minutes.

Cell lysis via lytic solution

- Each group will purify either the protein from the wild-type pellet or from the mutant pellet.
- Add 8 mL BugBuster reagent to your pellet, and mix by swirling, pipetting or <u>gentle</u> vortexing.
- Incubate the cell suspension on a shaking platform on the low setting for 15 min. at room temperature.

Removing insoluble debris

- Transfer your lysate to a small centrifuge bottle.
- Balance your centrifuge bottle with another pair's, adjusting with water if necessary.
- Remove the insoluble debris by centrifuging the suspension at 16,000 x g for 20 min. at 4º C in the floor centrifuge. Please make sure that you wait to run your samples until all of the slots in the centrifuge have been filled.

- Transfer the supernatant to a fresh 50 mL conical tube.

> Resuspend the pellet in 8 mL H_2O. Take a 10 μL sample of this resuspended pellet and combine it with 20 μL water and 10 μL SDS sample buffer. Heat the sample for 10 minutes at 95°C. Then put the tube in your freezer box to be stored for next week.
>
> Remove 10 μL of the supernatant and combine it with 20 μL water and 10 μL SDS sample buffer. Heat the sample for 10 minutes at 95°C. Then put the tube in your freezer box to be stored for next week.

Packing the column

- Assemble your column. The following video demonstrates the process, and is followed by step-by-step instructions.

A YouTube element has been excluded from this version of the text. You can view it online here:
https://wisc.pb.unizin.org/biochemistry551online/?p=116

- Cap the column and fill it with equilibration buffer. Yes, fill it all the way. Float the frit on top of the buffer and then push it gently to the bottom of the column with the wide end of a Pasteur pipette. Remove the end cap and run the buffer out of the column into a clean flask until there is approximately 1/8 inch of buffer above the frit and then re-cap the column. The buffer is clean, so you can reuse it for equilibrating the column.
- Your TA will give you 1 mL of the Ni-NTA resin for your column.
- Slowly add the resin down the side of your column and let it settle. Be careful not to introduce air bubbles. Once the resin has settled a little, remove the end cap and allow the buffer to flow to further pack the resin. You may want to add more buffer as it is running to help pack the resin.
- After the resin has packed, and while the column is running, add equilibration buffer until the buffer reaches the line near

the top of the column. If the resin is disturbed during the buffer addition, let it settle before going on to the next step.

- With the bottom of the column capped, place a small plug of glass wool into the column. Let the buffer soak into the glass wool so that there are no bubbles in the plug. Then push the glass wool into the column so that it is just above the resin but do not let the glass wool touch the resin bed. Make sure that there are no large bubbles between the glass wool and the resin.
- The glass wool will filter out any debris in your cell lysate that would otherwise clog the column. Equilibrate the column with 5 column volumes of equilibration buffer. (A "column volume" is the volume of resin you added to your column.) The following figure summarizes the column preparation steps:

1. Setup	2. Resin	3. Glass Wool	4. Equilibrate
Fill column with equilibration buffer, then insert frit	Add resin, let settle, pack by running more eq buffer	Fill with eq buffer. Be sure resin is settle. Add glass wool to just above resin	Flow 5 column volumes of eq buffer over column
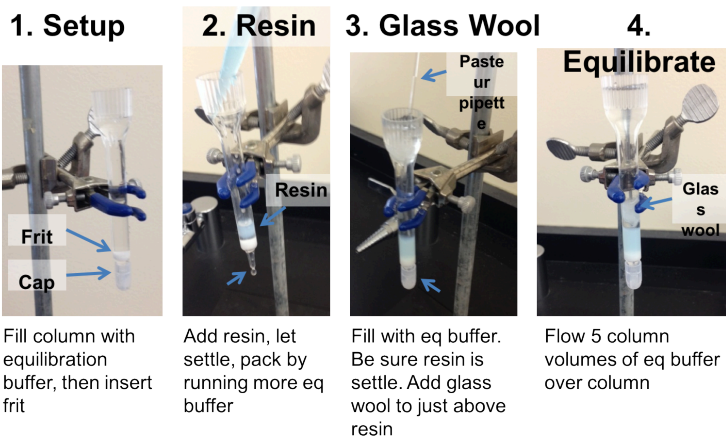
Fig. 7.13. Summary of steps involved in packing a column.

- Apply your cell lysate (supernatant) to the column and let it flow into the column. Collect the flow-through in a conical tube.
- Wash the column with 7.5 mL wash buffer of choice. Collect

the flow-through in 5 x 1.5 mL fractions into separate microfuge tubes.

- Elute the protein from the column with 5 mL of your elution buffer. Collect the flow-through in 5 x 1 mL fractions into separate microfuge tubes.

> **NOTE:** This is your purified protein — be careful with it!

> Remove 10 µL of liquid from each fraction you collected (flow-through fraction, wash fractions, elution fractions) and place it into a clean microfuge tube (be sure to label the tube). Dilute the sample with 10 µL of SDS sample buffer. Heat the samples to 95°C for 10 min. These samples can then be stored for next week.

Spectrophotometric determination of protein concentration

- Measure the protein concentration of each elution fraction by measuring the absorbance at 280 nm on the NanoDrop.
- Be sure to mix each fraction before taking a sample to measure on the NanoDrop.
- Use the A_{280} to determine the protein concentration of each of your fractions. The extinction coefficient for wild type HCAII is 50,420 cm^{-1}M^{-1}.

> **NOTE:** This is the extinction coefficient for WILD TYPE HCAII. The extinction co-efficient will be different for certain mutants. Do you have such a mutation? If so, you need to calculate its extinction coefficient.

- Decide which fractions contain the most protein, and dialyze those (directions in the next step). Typically protein is found in two concentrated fractions (see elution profile below) – but you could have more or less. As a general rule of thumb, use any fractions with $A_{280} > {\sim}0.3$ that have reasonable purity. Talk to your TAs if you are unsure.
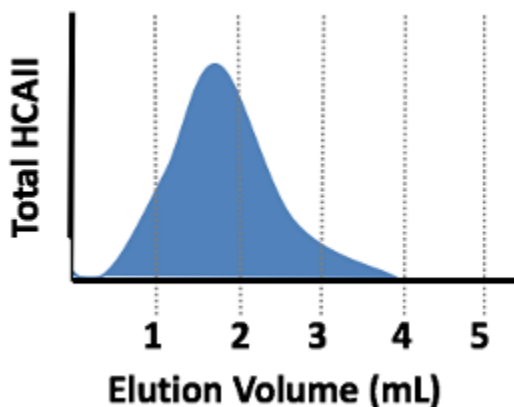
Fig. 7.14. Elution profile of HCAII being eluted into 5 x 1 mL elution fractions. In this case, most of the protein is in fractions 2 and 3, so those fractions should give the highest A_{280} readings. Fractions 2 and 3 should then be combined and dialyzed in the next step.

Dialysis of your elution fractions containing protein

- Prepare your protein sample for dialysis. The following video demonstrates the process, and is followed by step-by-step instructions.

A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/biochemistry551online/?p=116

- You should perform all of these steps over a clean, empty beaker in case you spill your protein. If the protein is in the beaker you can recover it. We also recommend that you practice the following steps with water before trying them with your protein to ensure that you do not lose your protein.
- Take a piece of tubing from the beaker and rinse it with dH_2O.
- Place a clamp on one end of the tube approximately ½ in. from the bottom of the tube. You should label the clamp.
- Add your most concentrated elution fractions (as decided on above) to the tubing and then place the second clamp on the tubing approximately ½ in. from the end that is not clamped. The second clamp should have a piece of string tied to it.

- Hold the filled tubing over your clean beaker and gently squeeze. If protein leaks out, try again.
- If you practiced with H_2O, you can simply unclamp one end of the tube, pour out the water, and reuse the tubing for your protein.
- After your protein is in the tubing and you know the tubing is not leaking, place your sample into the beaker containing the Protein Buffer (PB: 50 mM Tris, pH 7.7, 0.1 M K_2SO_4). Tape the string to the outside of the beaker, and label the piece of tape with your name so you will be able to easily find your tubing next week.
- A 551 staff member will exchange the Protein Buffer and you can collect your protein next week.

Lab 7: LAB ASSIGNMENTS

PRELAB QUIZ

Visit Canvas to take the quiz. The quiz questions are also listed below for your convenience:

Question 1

What experiments are being simulated this week? What is the purpose of those experiments? (Provide 2-3 sentences).

Question 2

What volume of IPTG will you add to *each* culture?

How many cultures do you need to induce with IPTG?

Given the two answers above, what *total* volume of IPTG do you need for this experiment?

Question 3

Which buffer will you use to equilibrate, wash, and elute during the column purification?

Buffer 1: 50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole

Buffer 2: 50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole

Buffer 3: 50 mM NaH2PO4, 300 mM NaCl

HINT: Thinking about the purpose of each step will help determine which buffer should be used:

- Equilibration: the purpose of this step is to prepare the Ni-NTA resin in a buffer similar to what you will wash and elute with, so that the HCAII will optimally bind
- Wash: the goal of washing is to remove proteins that might have a small affinity for the Nickel resin (such as those with several naturally occurring

histidines). Therefore the wash buffer must remove these proteins without removing HCAII.
- Elution: the goal of elution is to remove HCAII from the column so that you can collect it as it flows off.

IN-LAB ACTIVITIES

- After joining BBCU, you and your partner will join a breakout room.
- Your TAs will provide you a link to a Google doc. You and your partner will work together to complete it. It will include:
  - An analysis of each SDS-PAGE sample collected throughout the protocol.
  - A table where you will outline each step in the protocol, state its purpose, and write down any questions you have. Each pair is required to ask a TA at least one question about the protocol.
  - Based on the provided NanoDrop results, you will decide which elution fractions to dialyze.
- Before leaving BBCU, meet with a TA to ask them at least one question about the protocol. They will also discuss your elution fraction choices with you.
- Complete the lab/partner/self evaluation Google form.
- Get a sharable link to your Google doc and submit it on Canvas.

Lab 7: MINI LAB REPORT

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Reports

Draft Methods for Labs 2, 4, 5, and 7

This week, you will start to assemble a cohesive Methods section that describes the HCAII project to date. This Methods section will ultimately be incorporated into your Final Lab Report. Refer to the Final Lab Report assignment details on Canvas (link opens in a new tab): Final Lab Report

Please aim to keep your Draft Methods section to a maximum of five pages.

Be sure to incorporate feedback you received from your TAs on your previous mini reports.

A few general reminders for lab 7:

- State clearly whether you are purifying wt or mutant protein. Do not say "mutant" or "mutant 1"; instead, always write out the actual mutation you are testing (i.e. N62W).
- When mentioning buffers, include the actual recipes. Don't just say "Buffer 1"
- Include the equation you used to determine protein concentration and be sure to define the variables. (See note on equations on at Mini Lab Reports)

LAB 8: ANALYSIS OF HIS-TAGGED HCAII EXPRESSION AND PURIFICATION

Lab 8: Analysis of His-tagged HCAII expression and purification

Lab 8 Objectives

- Use SDS-PAGE to:

  - Determine expression of HCAII protein was induced in *E. coli*
  - Determine whether HCAII was successfully purified on a nickel column

- Measure the protein concentration of the final purified HCAII protein
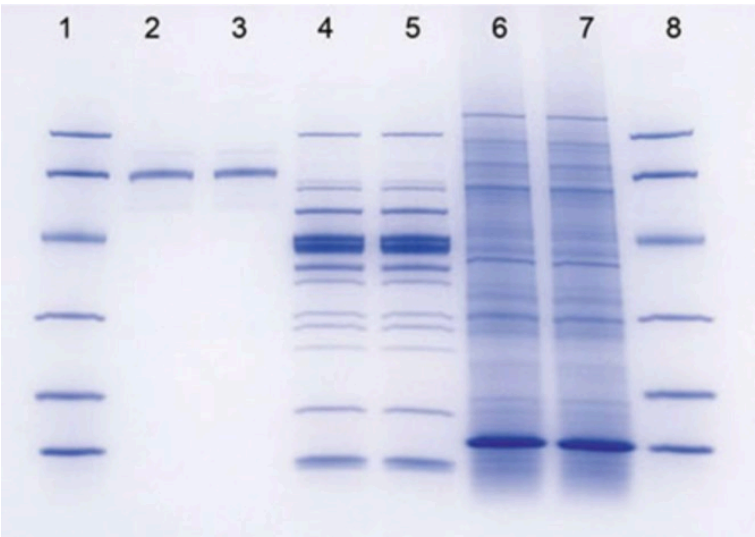
Fig. 8.1. A sample SDS-PAGE gel.

Lab 8: BACKGROUND

SDS-PAGE: The Gels

Gel electrophoresis is a powerful separation technique used for analyzing many biologically important molecules, including both proteins and nucleic acids. We have already used electrophoresis to examine DNA molecules, and now we will study proteins using a particular type of gel electrophoresis called SDS-PAGE:
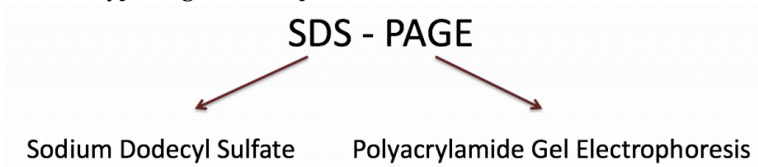
This type of electrophoresis relies on two components that allow proteins to run predictably through the gel:

- Sodium dodecyl sulfate (SDS) is a denaturing detergent. It binds proteins non-covalently and unfolds them. It also coats proteins with a uniform negative charge, creating a linear protein with a constant charge:mass ratio
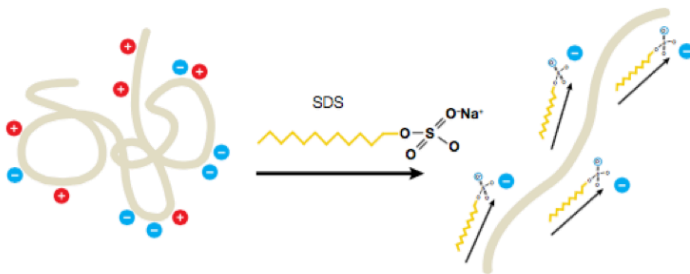
Fig. 8.2. Molecular interactions between SDS and proteins.

- A reducing agent, β-mercaptoethanol (BME), is also required to

break disulfide bonds and ensure proteins are fully linearized.
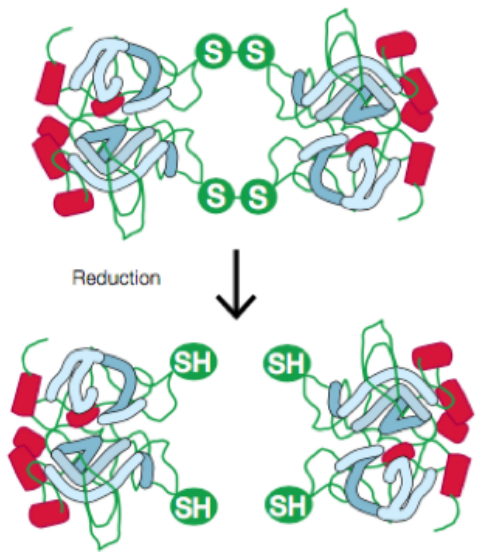
Fig. 8.3. BME breaks disulfide bonds in proteins.

The gel itself is made of acrylamide. A cross-linker, typically bis-acrylamide, is polymerized along with the acrylamide using free radicals to create a network of pores:
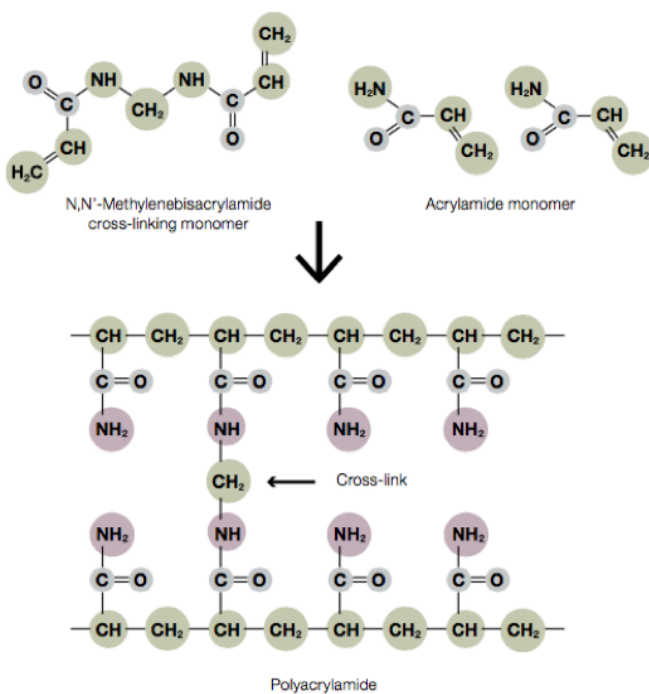
Fig. 8.4. Chemical composition of a polyacrylamide gel.

Our SDS-PAGE gels will have two layers (click on the plus signs to read about each):

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https://wisc.pb.unizin.org/biochemistry551online/?p=129

For more on SDS-PAGE, see the Appendix: Appendix 3: Additional laboratory details

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Visualizing Proteins in an SDS-PAGE Gel with Coomassie Blue

Once you load and run your samples through the gel, you will need to stain your samples in order to visualize the proteins. You will stain with a dye called Coomassie Blue. This is a dye that binds primarily to aromatic and positively-charged residues, making proteins appear blue in the gel.
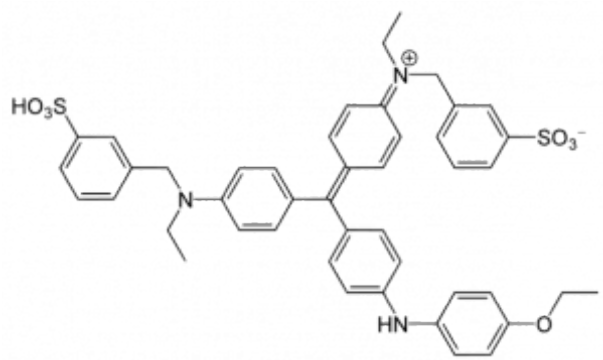
Fig. 8.5. Chemical structure of the Coomassie blue dye.
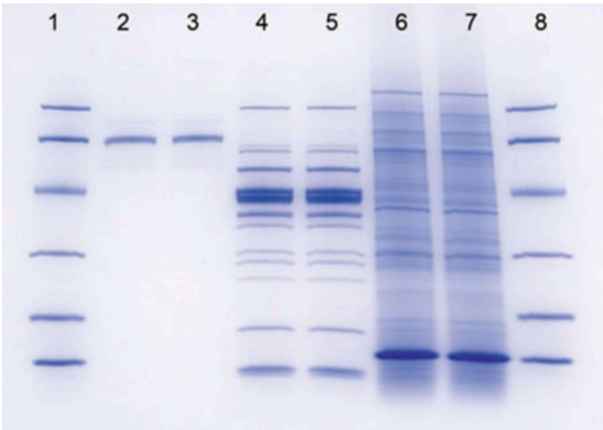
Fig. 8.6. Example SDS-PAGE gel stained with Coomassie blue.

Once you have stained your gel, you will need to calculate the molecular weight (MW) of any bands of interest, including HCAII. One way to determine the MW of unknown samples is to compare the unknown's relative mobility (Rf) with that of MW standards.
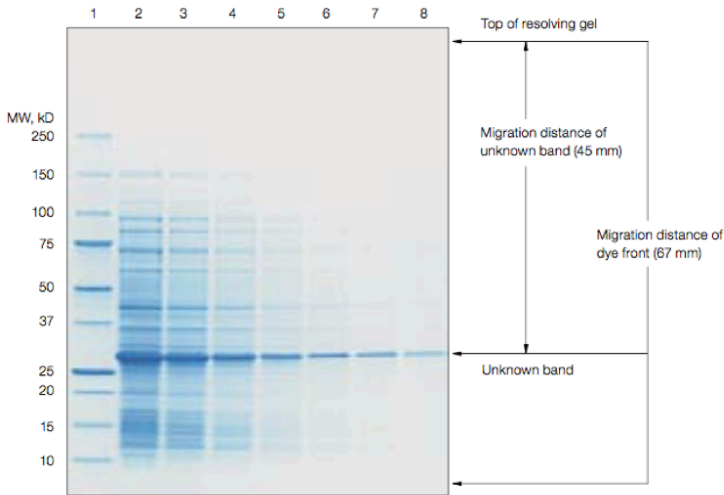
Fig. 8.7. *Important measurements for approximating the molecular weight of an unknown protein on a gel.*

$$R_f = \frac{\text{Distance (protein to top of resolving gel)}}{\text{Distance (dye front to top of resolving gel)}}$$

If you plot the Rf of the standards vs. the log of their MW, you should get a straight line for those standards that are in the linear range for the gel. You can then use this standard curve to determine the apparent molecular weight of any protein band on the gel, so long as the band falls within the linear range of the standards. Whether or not a particular size protein is within the linear range of the standard depends on the percentage acrylamide in the gel you

are using. For this lab, we will tell you what percentage to use so that HCAII falls within the linear range.

Please see the Data Analysis section, which follows the Lab 8 Procedure, for more information on this topic.

Lab 8: PROCEDURE

Preparation of the 15% resolving gel.

This video demonstrates how to set up a resolving gel:
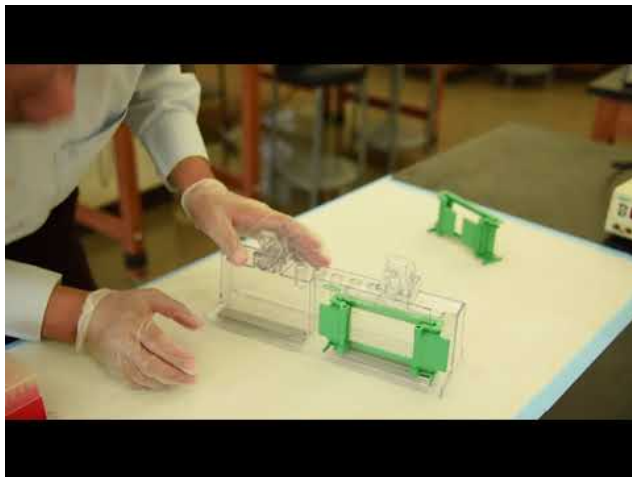
A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/biochemistry551online/?p=137

- Put on gloves. Clean the glass plates with EtOH and a KimWipe.
- Assemble a gel-pouring apparatus:
  - Start by placing a short plate on top of a tall plate, taking care that the tall plate is oriented with the writing at the top.
  - Next, place a casting frame on a flat surface with the pressure cams in the open position.

- Slide the plates in such that the taller plate is in back, and clamp the pressure cams in place to hold the plates.
- Check that the plates are flush across the bottom. Place the casting frame into the casting stand by positioning the frame on top of a grey gasket while engaging the spring-loaded lever onto the tall plate.
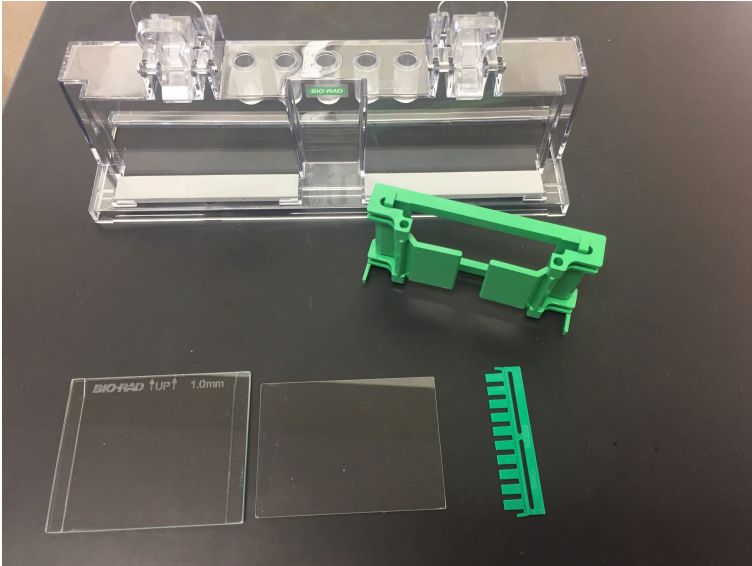
Fig. 8.8. These are the components you will need to pour your SDS-PAGE gel: casting tray, tall glass plate, short glass plate, green casting frame
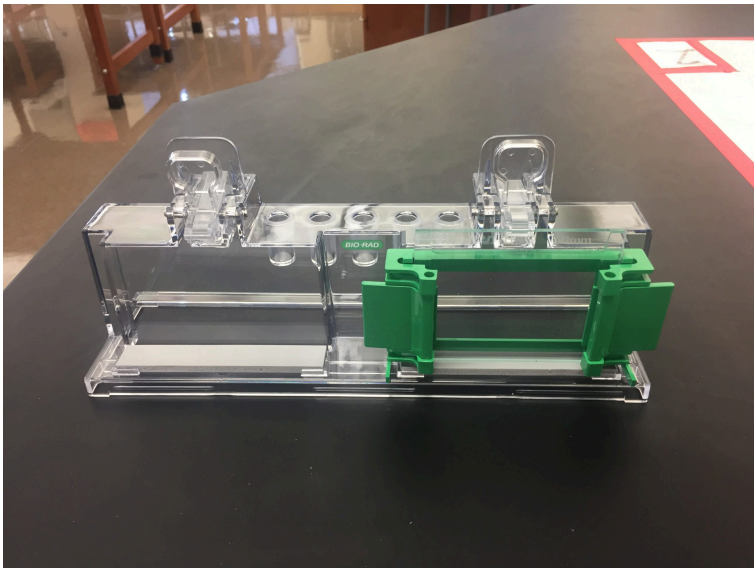
Fig. 8.9. The assembled pouring apparatus: Tall glass plate behind the short one, plates flush across the bottom, green pressure arms in the locked (outward) position, loaded into the casting tray.

- Check for leaks by filling the apparatus with 75% ethanol. Let it sit for ~5 minutes to check for slow leaks. If there are no leaks, pour the ethanol out and use a Kimwipe to dry the plates as much as possible without disassembling the apparatus. If it leaks, check the following:
  - Plates might be misaligned or oriented incorrectly.
  - The grey gasket might be torn or worn down. Try putting a strip of parafilm on top of the gasket.
- Insert a comb completely into the assembled gel cassette. Mark the glass plate 1 cm below the comb teeth. This is the level to which you will pour the resolving gel. Remove the comb.
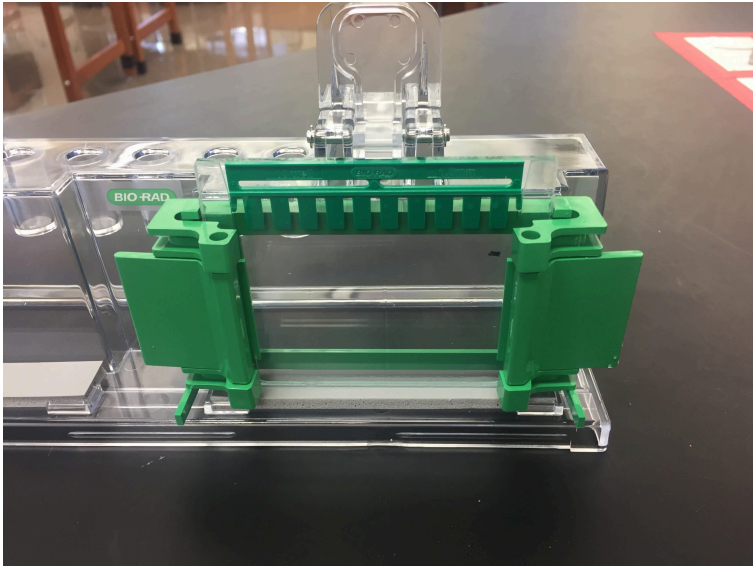
Fig. 8.10. Note the marking on the front glass plate ~1 cm below the bottom of the comb. This is how high you will pour your resolving gel.

- We will provide 3.75 mLs of 40% acrylamide in a tube labeled "R". Add 2.5 mLs of resolving gel buffer and 3.75 mLs of water to the vial. Mix by gently inverting several times. Take a tube of 10% APS and a tube of TEMED back to your pod – this is enough for you and your partner to pour two gels in total.

Lab Safety Notice

Liquid acrylamide is a neurotoxin! When working with it, please observe the following precautions:

- Wear gloves and eye protection.
- Return any unpolymerized acrylamide to your TAs.

Note that once it polymerizes, it is no longer toxic.

- Add 15 μL of TEMED and 50 μL of 10% APS to the acrylamide vial. Keep the tubes of TEMED and APS on ice – you will need them later for the stacking gel.

WARNING: After you add TEMED and APS to the acrylamide, it will immediately begin to polymerize. Work quickly!

- Mix the solution by inverting gently several times. Try to avoid foaming the SDS or mixing air bubbles into the solution.
- Using a long-tipped Pasteur pipette, immediately transfer the solution into the gel apparatus. Fill the gel to the mark you made on the glass.
- Using a squirt bottle, gently layer some ethanol on top of the acrylamide solution. This will help to form a smooth polyacrylamide surface to pour the stacking gel onto, as well as prevent the gel from drying out. Do not move the gel until polymerization has occurred which typically takes 15-45 minutes. While waiting for the gels to solidify, you can prepare your protein samples.

Collecting your protein from the dialysis tubing

- Remove your tubing from the beaker.
- Perform the following steps over a clean beaker (in case your protein spills!):
  - With one hand, firmly hold the tubing above and below the top clamp. With the other hand, gently unclamp the top clamp and set it aside.
  - Use a clean pipet to remove the protein from the tubing and place it into a clearly labeled 15 mL tube.
- Take a 10 μL sample, add 10 μL of SDS sample buffer, and heat at 95°C for 10 minutes.
- Measure the A_{280} of the dialyzed protein. Use Beer's law to determine the protein concentration from the A_{280}. Based upon the readings from last week, did you lose any protein during dialysis? (Note that this measurement and/or calculation can be done later, while your gel is running.)

> **NOTE:** You MUST plug your A_{280} into Beer's law to determine the protein concentration. DO NOT use the "concentration" in mg/mL given by the NanoDrop. The NanoDrop is not properly programmed to calculate protein concentration.

> *An interactive or media element has been excluded from this version of the text. You can view it online here:*
> *https://wisc.pb.unizin.org/biochemistry551online/?p=137*

Preparation of frozen SDS-PAGE samples from previous lab

- Thaw the samples, heat at 95°C for 5 minutes, and spin them in the microcentrifuge for 2 minutes to pellet any insoluble material.
- Also heat the MW markers at 95° C for 5 minutes and then spin the tube for a few seconds to ensure that all the marker is at the bottom of the tube.

Preparation of the 4% stacking gel – after the resolving gel has polymerized

The following video demonstrates how to prepare a stacking gel:
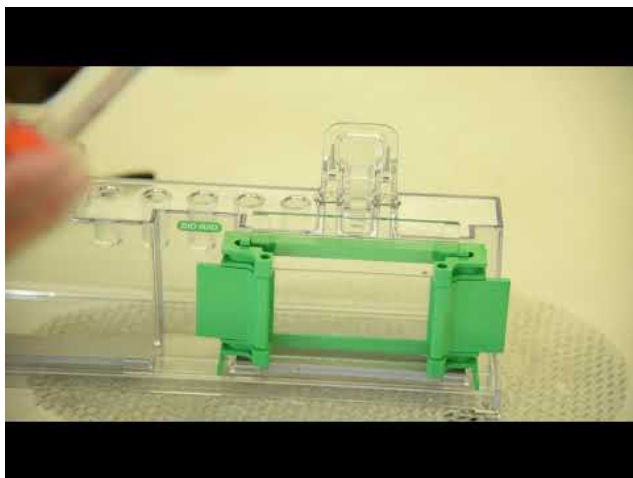
A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/ biochemistry551online/?p=137

- Put on gloves.
- Before preparing the stacking gel, make sure the resolving gel

has already polymerized. Watch for the appearance of a visible separation between the ethanol and the gel. When you think your gel is ready, carefully start to tip it to the side. It should be obvious that the gel is solid and will not pour out of the apparatus. If not, give it more time.

- Carefully invert the gel apparatus to pour off the ethanol layer. Remove any remaining ethanol from the corners using a Kimwipe.
- Insert the comb into the gel apparatus with one side sticking out enough to allow you to add your stacking gel solution with a Pasteur pipette (see figure below).
- Get a conical tube, labeled "S," containing 1.0 mL acrylamide. Add 2.5 mLs of stacking gel buffer and 6.5 mLs of water to the vial.
- At your bench, add 20 µL of TEMED and 100 µL of 10% APS and mix carefully.

> **WARNING:** Your acrylamide is now starting to polymerize. In fact, the stacking gel polymerizes even faster than the resolving. Work quickly!

- Using a long-tipped Pasteur pipette, immediately transfer the solution into the gel apparatus. Add stacking gel mixture until it fills the space between the resolving gel and your comb.
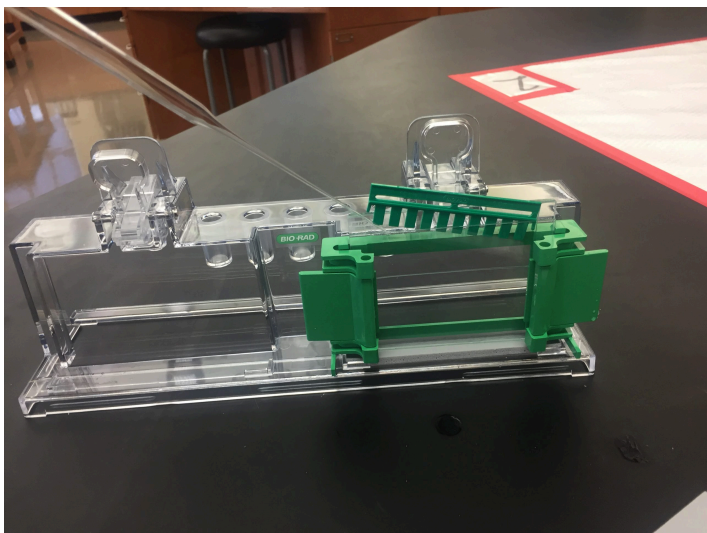
Fig. 8.11. Insert the comb while leaving enough space to pour the gel. Pour until the plates are completely full, then slide the comb all the way down.

- Gently slide the comb all the way into the acrylamide in the gel apparatus. Try to avoid splashing the acrylamide when the comb is inserted. Keep a Kimwipe handy to wipe up any overflow acrylamide. Do not move the comb too much after inserting it because the gel will begin to polymerize quickly.
- Wait for the stacking gel to polymerize.
- GENTLY pull out the comb.

The following video shows how to assemble and load the gel apparatus:

A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/biochemistry551online/?p=137

- Obtain 160 mL of 5X Running Buffer. Dilute it to 1X with diH2O from the tap and use that in steps that follow. You may be able to skip this step and re-use used 1X buffer, if available.
- Take the electrode clamping frame out of the gel box and drop the green clamps on either side. Slide the gels, one into each side. Gels should be slid in bottom first, so that the bottom of the gel is all the way down in the clamping frame. They also need to be placed so that the shorter glass plates face inward. Once the gels are in place, lift the green clamps on both sides until they lock into place.
- Place the electrode assembly into the electrode chamber, matching colors (red on electrode assembly to red on gel box). Fill the inner chamber completely with 1x SDS running buffer so that the current can flow through the gel. The current will not flow through the non-submerged part of the gel.

- Add enough running buffer to fill the outer box to the level marked "2 gels" on the outside of the box.

> An interactive or media element has been excluded from this version of the text. You can view it online here: *https://wisc.pb.unizin.org/biochemistry551online/?p=137*

- Load 8 µL of the markers and 10 µL of the samples you chose to run for the experiment. Load the gel as you drew it in your pre-lab. Note that the samples from lab 6 will probably be very viscous because of all the cell debris they contain. It is usually easiest to pipette these using the special gel loading tips. Let the TAs know if you're having a lot of trouble.
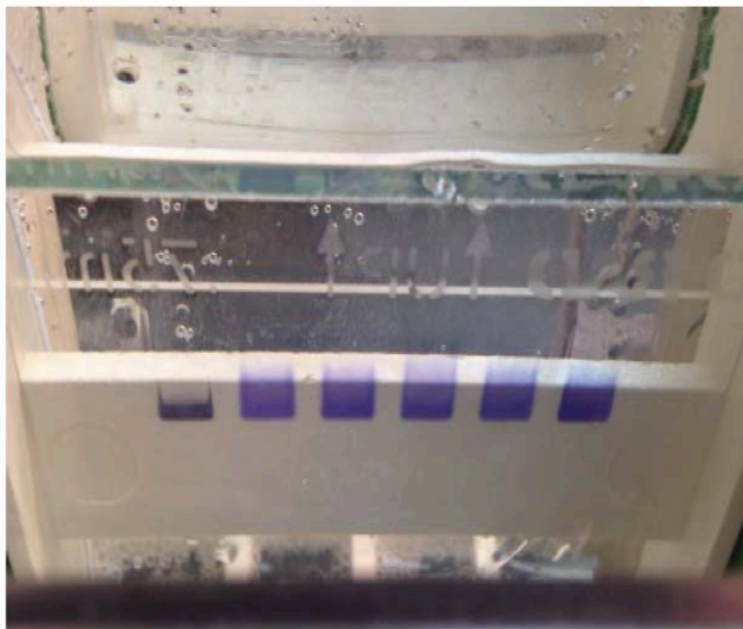
Fig. 8.12. An SDS-PAGE *gel with several loaded samples.*

Applying the voltage

- Place the cover on the electrophoresis unit. Be sure to connect each plug to the **like-colored** socket. Like when we ran DNA on agarose gels previously, it is possible to run an SDS-PAGE gel in the wrong direction. Pay attention to how you plug in your leads.
- Turn on the power supply and run at 100 V until the dye front is completely through the stacking gel (approx. 15 min.) and then turn the power up to 200V to run the samples through the resolving gel.

- Continue the electrophoresis until the dye front is almost completely run off the bottom of the gel. Do not allow the dye front to completely run off the gel, but try to get it very close, so that you get good separation of the protein bands. It is ok if you start to see some the dye front running off the bottom and floating away in the gel box.
- Turn off the power and unplug the leads from the power supply. Remove the cover. Pull the electrode assembly out, being careful not to dump buffer everywhere. Used 1x SDS running buffer should be poured into the "Used" carboy.

Staining the gel

- Add Coomassie Blue staining solution (re-use if used stain is available) to your staining box.

Fig. 8.13. BE CAREFUL! Coomassie blue stains proteins, like those in skin...and clothes and...floors...

- Open the green clamps of the assembly and remove the gels.
- Carefully pry apart the two gel plates. Apply pressure only at the center of the edge of the gel sandwich, **NEVER** near the "corners" as they will break off. You can use the green gel plate separator tool to help with this. The gel should adhere to one of the glass plates.
- Transfer the gel from the glass plate into the staining box containing Coomassie blue. Invert the plate with the gel over the box and gently lifting one corner of the gel with your finger. Once some air moves under the corner of the gel, it should slide off the plate.

> **IMPORTANT:** Gels are very delicate and will tear easily. Be careful!

- Heat the gel in stain for 45 seconds in the microwave. Shake for five minutes on a platform shaker.
- Pour off the stain into the used stain bottle — the acetic acid has a strong smell, so try not to breathe it in too much. Rinse the gel with dH2O and pour off in the sink.
- Place the gel in destain (enough so the gels float freely) and add two wadded Kimwipes to your staining box (this will help draw stain out of the destain solution). Microwave for 45 seconds. Shake for ten minutes.
- Replace the Kimwipes with new ones. Shake overnight.
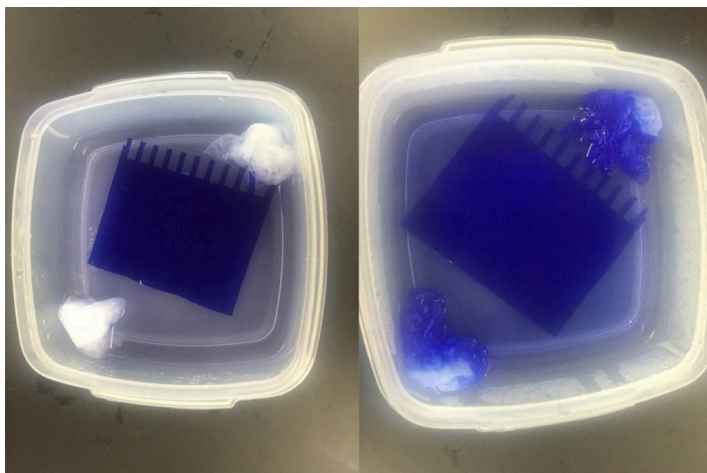
Fig. 8.13. Left: After staining but before destaining, the gel should appear uniformly dark blue. Right: After microwaving in the destain, you can see the gel start to get lighter. After shaking overnight, the gel should be completely destained so that only protein bands are still blue.

Clean up the gel apparatus, glass plates, and work area. All gel components should be rinsed thoroughly with water and left to dry on your tabletop.

Come into lab the day after your scheduled lab period. You will use the gel doc to image your gels. It is the same procedure as visualizing DNA gels, except we will use a different visualization tray. DNA gels utilize a UV light tray, while Coomassie gels utilize a "white light" tray.

Lab 8: LAB ASSIGNMENTS

PRELAB QUIZ

Question 1

What experiments are being simulated this week? What is the purpose of those experiments? (Provide 2-3 sentences).

Question 2

This lab involved a chemical hazard. Describe the chemical, the potential risks, and steps you would take to protect yourself.

Question 3

If you assemble your gel apparatus and find that it leaks, describe one way you can try to fix it.

Question 4

Which is likely to polymerize faster: Resolving gel (bottom, poured first), or Stacking gel (top, poured second)?

IN-LAB ACTIVITIES

- After joining BBCU, you and your partner will join a breakout room.
- Your TAs will provide you a link to a Google doc. You and your partner will work together to complete it. You will:
  - Choose samples to run on your SDS-PAGE gels
  - Answer questions about the procedure
  - Discuss how to troubleshoot several example cases where something went wrong
  - Determine the protein concentrations of the final, dialyzed protein samples
  - Meet with your TAs to discuss. Once they are happy with your work, you will receive a picture of the gel you can analyze for your results.

- You are welcome to use any remaining in-lab time to analyze your gel and start working on your lab report.
- Complete the lab/partner/self evaluation Google form.
- Get a sharable link to your Google doc and submit it to Canvas.

IMPORTANT NOTE FOR LAB 9

For lab 9, you will need to complete an at-home experiment _before_ your lab time. Please plan out your next week accordingly!

The at-home experiment requires 2-4 eggs and 1/3 cup rubbing alcohol.

Lab 8: DATA ANALYSIS

Download your gel results here: Results: Lab 8

You will use these gel images to determine Relative Mobility (Rf), or the ratio of the distance a band of interest migrated compared to the distance the dye front migrated.

Start by measuring the distance from the top of the resolving gel to the edge of the dye front. The edge of the dye front should appear as a faint line towards the bottom of the gel, and is the farthest that anything could have run on the gel. This can be difficult to see, so estimate if necessary. This value will be the denominator in each Rf calculation below.

$$R_f = \frac{\text{Distance (protein to top of resolving gel)}}{\text{Distance (dye front to top of resolving gel)}}$$

Now measure the distance from the top of the resolving gel to each of the MW ladder bands. Divide each of these by the total length of the resolving gel, as measured above, to calculate Rf.
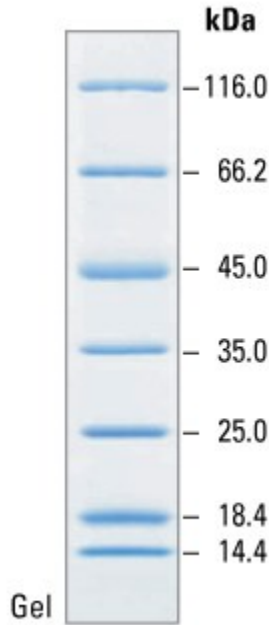
Fig. 8.13. MW ladder

The masses of each band in the MW ladder are shown above. Use this information to create a mass calibration curve by plotting Rf on the x-axis and log(MW) on the y-axis. If any points appear to be outside the linear range, you can discard them. Add a best-fit line and be sure to show the equation, as shown in the image below.
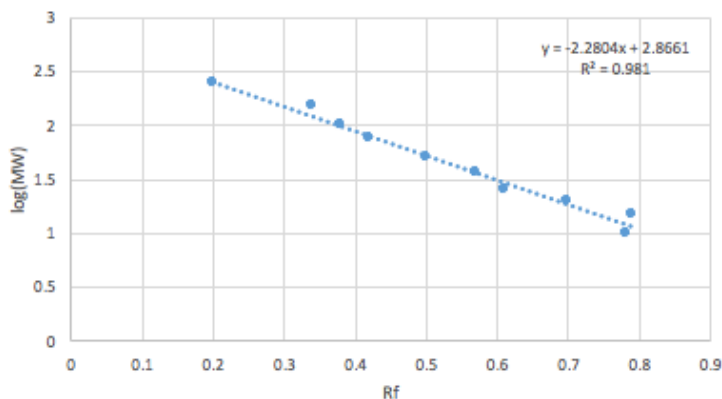
Fig. 8.16. Example Rf calibration curve.

Use this equation to determine the apparent molecular weight of any other bands of interest. Measure the migration distance of such bands and calculate the Rf, then plug into the equation to determine the apparent MW.

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Lab 8: MINI LAB REPORT

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Reports

Methods (just lab 8)

- State exactly which samples were run on the gel
- Be sure to include any equations you used to calculate Rf values

Results

- Include a picture of your gels, indicating the following:
  - The identity of the sample in each lane
  - The electrode polarity (positions and charge of anode and cathode)
  - The position of the top of the resolving gel
  - The position of the dye front
  - The size of the molecular weight markers in the gel
  - Highlight and label any bands of interest (some suggestions below)

- A molecular mass calibration curve for the standards by plotting the Rf versus the log of the MW. What is the linear range of the molecular weight marker? Include the best-fit line on the graph. Describe the graph including the shape of the line and what it tells you about the size range of the markers.

- From your examination of the expression induction samples: identify and label on the gel any induced protein bands. Calculate Rf-values for these bands and determine the molecular weight of these proteins by comparing the Rf for this band to the molecular weight standards. Based upon your molecular weight calculation of the most highly induced

protein band, did you induce a protein with the same MW as HCAII? At what time, and under what conditions, did you achieve maximum expression of this protein according to SDS-PAGE?

- For the purified HCAII samples: describe the gel results and what they tell you about the protein purification process. Calculate the Rf value for the brightest purified band; does its MW match that of HCAII?

- The concentration of your protein after dialysis, in μM

Discussion:

- Which samples were run on the gel and why? What information would you expect to get from those samples? Did you get the information you expected? If not, why not?

- Do you think HCAII expression was induced in *E. coli*? Use your data to support your conclusion. Did you induce expression of any other proteins? If so, what were their MWs, and what might they be?

- Do you think you HCAII protein was purified? How well did the purification column work? Use data to support your conclusion.

- If anything appears to have gone wrong in the purification, describe what is was based on the gel, and how you might be able to correct the problem if you were to repeat the purification.

- Although your protein is not completely pure, it is pure enough for the rest of the experiments in this class. Describe what you would do as a next purification step if you needed very pure protein (e.g.. pure enough for X-ray crystallography or NMR).

- Briefly describe what you will do next with the purified HCAII.

LAB 9: DETERMINATION OF PROTEIN STABILITY USING CHEMICAL DENATURATION AND INTRINSIC FLUORESCENCE

Lab 9: Determination of Protein Stability using Chemical Denaturation and Intrinsic Fluorescence

Lab 9 Objectives

- Gain hands-on experience with protein denaturation
- Determine whether your mutation affects protein stability based on measurements of the free energy of unfolding ($\Delta G_{unfolding}$) of wild type and mutant HCAII

Before lab, you will complete a brief at-home experiment to study protein denaturation using egg whites. This should take no more than 60 minutes. During lab, you will work with your partner to connect that experiment to the HCAII experiment. (Note that it may help to view the associated lecture material before lab as well!) The in-lab time will be relatively short this week, since you will be doing some lab work before lab.

This is the first of three Biochem 551 labs in which you will be determining whether or not your mutation of interest has a measurable effect on the function of HCAII. Before directly measuring any functional parameters, it is important to determine whether or not your mutation caused any changes in structural

stability of the protein. If, for example, a mutation destabilizes the protein to such an extent that it is completely unfolded, that mutant will not be able to catalyze any reactions. However, this would be a very different case than a mutant protein that is well-folded and stable, but catalytically dead. In order to differentiate between these possibilities, in lab 9 we will compare the protein stability of wild type and mutant protein.

Lab 9: AT-HOME EXPERIMENT

Expected time requirement: About 60 minutes.

Protein Folding and Denaturation

Proteins are composed of secondary structures that primarily result from non-covalent, intramolecular interactions between the backbone amides. Additionally, entropy favors the reduction of solvent-accessible surface area of hydrophobic amino acid residues. This promotes hydrophobic residues being pushed into the center of a protein, thus organizing the hydrophobic core that stabilizes globular protein structures.

Some <u>chemical agents</u> can compete for those weak intramolecular interactions and reduce the entropic penalty of organizing water near hydrophobic residues. This disrupts protein structure, leading to denaturation. <u>Heat</u> can also disrupt protein structure, by increasing kinetic energy and causing the molecules to vibrate so rapidly that the weak interactions are broken.

Protein denaturation is an essential part of food science, and an everyday part of cooking. In this experiment you will get to explore the phenomenon yourself using eggs. Egg whites contain ~90% water and ~10% protein. The most abundant protein in egg whites is ovalbumen, shown below. It is a 386 amino acid protein with a fairly compact structure containing both beta sheets and alpha helices. You will study the denaturation of ovalbumin in the experiment below.
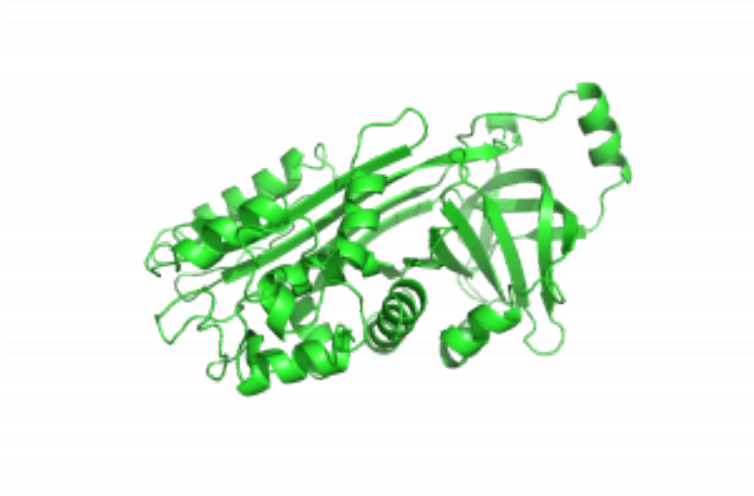
Fig. 9.1. Structure of ovalbumin. PDB ID 1OVA

Denaturation of Albumin

> If you are vegan or otherwise unable/unwilling to purchase eggs, please reach out to Dr. Prost for an alternative activity!

<u>Materials</u>

- Stove and pot OR microwave and microwave-safe bowl
- 1 fork
- 1 pair scissors
- 1-4 eggs
- 1 bowl
- 4 small containers of about the same size

- 2/3 cup water
- 1/3 cup rubbing alcohol

Procedure

1. Pour 1/3 cup rubbing alcohol into one small container, room temperature water (1/3 cup) into another, and the rest of the water (1/3 cup) into a microwave-safe container (or into a pot).
2. Crack egg into the bowl, removing the yolks. (This is not super easy if you've never done it before. Dr. Prost's preferred approach is to use one half of the broken egg shell as a little cup for the yolk while letting the whites run out into the bowl. You can search YouTube for "how to separate egg whites and egg yolks" for more help.)
3. If using 1 or 2 eggs, cut the egg white into pieces using scissors so you can more easily add ¼ of the total into each glass container. You can alternatively just use 4 eggs.
4. Heat up the water for your hot water treatment and pour into one of the empty glass containers.
5. Quickly put ¼ of the egg whites into the boiling water. Then put ¼ into the alcohol, ¼ into the room temperature water, and the remainder into the final, empty glass container.
6. Observe any immediate changes that occur in terms of egg white color and consistency. If you try stirring the different treatments, rinse your fork between stirs.
7. Wait for 30 minutes. While you're waiting, check out the following protein folding simulations:

   1. Simulation 1: observe where polar and hydrophobic amino acids reside in different proteins (including albumin!)
   2. Simulation 2: observe how protein folding is affected by the hydrophobicity/hydrophilicity of its amino acids
8. Use the fork to inspect the state of the egg whites in each treatment and note how they may have changed over time.
9. Take a photo of your completed experiment (you will have to submit it on the prelab quiz).

Conclusions

So, what happened to the ovalbumin? You will need to answer the following questions on the prelab quiz, and you will discuss them with your partner in lab.

1. For each condition tested, answer the following:
    1. Did you observe protein denaturation?
    2. If yes, what caused the denaturation at the molecular level?
    3. If not, why not?
2. Describe another method for denaturing proteins in egg whites. What causes the denaturation in this case?
3. Can you re-fold proteins that have been denatured? Watch the video below called "How to unboil an egg" and summarize what you learn.

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text. You can view it online here: https://wisc.pb.unizin.org/ biochemistry551online/?p=597

Lab 9: BACKGROUND

As you experienced with albumin, it is not very difficult to denature proteins. In a research setting, we want to denature proteins in a more controlled, quantitative way so that we can gain insight into the thermodynamics of protein unfolding, which can tell us about the stability of the protein. To quantitatively measure protein denaturation of HCAII we will take advantage of two biochemical phenomenon: chemical denaturation and fluorescence spectroscopy.

In the case of boiled egg whites, the proteins were denatured, but then formed larger bond networks that knitted many proteins together, causing the change in color and firmness. In contrast, when denaturing proteins in a research setting, <u>chemical denaturants</u> called chaotropes (ex. urea and guanidinium HCl) are used. These agents essentially outcompete for hydrogen bonding to water, thereby weakening the hydrophobic effect to the point that proteins no longer fold. These agents also prevent the formation of larger aggregates, resulting in fully denatured protein molecules floating freely in solution. In 551 lab, urea will be used to incrementally reduce the fraction of folded HCAII in a sample at room temperature, in such a way that the fraction of folded and unfolded proteins can be measured, enabling thermodynamic analysis of protein stability (Bennion and Daggett, 2003).

To measure how much folded and unfolded protein is in each sample, we will use <u>fluorescence spectroscopy</u>. This is a technique used to measure a fluorescent signal inherent to fluorescent molecules. When a fluorophore absorbs a specified wavelength of light, its electronic state is excited to a higher energy state. The higher state subsequently drops in electronic state, which coincides with emitting photons at a lower energy wavelength (Figure 9.2).
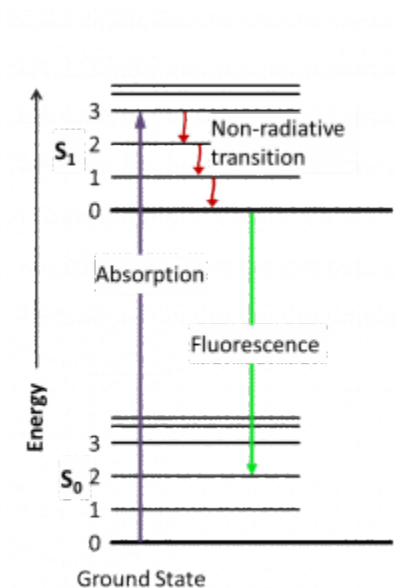
Fig. 9.2. Jablonski diagram illustrating the change in energy state of a fluorescent molecule.

Fluorimeters can detect this emitted wavelength, which can then be used to infer various properties of the fluorophore and of its surroundings. This technique can be applied to study, for example, protein stability, enzymatic activity, and ligand binding. Advantages of this technique include the ability to use a wide range of experimental conditions, the requirement for a small amount of sample, and the ability to detect fast changes.

In this lab, we will exploit the fact that a fluorescent amino acid (tryptophan) emits fluorescence at a different wavelength depending on whether the protein is folded or unfolded. Because tryptophan is already present in protein, we refer to this phenomenon as "intrinsic fluorescence" (to differentiate from a case where a fluorophore has been added chemically to a protein). This intrinsic protein fluorescence due to tryptophan is observed when

the protein is excited at 280nm and its emission is observed at a different wavelength (e.g. 310nm-370nm).

HCAII contains seven tryptophans; you examined them during Lab 1. Tryptophan fluorescence depends on the polarity of the environment surrounding the tryptophan and can therefore indicate changes in the structure of the protein (Muino and Callis, 2009). Upon denaturation, the Trp fluorescence spectrum shifts to longer wavelengths and the fluorescence intensity decreases because the tryptophan residues – which are mainly buried inside the protein – become exposed to the more polar environment of the protein buffer (Fig 9.3). We can use this change to determine how much protein is folded in a sample.
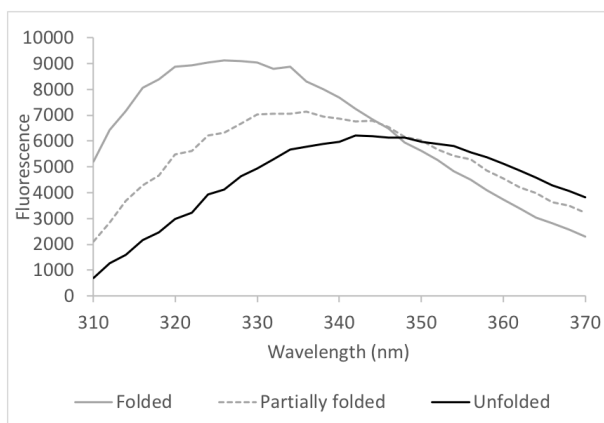
Fig. 9.3. Difference in fluorescence emission spectra of folded, partially folded, and unfolded protein samples. Emission spectra are shown of wt HCAII (2 uM) following excitation at 280 nm. The samples were incubated for one hour at room temperature in 0M (folded), 4.5M (partially folded), or 9M (unfolded) urea.

A simplified view of protein folding assumes that the protein will occupy either a completely folded or unfolded state (this view applies to most smaller, globular proteins, such as HCAII). Exposure to increasing concentrations of a denaturing agent, such as urea, gradually increases the amount of unfolded protein in the sample. This change, in turn, gradually shifts the emission spectrum from one that is typical of a folded protein (grey curve in figure 9.3) to one that is typical of an unfolded protein (black curve). We can plot this change and obtain a sigmoidal denaturation curve, i.e. a curve that estimates the fraction of folded protein (Xf) as a function of concentration of the denaturant.

Denaturation midpoint is defined as "the denaturant concentration (C_M) at which both the folded and unfolded states are equally populated at equilibrium" (i.e. 50% of the protein is folded and 50% is unfolded) (Munoz and Sanchez-Ruiz, 2004). This gives an initial quick estimate of protein stability, since a more stable protein generally requires higher concentrations of denaturant in order to reach the mid-point concentration of unfolding. Note that this is not a strict rule, since different proteins have different dependency between denaturant concentration and stability.

A thermodynamic analysis can be applied to the denaturation curve to determine the Gibbs free energy of unfolding ($\Delta G_{unfolding}$). The $\Delta G_{unfolding}$ is a direct measure of protein stability and will allow you to determine quantitatively whether your mutant protein is as stable as the wild type. This analysis is based on a known linear relationship between the concentration of denaturant and the $\Delta G_{unfolding}$.

References

Bennion BJ and Daggett V. 2003. The molecular basis for the chemical denaturation of proteins by urea. *Proc. Natl. Acad. Sci.*100(9):5142-5147

Muiño PL and Callis PR. 2009. Solvent effects on the fluorescence quenching of tryptophan by amides via electron transfer.

Experimental and computational studies. *J. Phys. Chem. B.* 113(9):2572-7

Munoz V and Sanchez-Ruiz JM. 2004. Exploring protein-folding ensembles: A variable-barrier model for the analysis of equilibrium unfolding experiments. *Proc. Natl. Acad. Sci.* 101:17646-17651

Lab 9: PROCEDURE

Assay overview:
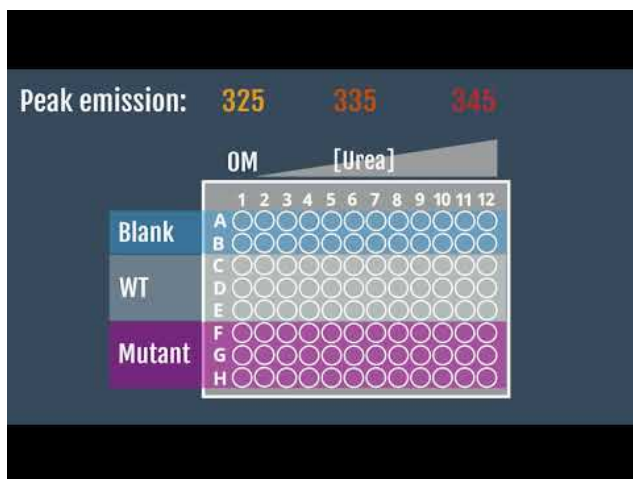
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Plan the experiment

   Note: This section walks through how to plan the experiment. You should review this before coming to lab, but you will have time to complete the calculations with your partner in lab. There will also be a video to walk through this planning process.

- You will be using a 96-well plate, and each well will contain a single test condition. A picture of a 96-well plate is shown

below, along with a drawing that highlights the standard
labeling of rows and columns:

Fig 9.4. A *black 96-well plate.*
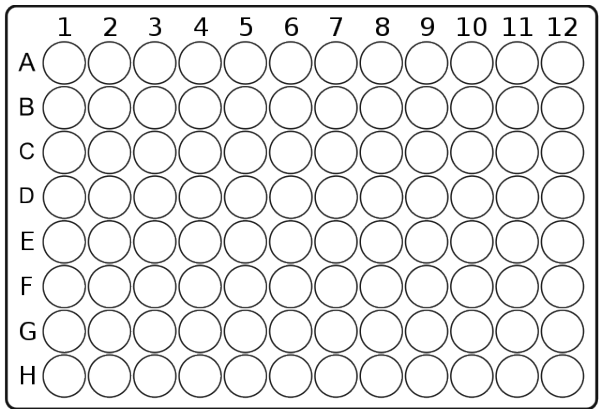
Fig 9.5. A *drawing of a 96-well plate with rows and columns
labeled.*

- Create a detailed chart like the one below to keep track of the volumes of solution you are adding to each well (again, this will be completed in lab).

Plate Row	Plate Column	Protein	Protein Stock Conc	Protein Volume	Urea stock Conc	Urea stock Volume	Protein Buffer	Final Urea Conc
A–B	1	Blank	0	0	0	0	100 μL	0
C–E	1	Wt	5 μM	10 μL	0	0	90 μL	0
…								
A–B	2	Blank	0	0	0.56M	90 μL	10 μL	0.5 M
C–E	2	Wt	5 μM	10 μL	0.56M	90 μL	0	0.5 M
…								

- Organize your experiment to minimize pipetting: add the same concentration of urea in each column and add equal volumes of the same component (i.e. buffer blank or protein) to each row.
- Suggested rows: A and B – Control, C to E – wild type, and F to H – mutant HCAII
- Plan for a final volume of 100 μL per well.
- Choose final protein concentrations. The final concentration of protein in each well should be 0.8 – 2.0 μM. The higher the protein concentration, the better your data will be; however, you still need to have protein for future labs. If you have at least 1 mL protein at a concentration of 50 μM or higher (or the equivalent), use a final protein concentration of 2.0 μM. If you do not have that much protein, use a lower concentration. Talk to the teaching staff if you are not sure what to do. Note that you can use different concentrations of protein for wild type vs mutant, if necessary.
  - Note: in a virtual setting, we don't have to worry about volume of available protein, so go ahead and plan for 2.0

µM final protein concentration.

- Once you've decided on final protein concentrations, plan to make 500 µL stocks that are 10x concentrated. For example, for a final protein concentration of 2.0 µM, you will make a 20 µM stock.

  ◦ Refer back to lab 8 for the concentration of each of your protein stocks.

- Using a multichannel pipette, add 10 µL of this stock of wild type or mutant, or protein buffer, to the designated rows.
- The remaining volume to 100 µL will be different concentrations of urea solution. You should test 11 concentrations of urea between 0.5M and 9M (plus 0M). Figure out the volume of urea you can add to each well, and then what urea stock concentrations you want to use.
- Test each urea concentration in triplicate.

Prepare solutions

- You will be provided with 10 mL of 10M urea in protein buffer. Use this stock urea solution to make several 1 mL dilutions in Protein Buffer, creating the stock solutions you planned in your chart. Again, you should test 11 concentrations between 0.5M and 9M.
- Create the stocks of wild type and mutant protein, as you planned above. For most people, these will be 500 µL stocks at 20 µM.

Assemble the experiment

- Begin by using a multichannel pipette to add 10 µL protein buffer to rows A and B, and 10 µL protein stock to the remaining rows (wild type in C-E and mutant in F-H).
- Next, use the mulitchannel to pipette the appropriate urea

solution into each column. Conduct pipetting with increasing urea concentrations and rinse/dry the reservoir prior to adding the next stock.

- Be careful not to create bubbles when pipetting.
- Cover your plate with labeled plastic wrap and incubate at room temperature for 60-90 minutes.

Assembling an unfolding experiment in a 96-well plate

- The following video demonstrates how to use multichannel pipettes to set up a 96-well plate:

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Read the fluorescence

- Use the plate reader to excite at 280nm and measure emission

from 310nm-370nm in each well.

- The following video shows how to operate the plate reader:

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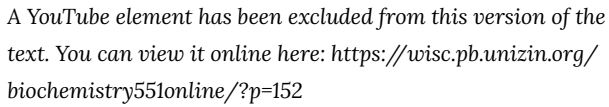
Lab 9: LAB ASSIGNMENTS

PRE-LAB QUIZ

Question 1

Upload a photo of your completed at-home experiment.

Question 2

For each condition you tested in the at-home experiment, did you observe protein denaturation? If yes, what caused the denaturation at the molecular level? If no, why not?

Question 3

Describe another method for denaturing proteins in egg whites. What causes the denaturation in this case?

Question 4

Can you re-fold proteins that have been denatured? Summarize what you learned from the video "how to unboil an egg."

Question 5

What experiment with HCAII are we simulating this week? What is the purpose of that experiment? (Provide 2-3 sentences).

IN-LAB ACTIVITIES

- After joining BBCU, you and your partner will join a breakout room.
- Your TAs will provide you a link to a Google doc. You and your partner will work together to complete it. You will:
    - Discuss the at-home experiment and compare/contrast with the HCAII experiment
    - Answer several questions about the HCAII experiment procedure
    - Analyze the tryptophan residues in HCAII and how they affect the experiment

- ◦ Create a detailed plan for setting up the HCAII experiment. There will be a video that helps walk through the experimental planning process.
  - ◦ Meet with your TAs to discuss. They will provide you with the results of the experiment from the plate reader (it will be an Excel file).
- You are welcome to use any remaining in-lab time to work on the data analysis.
- Throughout the lab time, TAs can be available for questions, and will circulate to check in on each group at least once.
- Complete the lab/partner/self evaluation Google form.
- Get a sharable link to your Google doc and submit to Canvas.

Lab 9: DATA ANALYSIS

Your data will be provided by your TAs after you complete the in-lab activities.

  During data analysis you will perform these major steps:

- Blank and normalize your fluorescence data
- Fit the data directly in GraphPad Prism to determine:
    - the $\Delta G_{unfolding}$
    - the C_M
- Manually manipulate the fluorescence data to create two plots:
    - fraction of folded protein (Xf) vs. [urea]
    - $\Delta G_{unfolding}$ vs. [urea]

Part 1: Blank and normalize your fluorescence data

- The following video helps get you oriented to your data and demonstrates how to average your blanks and subtract them from your experimental values. NOTE: For all videos, you should be able to actually read the numbers being shown. You may need to view full-screen, and/or manually increase the video quality. You can also turn on closed captioning for all videos.

    - NOTE: The urea concentrations tested are included in the "Setup" page of the data file. These may not be the same concentrations you were planning to use! *Be sure to take the urea concentrations from the data file, not from your plan.*
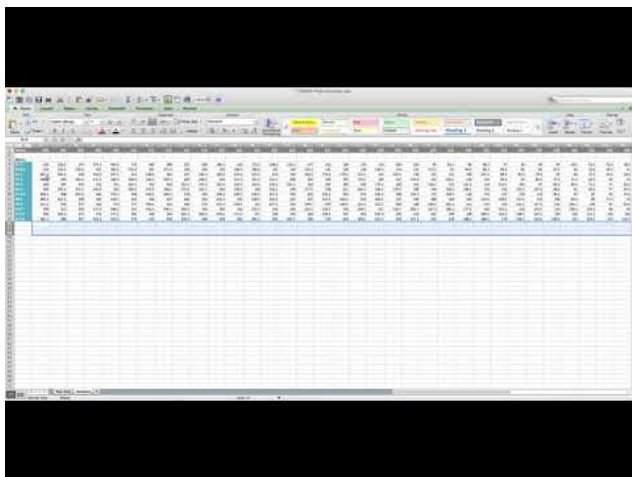
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- For each protein (wt and mutant), create plots that overlay the emission spectrum of your protein at each urea concentration, as demonstrated in the following video.
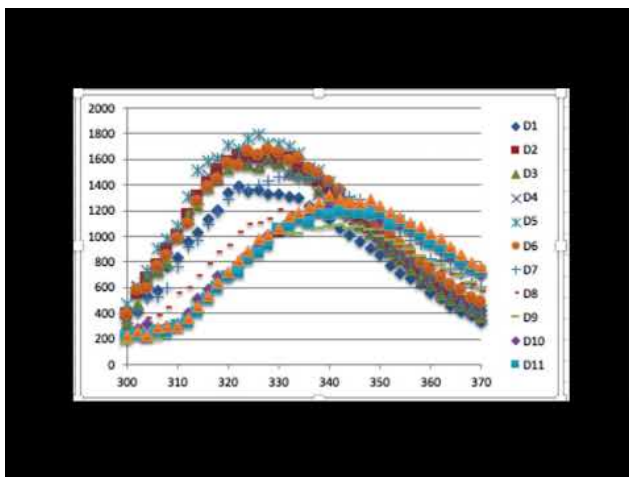
A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/biochemistry551online/?p=165

- The plate reader records values in relative fluorescence units (RFU), which depend on the instrument settings. All samples should have the same protein concentration, but pipetting errors can cause slight (or large) variances. Therefore, it is helpful to normalize the data, using the fluorescence at a wavelength that is not very affected by the state of the protein (340 nm) as a reference point. First, look at the plots you created in step 2 and be sure the folded curves have a peak around 324 nm, while the unfolded curves shift to higher wavelengths. If not, talk to the teaching staff before proceeding with the data analysis.
- Normalize the data by calculating the ratio between two emissions: 324/340. 324 nm is the point where you should observe a peak in the folded protein curves. 340 nm is the approximate point where all the curves come closest to

intersecting.
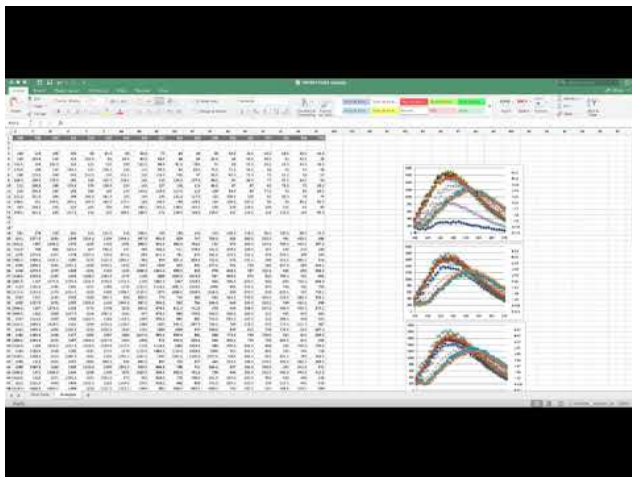
A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/ biochemistry551online/?p=165

- Discard data from any wells that appear to have no (or too little) protein. These would appear as approximately a straight line in the fluorescence vs wavelength plot, and/or give a negative or otherwise unreasonable 324/340 ratio. DO NOT use data from these wells in any of the remaining analyses.

Before continuing, you must download the free 30-day trial of GraphPad Prism: http://graphpad.com

> Note that you should only have one partner download the Prism software now. The 30-day trial may end before you finish your 551 assignments, in which case the other partner could then download the trial.

Part 2: Fit the data directly in Prism to determine the $\Delta G_{unfolding}$ and C_M

- This video walks through the basics of fitting data in Prism. Download the sample data file so that you can follow along with the video. Sample file can be downloaded from Canvas here: Sample Lab 9 Data Analysis File

  - PLEASE NOTE: This video was made using Prism 7 for Mac. Your version may differ slightly.
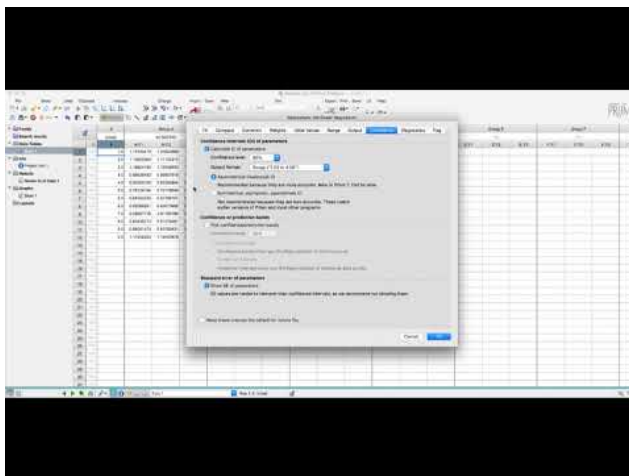
A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/biochemistry551online/?p=165

- The first thing you need to do is download the correct fitting equation from the sample data file. Unfortunately you can't directly download a Prism equation, but this is a workaround:
  - Open the sample file and navigate to the "Nonlinear fit of…" page under the "Results" heading.
  - Look for the cell with a blue link "Nonlin fit" in the top left corner of the sheet. Click on this link.
  - Without changing any parameters, click "OK"
  - Your computer has now run the analysis and downloaded the correct fitting equation into your Prism. You can now close the sample data file.
- Open a new project file in Prism and select "Enter 3 replicate values in side-by-side subcolumns," hit "Create"

- Paste the urea concentrations used on the X column and the calculated 324/340 emission ratio values for wild type on the "Group A" columns and for mutant on the "Group B" columns. Rename the titles.
- Analyze the data using the "Analyze" button. Select "Nonlinear regression (curve fit)" under "XY analyses"

- Under both "Recently used" and "User-defined equations" you should see the equation from the sample data: "deltaG fit Prism9". Select this equation but DO NOT click "OK" yet.

Mathematical Description of the ΔG$_{unfolding}$ Fit

Determining the ΔG$_{unfolding}$ depends on the linear relationship between [urea] and deltaG.:
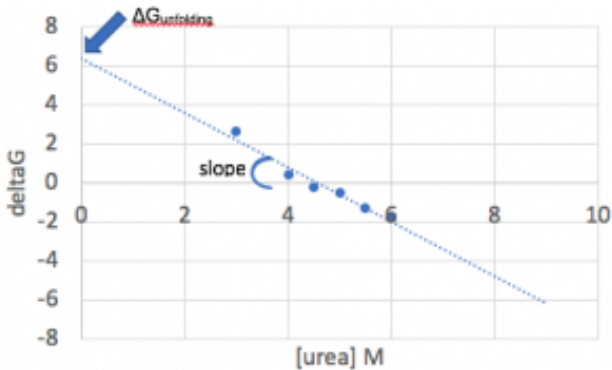
As discussed in lecture, there is a logarithmic relationship between the ΔG$_{unfolding}$ and the fraction folded (Xf), meaning a plot of [urea] vs Xf is sigmoidal:

$$\Delta G_{unfolding} = -RT \ln K_{eq}$$

and K$_{eq}$ is related to Xf because:

$$K_{eq} = [U] / [F]$$
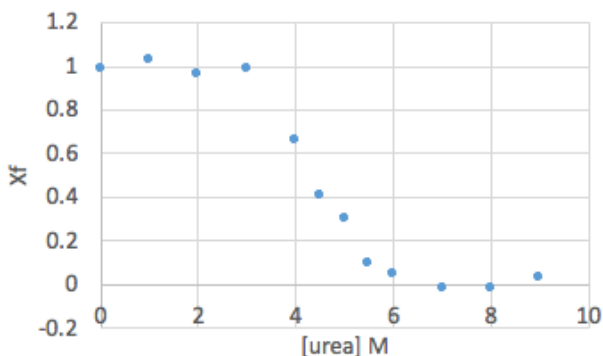
Going back one step further, the fluorescence data you just entered into Prism is also sigmoidal, but is spread between a "top" value (fluorescence representing fully folded protein) and "bottom" (fully unfolded) rather than between 1 and 0.
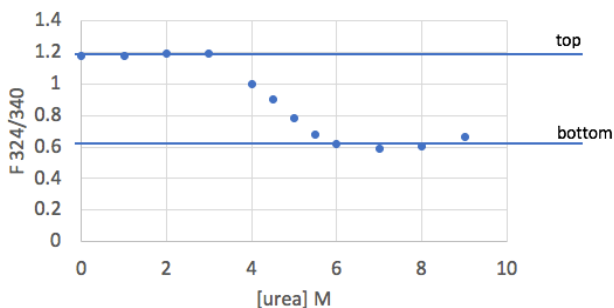
Since we know the mathematical relationships relating these plots, we can combine them to derive a function that relates the normalized fluorescence directly to $\Delta G_{unfolding}$. This equation is shown below, and is what you will fit in Prism:

$$y = bottom + \frac{top - bottom}{1 + e^{\frac{ax + dGo}{-RT}}}$$

where x is [urea],

bottom and *top* are fits of the bottom and top baselines for the sigmoidal curve,

a is the slope of the linear relationship between [urea] and deltaG,

0.59 is used as the value of RT, and

dGo is the $\Delta G_{unfolding}$ value to be determined from the fit.

- Click on the "Compare" tab and select "Do the best-fit values of selected unshared parameters differ between data sets?" Then choose "dGo". This will force Prism to do a t-test to compare your wild type and mutant results (see appendix for more information on statistics).

- **Error reporting has changed slightly in the new version of Prism.** Click on the "Confidence" tab. In older versions, you were able to check box a for "Show SE of parameters." In order to select this option in the newest Prism, you have to first select "Symmetrical (asymptotic) approximate CI" then select "Show SE of parameters." If you are not able to show standard error in your version of Prism, that is ok. You can check "Calculate CI of parameters" using the option "Asymmetrical CI" instead.

  - ◦ Note: Prism doesn't recommend fitting this way, but Standard Error values are useful for us. Based on a few initial trials, this type of CI calculation only differs very slightly from the recommended type.

- Now press OK.
- The program will run a fit of your data according to the equation as described above. You are interested in the parameters "dGo" and "Cm" in the output. You will also see 95% confidence values and/or standard error values for the dGo and Cm; you should report those.
- Prism will also tell you whether or not the dGo ($\Delta G_{unfolding}$) is statistically different for the two data sets, including a p-value. You should also report this.

  - Note that what Prism runs this analysis, it does it in two ways: allowing the values for wt and mutant to be different, and forcing them to be the same. Even if Prism determines that the p-value is high and the values are therefore not statistically significant, you should report the values from the analysis where the two were allowed to be different (i.e. don't report the exact same value for wt and mutant).
- Under "Best-fit values," you are also given a "Top" and "Bottom." Note each of these values for both wt and mutant – you will need them in the next step of the data analysis.

Example output (note that depending on your version of Prism, there may or may not be standard error reported):

Nonlin fit

		A	B	C	D	E
		wt 324/340	T199S 324/340	Global (shared)	Title	Tit
		Y	Y	Y	Y	Y
1	Comparison of Fits					
2	Null hypothesis			dGo same for all data sets		
3	Alternative hypothesis			dGo different for each data set		
4	P value			0.0015		
5	Conclusion (alpha = 0.05)			Reject null hypothesis		
6	Preferred model			dGo different for each data set		
7	F (DFn, DFd)			11.07 (1, 64)		
8						
9	dGo different for each data set					
10	Best-fit values					
11	bottom	0.6216	0.62			
12	top	1.192	1.221			
13	a	1.155	0.8852			
14	dGo	5.101	3.797			
15	Cm	4.418	4.289			
16	Std. Error					
17	bottom	0.006093	0.008588			
18	top	0.006214	0.009251			
19	a	0.06929	0.06535			
20	dGo	0.3117	0.2918			
21	Cm	0.03224	0.05196			
22	95% CI (profile likelihood)					
23	bottom	0.6092 to 0.6337	0.6053 to 0.64			
24	top	1.18 to 1.205	1.203 to 1.24			
25	a	1.033 to 1.294	0.7745 to 1.014			
26	dGo	4.553 to 5.727	3.305 to 4.364			
27	Goodness of Fit					
28	Degrees of Freedom	32	32			
29	R square	0.9945	0.9907			
30	Absolute Sum of Squares	0.01157	0.01954			
31	Sy.x	0.01901	0.02471			

Annotations:
- This tells you that the dG values are significantly different
- Top and Bottom
- dG and Cm values
- Std Err on dG and Cm values

Nonlin fit of Data 1 — Table of results

Part 3: Manually manipulate the fluorescence data and create plots of interest

The following analyses can all be completed in Excel. You will manually approximate the same fit that you just ran in Prism, by calculating the Xf and then the $\Delta G_{unfolding}$ values for your data set. Doing so will help you assess the quality of your data, and allow you to create useful plots to include in your lab report.

- Return to your fluorescence ratios, and further normalize this data by calculating the fraction of folded protein Xf at each urea concentration. This will essentially spread the data between 1 (fully folded, which corresponds to the points near 0M urea) and 0 (fully unfolded, which corresponds to the points at very high urea concentrations).

- Calculate *Xf* for each data point, using the experimental emission ratio at each urea concentration. Do this calculation separately for wt and mutant, as they probably have different top and bottom values.

Xf = (Experimental fluorescence ratio − Bottom) / (Top − Bottom)

- Plot *Xf* against urea concentration, including both wt and mutant on the same graph.

Sample data:

val	
124/340	XT
1.178492	0.923701
1.21991	1.00195
1.216472	0.99705
1.214398	0.993346
1.133912	0.837605
0.988657	0.553758
0.900862	0.387528
0.803183	0.198885
0.705308	0.009865
0.733953	0.065185
0.671084	-0.05627
1.190203	0.946317
1.166788	0.901597
1.223617	1.010848
1.197479	0.960369
1.244718	1.0516
1.170955	0.909145
0.985991	0.551934
0.933647	0.450844
0.791045	0.175444
0.703552	0.006476
0.696918	-0.05634
0.672624	-0.05326
1.147615	0.864369
1.240079	1.042641
1.276833	1.113599
1.214398	0.993346
1.245657	1.053412
1.13787	0.845248
1.011281	0.600776
0.925664	0.435428
0.787234	0.168084
0.751543	0.062465
0.685782	-0.02785
0.689613	-0.02345
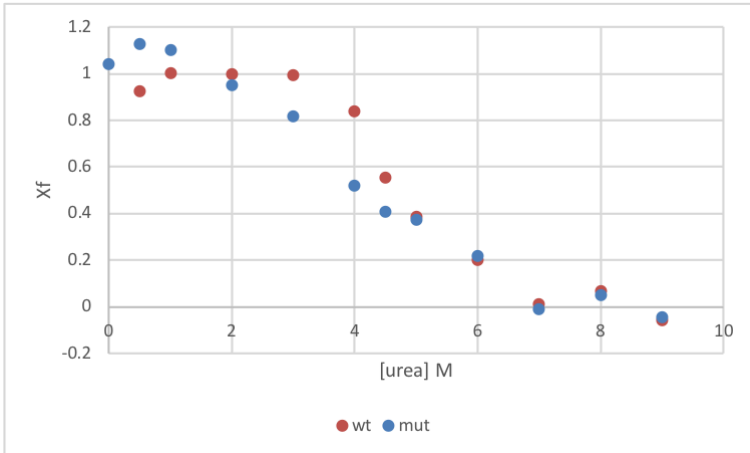
Sample Xf plot

This is an important place to pause and take a thoughtful look at your data. Do your curves have the expected shape? Are they spread between 1 and 0? Imagine a protein that is completely unstable – what would the *Xf* plot look like for such a protein? Do you see evidence of that in your own plot? Below are two example plots that contain problematic data. Look out for similar problems in your own data.

An interactive or media element has been excluded from this version of the text. You can view it online here: *https://wisc.pb.unizin.org/biochemistry551online/?p=165*

- The next step is to determine the $\Delta G_{unfolding}$ from the fraction folded (Xf) at each urea concentration, using the following equations:

$$Keq = (Xf)^{-1} - 1$$
$$\Delta G_{unfolding} = -RT \ln(Keq)$$

 Use RT values to give units of kcal/mol. Omit any data points where $Keq<0$.

- Create a plot of the $\Delta G_{unfolding}$ vs [urea]. Notice that increasing the urea concentration favors unfolding (reduction in $\Delta G_{unfolding}$) and that $\Delta G_{unfolding}$ becomes zero and eventually negative, as shown below.
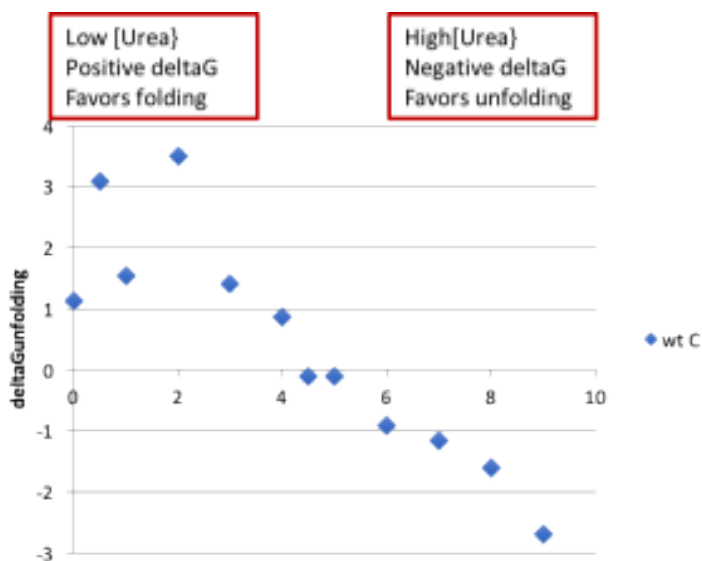
There are more important questions to ask yourself here! Is your curve linear in the expected region? Based on your plot, what urea concentration is the apparent C_M? Does this match the value Prism determined?

- With an ideal data set, you would be able to fit the linear region of this plot, and the y-intercept of that fit line would match the $\Delta G_{unfolding}$ value from the Prism fit. However, in practice, large amounts of error where Xf is either small or large make this sort of fit unreliable.

Lab 9 MINI LAB REPORT

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Reports

Methods

- Describe all the experimental conditions tested (using concentrations, not volumes)
- Describe all the data analysis and equations you used to determine the values you report in the Results

Results

- For both wild type and mutant, show one representative set of fluorescence emission curves (wavelength on the x-axis, fluorescence on the y-axis, one curve per [urea]).
- Show the Xf and $\Delta G_{unfolding}$ plots you generated in Excel. Include error bars.

    - Note: error bars in Excel are actually kind of hard. Here is a link to some documentation that may help. You will want to do custom error bars, and be sure to use only vertical bars (not horizontal): link to Excel error bars help
    - Compare the shapes of these curves. Are the wt and mutant the same or can you observe some differences?
- Use a table to report the C_M and $\Delta G_{unfolding}$ (**as determined in Prism**) for both wt and mutant protein. Be sure to include measurements of error, and state clearly what measurements you are using.
- Report statistical significance and p-values where possible.

Discussion

- How do your C_M and $\Delta G_{unfolding}$ values compare to published values? What are some reasons why your values might differ

from what has been published?
- Point out any abnormalities in the data and provide possible explanations.
- What did you learn about the effect of your mutation on the stability of HCAII?

LAB 10: INVESTIGATION OF WILD TYPE AND MUTANT HCAII ENZYME ACTIVITY

Lab 10: Investigation of the wild type and mutant HCAII enzyme activity

Before lab, you will complete a short at-home experiment on enzyme kinetics from Carolina Distance Learning. In lab, you and your partner will discuss this experiment, then work on the HCAII experiment.

Lab 10: AT-HOME EXPERIMENT

This week you will complete the Carolina Distance Learning at-home lab "Enzyme Catalysis." You should have received a hard copy of the background and lab procedure with your lab kit. If not, please refer to the Canvas announcement on lab 10, which includes a pdf of the procedure as an attachment.

This lab has two parts, labeled Activity 1 and Activity 2. You and your partner can split the activities up so that you each only need to do one (though you can do both yourself if you want to!).

Whichever activity you are doing, everyone should review the first six pages of the instruction manual (Background, Safety, and Preparation). Then, complete your activity. Be sure to fill out the provided data table. Then plot your results using graphing software, such as Excel.

For this week's pre-lab quiz, you will need to submit:

- a photo of your completed data table, and
- a plot of your results

When you meet with your partner during lab, you will review each other's results and answer a few questions about these experiments.

Lab 10: BACKGROUND

Enzyme Kinetics

The standard model to describe enzyme kinetics was first put forth by Michealis and Menten to describe initial velocities as a hyperbolic function of enzyme and substrate concentrations (Eqn 1). This is known as the Michealis-Menten equation and describes enzyme catalyzed reactions as shown (Eqn 2).

(1) $v_o = k_{cat}[E] \frac{[S]}{[S]+K_M}$

(2) $E + S \rightleftharpoons ES \rightarrow E + P$

Two important parameters k_{cat} and K_M are used in the Michealis-Menton equation. The first, k_{cat}, is the first order rate constant of the conversion from substrate to product. When the enzyme is saturated with substrate, the initial velocity will be directly proportional to the enzyme concentration ([E]) and k_{cat}. This is a special case and the initial velocity under this condition is called the maximal velocity (V_{max}) (Eqn 3). This is a theoretical velocity which cost and solubility often prevent from being observed experimentally. Notice, V_{max} is a function of enzyme concentration, which can make comparing this value across different experiments problematic.

(3) $V_{max} = k_{cat}[E]$

In contrast, at lower substrate concentrations, the initial velocity cannot be expressed as simply proportional to [E] and k_{cat}; the Michealis-Menton constant (K_M) must also be included to account for substrate binding. The Michealis-Menton constant is often thought of as the affinity of the substrate for the active site; however, it also includes k_{cat} so it is not synonymous with the equilibrium dissociation constant (K_d) even though they share the same units (Eqn 4). The Michealis-Menton constant also has a special significance. It is the concentration of substrate at which the initial velocity is half of V_{max}.

(4) $K_M = \frac{k_{off} + k_{cat}}{k_{on}}$

Another interesting condition to consider is when the substrate concentration is much lower than the Micheails-Menton constant ($K_M/[S] > 20$). The initial velocity exhibits a second-order relation to the enzyme and substrate concentration, first-order to both enzyme and substrate concentration. The Micheails-Menton can be rewritten in a linear form to describe the region of initial velocity plotted as a function of substrate concentration near the origin (Eqn 5).

(5) $v_o = \frac{k_{cat}}{K_M}[E][S]$

Finally, the value of k_{cat}/K_M has a special significance in enzymology. It is the second order rate constant (units of $M^{-1}s^{-1}$) to describe enzyme-catalyzed reactions, and is referred to as the catalytic efficiency. Catalytic efficiency is the most commonly used criteria to judge enzymes as catalysts. Enzyme-catalyzed reactions have an inherent speed limit imposed by diffusion in aqueous solutions. The limit of diffusion is ~$10^{9\text{-}10}$ $M^{-1}s^{-1}$ which catalytic efficiencies of enzymes cannot typically exceed. HCAII is among the most efficient enzymes known and is considered a diffusion-limited catalyst (Khalifah, 1973).

Kinetic Assays for HCAII

While the *in vivo* function of HCAII is to hydrolyze CO_2, the enzyme also has an esterase function that allows it to catalyze the hydrolysis of p-nitrophenyl acetate (PNPA) to form PNP.
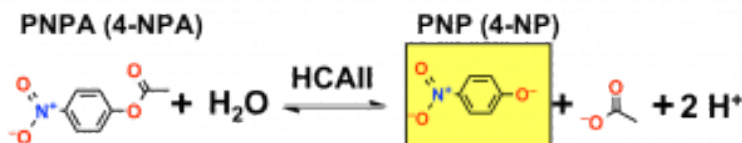
Fig. 10.1. Esterase activity of HCAII can be measured via the conversion of PNPA to PNP, which is yellow.

Although the hydrolysis of PNPA is not the biological function of HCAII, the esterase activity is much easier to assay than the hydrolysis activity so this is the assay we will use to study the kinetic parameters of the enzyme. Since the esterase activity utilizes the same active site as the hydrolysis of CO_2, we will use this assay to investigate the effect of the mutations on HCAII. The reaction will be done in protein buffer (PB: 50 mM Tris, pH 8.5, 0.1 M KSO_4) causing the PNP released to adopt its yellow-colored ionized form, which we can measure at 405 nm with the spectrophotometer.

In an aqueous buffer, PNPA will also gradually undergo spontaneous hydrolysis, meaning some yellowing will appear over time even in the absence of any enzymatic reaction. For this reason, it is essential to include a blank reaction. The blank should contain buffer and PNPA at the same concentration that is being tested, but no enzyme. The absorbance at 405 nm of this blank can then be subtracted from the sample to yield the amount of hydrolysis that is due to any enzymatic reaction.

We will conduct a Michaelis-Menten kinetics experiment to compare the esterase activity of mutant HCAII to that of wild type. By using a constant concentration of enzyme and measuring esterase activity across a range of substrate concentrations, you will determine a number of kinetic parameters.

An interactive or media element has been excluded from this version of the text. You can view it online here:
https://wisc.pb.unizin.org/biochemistry551online/?p=170

Reference

Khalifah RG. (1973). Carbon dioxide hydration activity of carbonic

anhydrase: paradoxical consequences of the unusually rapid catalysis. *Proc. Nat. Acad. Sci.* USA 70 (7): 1986-1989.

Lab 10: PROCEDURE

Assay overview and technical considerations

Here is a link to two videos created by one TAs that provide an overview of the HCAII kinetics assay:

Lab 10 assay overview video

Lab 10 assay set up details video

📄 *An interactive or media element has been excluded from this version of the text. You can view it online here:*
https://wisc.pb.unizin.org/biochemistry551online/?p=171

Assembling and running a kinetics assay

This video demonstrates how to:

- set up a kinetics assay in a cuvette
- run the spectrophotometer
- find your data when the read is finished
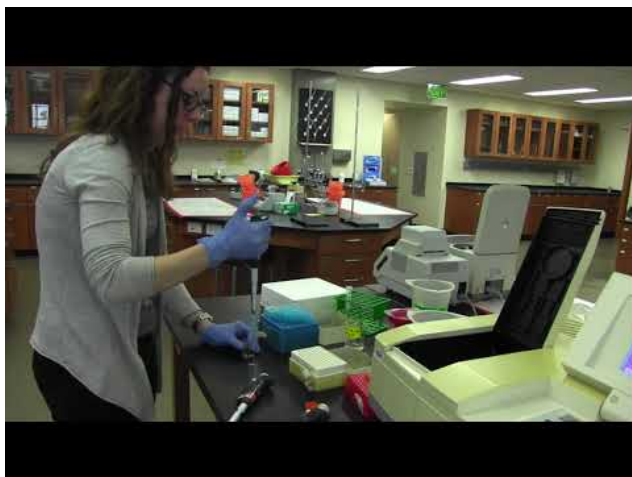
A YouTube element has been excluded from this version of the text. You can view it online here: https://wisc.pb.unizin.org/biochemistry551online/?p=171

Setting up the spectrophotometer

- Turn on the Shimazdu Spectrophotometers and allow it to warm up (this takes about 15 min.)
- This is a dual-beam spectrophotometer. It has two slots for cuvettes so that it can measure sample and blank at the same time.
- Set the following parameters on the Shimazdu Spectrophotometers:
  - From the main menu, select Kinetics (# 4)
  - From the next menu, select 1 cell kinetics/λ (#1)
  - Measure time = 40 seconds
  - Next hit the "Goto WL" button. Then type in 405. The

spectrophotometer should change the wavelength to 405 nm.

- Note there are also spectrophotometer instructions in the Appendix (link opens in a new tab): Appendix 5: Operation of the Shimadzu spectrophotometers

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Pilot reactions

The purpose of a pilot reaction to is be sure that your experiment is working (i.e. you are able to measure something), and that your experimental conditions result in a signal within the range that the spectrophotometer can reliably read. This has been somewhat optimized over several years of 551 labs, but the exact optimal conditions may vary a bit depending on your protein sample and your mutation. You will test one condition to start, and if the resulting signal falls within a good detectable starting range, you can proceed with the experiment using those conditions. If, however, the signal is too high, you will need to decrease the protein concentration used. Conversely, if the signal is too low, you will need to increase the protein concentration used.

- The pilot reaction should contain:
    - A final protein concentration of 0.1 µM
    - 20 µL of 50 mM PNPA (for a final concentration of 1 mM)
    - Buffer to a total of 1 mL
- We recommend assembling the reaction directly in the cuvette (this also saves plastic). Add buffer first, then protein. Always add PNPA last, when you are ready to measure.
- Once the PNPA has been added to the reaction you will need to act quickly. Make sure your reaction is well mixed before measuring – mix by gently pipetting up and down with a p1000.
- Your reaction should be in the front slot of the spec and your blank should be in the back.

- ◦ The blank should contain protein buffer and the [PNPA] you are testing, but no protein.
- Once both samples are in the spectrophotometer hit "START" immediately.
- Once the reaction has finished, look at the graph. If the line runs off the top of the graph (i.e. the absorbance went above 2) within the first 10 seconds, the reaction rate is too fast and you need to reduce the amount of enzyme you are using. Next, press the "Datalist" button to see the slope of your reaction. Your slope should be around 0.1. If it is not, increase or decrease the amount of enzyme you are using. (If you are in the correct range, you can repeat the assay twice more and use this as one of the substrate concentrations in your data set.)
- **Important notes:**
  - ◦ If you do not have enough enzyme to do the remaining assays, reduce the amount of enzyme you are using as much as possible. Alternatively, you may have to collect fewer data points. Discuss with a TA if you are unsure what to do.
  - ◦ Be sure to write down the slope of the line (i.e. $\Delta A/min$) – you will use this data to calculate the reaction rate. The spectrophotometer will not save your data.
  - ◦ It is possible that you will need to use different protein concentrations for wt vs. mutant in this assay. Pilot experiments should be conducted separately for wt and mutant.

Full Experiment

- Once you have determined the amount of enzyme you will use for each reaction, plan the rest of the assays.
- Since you are going to be calculating the K_M and V_{max} for your

mutant and wild-type enzymes, you will want to test a range of PNPA concentrations above and below the K_M. In lab you will work with your partner to find the published K_M. You can then select 8 substrate concentrations to test.

- ◦ Note that for technical reasons <u>you cannot test [PNPA] >5 mM</u>.
- ◦ You will need a separate blank for each [PNPA].
- ◦ You will run each [PNPA] in triplicate. It is possible to use the same blank for all three replicates as long as the PNPA in the blank has not hydrolyzed too much (i.e. turned yellow); however, it is challenging to move quickly enough (especially at high [PNPA]), and you may get cleaner data by making a fresh blank for each measurement.

- **At the end of lab, SAVE YOUR PROTEIN SAMPLES AT 4 ºC**.

Lab 10: LAB ASSIGNMENTS

PRELAB QUIZ

Question 1

Upload a photo of your completed data table

Question 2

Upload a photo or file containing a plot of your data

Question 3

What experiment with HCAII are we simulating this week? What is the purpose of that experiment? (Provide 2-3 sentences).

IN-LAB ACTIVITIES

- After joining BBCU, you and your partner will join a breakout room.
- Your TAs will provide you a link to a Google doc. You and your partner will work together to complete it. You will:
    - Discuss the at-home experiments and connect with the HCAII experiment
    - Plan the HCAII esterase pilot reaction
    - Complete a simulation of the pilot reaction, found below. It is a branching case scenario that walks through the process of a pilot assay for wt protein. It will present you with data and then ask you to choose what to do next. Once you and your partner successfully work through the simulation, you will figure out the optimal protein concentration to use in the assay. In the lab 10 in-lab assignment doc, you will be asked what protein concentration you found was optimal.

- ◦ Find the published K_M of HCAII for PNPA
- ◦ Plan the full HCAII esterase experiment. There will be a brief video to help walk you through the process.
- ◦ Meet with your TAs to discuss. They will provide you with the results of the experiment from the plate reader (it will be an Excel file).
- You are welcome to use any remaining in-lab time to work on the data analysis.
- Throughout the lab time, TAs can be available for questions, and will circulate to check in on each group at least once.
- Complete the lab/partner/self evaluation Google form.
- Get a sharable link to your Google doc and submit it to Canvas.

Lab 10: DATA ANALYSIS

Your data will be provided by your TAs after you complete the in-lab activities.

Complete the following steps to calculate V_{max}, K_M, k_{cat} and k_{cat}/K_M for wt and mutant HCAII.

- Make a table of of initial velocities (in μM product/time) and substrate concentrations. To calculate μM product/time, you need to know: The molar absorption coefficient of PNP is 1.73 x $10^4\,M^{-1}cm^{-1}$ at 405 nm, and the effective path length (*b*) of the spectrophotometer cuvettes is 1.0 cm.

Michaelis-Menten Fit:

> **NOTE:** The fit described here is very similar to the one from Lab 9. If you need more guidance within Prism than what is written here, refer back to the data analysis from Lab 9.

- Open GraphPad Prism and select "Enter 3 replicate values in side-by-side subcolumns," hit "Create"
- Paste your data in. You can put replicates in adjacent columns.
- Select "Analyze"
    - Under "XY analyses" select "Nonlinear regression (curve fit)" Press OK
    - Under "Enzyme kinetics – Velocity as a Function of Substrate" choose "Michaelis-Menten."
        - Go into the "Compare" tab to have Prism compare the K_M for wild type versus mutant.

- Go to the "Confidence" tab and select "Symmetrical (asymptotic) approximate CI" then select "Show SE of parameters." Press OK.
- You should now have values for V_{max} and K_M with standard errors, a determination of statistical significance for the K_M including a p-value, and a plot showing the fit.
  - Also make note of the "Degrees of freedom" provided for both wt and mutant. You will need this number in a future analysis.
- Note that you also now have a plot of V_o vs [S]. You can play with the plot in Prism to make it look nice, just as you would in Excel: label axes, remove title, add error bars, etc.

k_{cat} Calculation:

- Calculate k_{cat} for your wt and mutant enzymes based on your Michaelis-Menten fit using Equation 3. Pay attention to units: the units of k_{cat} are sec^{-1}.

$$(3) \; V_{max} = k_{cat}[E]$$

- You are manipulating values that all have their own associated error. You will need to report standard error values for k_{cat} using the principles of propagation of error. Refer to the Appendix for additional help (link opens in a new tab): Appendix 2: Statistical methods used in 551 data analyses
- Once you have determined the standard error for k_{cat} for both wild type and mutant, you can utilize a different type of Prism analysis to determine p-values.
- Open Prism and select from the far left menu "Column" (by contrast, the default is "XY")
- Select "Enter and plot error values already calculated elsewhere" and from the dropdown menu choose "Mean, SEM, N"
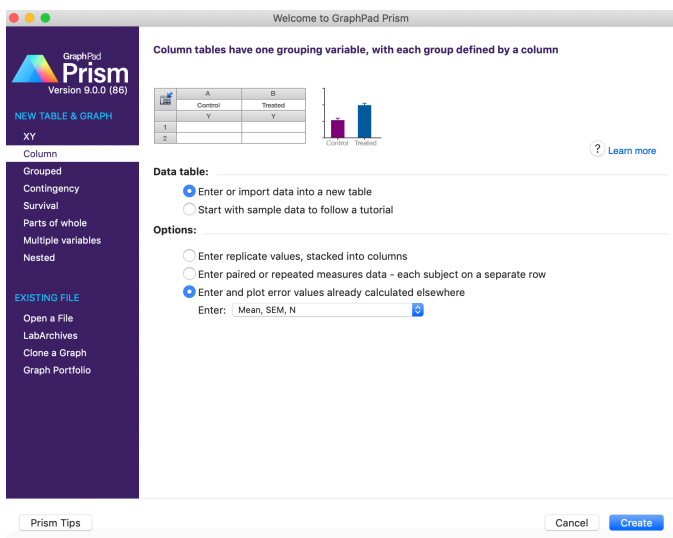
- Enter the following for wt and mutant:
  - Mean: the k_{cat} values you determined
  - SEM: the standard error of the k_{cat} values (determined by propagation of error)
  - N: the degrees of freedom (determined by Prism above) + 1
- Go to "Analyze" and select, under Column analyses, "t tests (and nonparametric tests." Press OK. (Leave all the parameters and options as the default.)
- The Results sheet should provide a p-value.

k_{cat} /K_M Calculation:

- Now take the k_{cat} values you calculated and divide them by K_M to determine the catalytic efficiency.
- You will again need to use principles of propagation of error to determine standard errors for each k_{cat} /K_M value.
- Repeat the Prism analysis above to determine a p-value for k_{cat} /K_M.

Lab 10: MINI LAB REPORT

Refer to the general guidelines for Mini Lab Reports on Canvas, found here (link opens in a new tab): Mini Lab Reports

Methods

As always, describe the assays in enough detail that someone with scientific training could repeat it. Include the assay conditions and what you were assaying. Describe how the assay was set up including a description of the blank. State the **concentrations** of PNPA you used. State how many replicates were done.

Note that you **do not** need to explicitly describe the pilot experiments. Generally methods sections don't include details on how an assay was optimized, or any troubleshooting that may have been done. Simply report what was actually done to generate the data you are showing in the report.

State how you calculated all the values you need to report in the results section and be sure to include equations.

Results

- Plot of V_0 vs. [S] from Prism. Be sure to label your axes.
- In the text, state the results of the fit including the V_{max} and K_M. Include error measurements.
- Table of relevant values, including k_{cat} and k_{cat}/K_M
- Always remember to include error measurements and p-values where applicable.

Discussion

- Discuss the k_{cat}/K_M values – what do they tell you about the enzyme?
- Compare the K_M, k_{cat} and k_{cat}/K_M of the wild type and mutant HCAII. Discuss how the mutation may be affecting the activity of the protein. Be specific. Include information about the mutated amino acid in your answer. Was the result expected

based upon the structural and biochemical properties of the mutation? Why or why not?

- How could you improve this experiment? For this discussion point, consider the concentration range of the substrate, the concentration of wild type and mutant HCAII, the HCAII mutant you assayed, the order in which you assembled your samples, etc.

LAB 11: FORSTER RESONANCE ENERGY TRANSFER (FRET) TO DETECT LIGAND-BINDING TO HCAII

Lab 11: FRET to detect ligand binding to HCAII

- Determine the binding constant (K_D) of a ligand (dansyl amide, DNSA) and of an inhibitor (acetazolamide, AZ) to wild type and mutant HCAII

Lab 11: BACKGROUND

In this lab, we will be using a specialized fluorescence technique called FRET to study the binding of HCAII to two ligands: dansylamide (DNSA) and acetazolamide (AZ) (structures shown below).
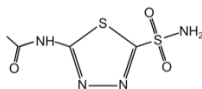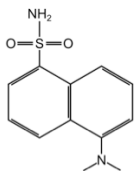
Fig. 11.1. Structure of DNSA (left) and AZ (right).

A ligand is defined as anything that binds a protein. Understanding how HCAII binds to ligands, and determining the effect of mutations on ligand binding, are important components of understanding protein function. AZ is used medically to inhibit HCAII (you will research more on this for your pre-lab quiz). It is useful to know how mutations in HCAII might affect drug binding, as they may make the drug more or less effective. More broadly, identifying key residues involved in the interaction between HCAII and AZ may help us better understand the mechanism of drug action and lead to the design of better drugs.

We have already used fluorescence spectroscopy in Lab 9 to study protein stability. Another specific application of fluorescence spectroscopy is Förster Resonance Energy Transfer (FRET). FRET results from the proximal interaction between two fluorescent molecules. A donor molecule absorbs light at a specific wavelength and is excited. Once excited, the donor transfers energy to a neighboring acceptor molecule, which then becomes excited and emits light at a specific lower energy wavelength. In order for FRET to occur, the emission spectrum of the donor must overlap with the

absorption spectrum of the acceptor. In addition, the two molecules must be within a distance range of 10-100 Å.

Since the 10-100 Å range is typical for biological macromolecule interactions, FRET can be applied to measure ligand binding affinity if a protein and its ligand have appropriate fluorescent properties. In this experiment, increasing concentrations of the ligand (a fluorophore, the acceptor in our FRET experiment) are titrated into a solution of the protein (our FRET donor) resulting in a change of fluorescence emission by the fluorophore. This change in emission can be used to calculate the ligand-binding affinity (*i.e.* the thermodynamic binding constant, K_D). This value is an equilibrium constant (given in units of Molarity) of the ratios of the concentrations of species (protein, ligand, and ligand-protein complex) in a reaction.

The accuracy range of the fluorimeter and the efficiency of the fluorophore limit FRET measurement. Generally, 1 nM to 1 μM fluorophore can be accurately detected in conventional instruments. However, in order to calculate a binding constant, the protein concentration cannot be higher than the K_D. Therefore, if the binding interaction is very tight (indicated by a very low KD), the protein concentration, and in turn fluorescence, may be too low to be measured. Similarly, if one was detecting a fluorescent ligand rather than the protein, the concentrations needed to measure the high affinity will be too small to detect. Conversely, if the interaction is too weak, one might not have enough ligand to reach saturation of the protein.

For this experiment, your goal is to determine the binding affinity of AZ to your wild type and mutant HCAII. Because AZ is not a fluorescent molecule, is is challenging to measure its binding directly. The fluorescent ligand DNSA will allow us to use FRET to measure ligand binding.

First, you will calculate the binding affinity of DNSA to HCAII using the FRET that occurs between the tryptophan residues in the active site of HCAII and the small molecule DNSA. The Trp residues in the protein absorb at 280 nm and emit at 340 nm. DNSA absorbs

at 340 nm and emits light at 470 nm. Therefore, when we excite at 280 nm, we will only observe fluorescence at 470 nm if DNSA is proximal to the Trp residues of the active site. You will confirm this with controls. You will then determine the binding constant of DNSA to both mutant and wild-type HCAII.

We will then measure the binding constant of AZ by competing off the DNSA binding. You will use the K_D of DNSA that you just determined and create a competition between DNSA and AZ to calculate the K_D of AZ binding. This will be done for both wt and mutant HCAII.

Lab 11: PROCOTOL

Assay overview and technical considerations
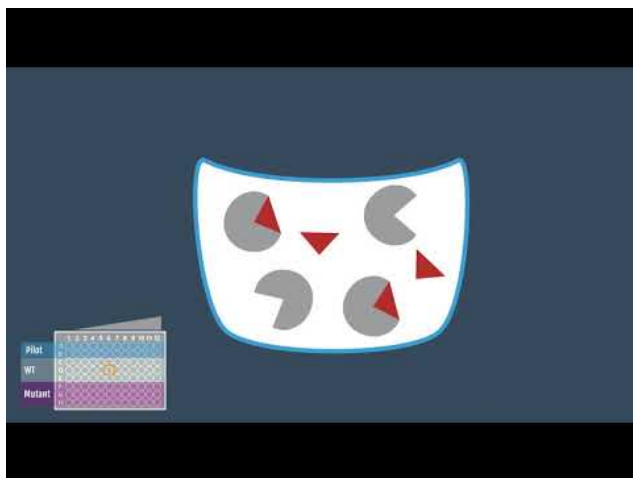
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Overall Experimental Setup:

You will be given two 96-well plates to complete four steps:

1. A pilot experiment for DNSA binding
2. A DNSA titration to determine K_D for DNSA
3. A pilot experiment for AZ binding
4. An AZ titration to determine K_D for AZ

Each plate will look something like this:
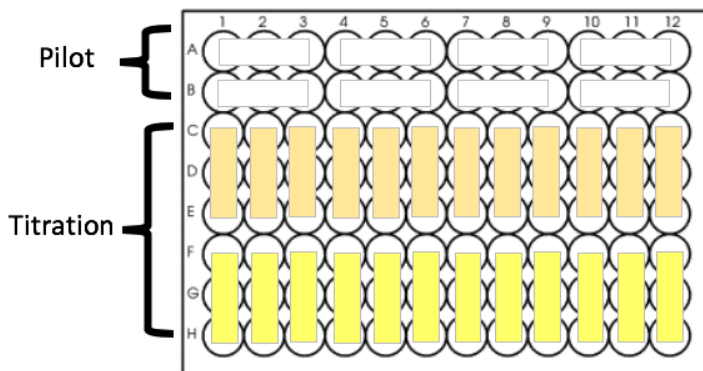
Fig. 11.1. General layout of a 96-well plate for lab 11. You will have two such plates: one for DNSA and one for AZ. The top two rows are used for the pilot. The rest of the plate is used for the titration. Wild type triplicates are shows as orange boxes, mutant as yellow boxes. The ligand concentration will increase moving from left to right, with the left-most column containing the lowest concentration of ligand.

GENERAL RECOMMENDATIONS:

- Do everything in triplicate (even the pilots!), unless you do not have enough protein.
- Be sure to include a no-protein blank on every plate, and remember to collect data for both wt and mutant HCAII.
- Plan for a final protein concentration of 0.25 μM protein.
- Use multichannel pipettes whenever possible! They save significant time and reduce error.
- To make planning and pipetting easier, your final volumes can differ by up to ~5 μL.

  - What this means is that you will pipette 100 μL of a protein + buffer stock *into every well*. You will then add up to 5 μL of ligand or inhibitor.
  - Different conditions will therefore end up with different

final volumes. You will adjust for that volume difference during data analysis.

- Don't add more than 5 µL DMSO to 100 µL total volume. Minimize the amount of DMSO added to the protein at each step by switching to a more concentrated stock as soon as you can, but don't pipette less than 0.5 µL.
- The plate reader generates data in arbitrary units. This means you cannot directly compare the raw data you get from reading different plates.

Pilot 1: DNSA

- The DNSA stock will be prepared for you as follows: 1-4 mg of DNSA will be dissolved in 1 mL DMSO. In lab, we will tell you the amount used and you will need to calculate the concentration of the stock.
- Use your DNSA stock to make additional stocks in DMSO at the following concentrations: 1 mM, 100 µM and 10 µM.
- Design a pilot experiment to determine whether the protein is functional and your assay is working before you use up all of your protein setting up a full titration. Like you did in Lab 9, create a detailed table to plan your experiment. Include the following in the pilot:
  - A blank
  - A control for protein autofluorescence
  - A control for DNSA autofluorescence
  - Two experimental conditions to determine where DNSA binding is saturated: protein plus two high concentrations of DNSA. We suggest final concentrations of 20 and 40 µM DNSA.
- Measure the fluorescence of your pilot conditions on the plate reader (*what are the excitation and emission wavelengths?*).

- THINK ABOUT THE RESULTS FROM THE PILOT BEFORE YOU
  MOVE ON. If you are not confident that your assay is working,
  talk to your TA. You could try a higher protein concentration
  and/or more DNSA.

Binding of DNSA to HCAII: Full Experiment

- Design your full titration experiment such that the
  concentration of HCAII is constant and you add increasing
  concentrations of DNSA. You will then mix your decided
  amounts of DNSA with HCAII in a 96 well plate.

> **RECOMMENDATION:**
>
> In a research setting, you would run multiple pilots
> and perhaps full titration experiments to determine a
> good range of DNSA concentrations to test. We will save
> time by providing you a range.
>
> We have found an optimal range for measuring DNSA
> binding to HCAII is approximately 50 nM – 20 µM DNSA.

- Dilute your protein in protein buffer to the desired final

concentration. Pour it into a buffer trough, and use a
multichannel pipette to add 100 μL protein to the appropriate
wells.

- Next, pipette the varying amounts of DNSA into the designated
  wells – again using a multichannel.
- After pipetting components into the wells, you can mix them
  by gently tapping the side of the plate.
- Measure fluorescence using the plate reader. Note that no
  incubation time is necessary.
- Take a quick look at the data to make sure that you have
  reached saturation. If you don't think you have, you may want
  to quickly plot [DNSA] vs. Fl. If your fluorescence is still rising
  dramatically at the highest [DNSA] tested, you should re-do
  the experiment with higher [DNSA].

Pilot 2: AZ

- Stocks of the inhibitor AZ will be provided at 1 mM. Keep in
  mind that you may need to dilute the stocks further. DMSO will
  also be provided for such dilutions.
- Design another pilot experiment to make sure that inhibition
  with AZ is measurable. Choose what you think would be
  reasonable highest and lowest [AZ] to test in this experiment.
  You will include these two conditions in the pilot. Create a
  chart like you did for the DNSA binding experiments. Include
  the following:

  ◦ A blank
  ◦ A control for AZ autofluorescence (you already ran
    controls for protein and DNSA autofluorescence so you
    don't have to re-do them)
  ◦ Protein plus a saturating concentration of DNSA (this is a
    positive control for fluorescence signal associated with
    DNSA binding)

- ◦ Protein plus DNSA plus your highest [AZ]
- ◦ Protein plus DNSA plus your lowest [AZ]

📰 *An interactive or media element has been excluded from this version of the text. You can view it online here:*
https://wisc.pb.unizin.org/biochemistry551online/?p=180

- THINK ABOUT THE RESULTS FROM THE PILOT BEFORE YOU MOVE ON. If you are not confident that your assay is working, talk to your TA. You could try using less DNSA or adjust your concentration range of AZ.

Binding of AZ to HCAII: Full Experiment

- Once you determine the best concentration range, design your full AZ titration.
- This time you can create your protein stock by mixing your protein with both protein buffer *and* DNSA. Pipette 100 µL of the mixture per well with a multichannel pipette. Then add AZ to wells as you've decided.
- Measure fluorescence using the plate reader.
- Take a quick look at your data to make sure you tested reasonable concentrations of AZ.

Lab 11: LAB ASSIGNMENTS

PRELAB QUIZ

Question 1

What experiment with HCAII are we simulating this week? What is the purpose of that experiment? (Provide 2-3 sentences).

Question 2

We will be studying acetazolamide (AZ) in this lab. What is AZ and why is it interesting? What medical applications does it have? (This will require a bit of research.)

Question 3

Use the BRENDA database again to find published Ki values for acetazolamide inhibition of HCAII. This should give you a general idea for how strongly AZ binds HCAII. Which of the following is the closest approximation? (Note that in lab 11 you are actually measuring K_D, not Ki. However, the Ki should still give you an idea of the strength of binding.) Here is the link to BRENDA (link opens in a new tab): http://www.brenda-enzymes.info/

- 5 nM
- 12 nM
- 12 mM

IN-LAB ACTIVITIES

- After joining BBCU, you and your partner will join a breakout room.
- You will use the in-lab time to work with your partner to complete the planning template file for this experiment.
    - Start with the pilot for DNSA. Once you have this ready, a TA will share and discuss some data with you.

- Next, plan the full DNSA experiment.
- The next step is to plan the pilot for AZ. A TA will take a look at your suggested high and low AZ concentrations and give you estimated AZ values. Based on these, you will likely need to adjust your AZ concentrations and try again.
- Once you and your TA are satisfied with your AZ concentrations, you can complete the planning document by filling out the full AZ experiment.

The following videos were made to help you understand how to plan these experiments, which are quite complicated:

Part 1: DNSA
TA video will be added when available
Part 2: AZ
TA video will be added when available

- You can use the remaining lab time with your lab partner to analyze the lab 11 stock data.
- TAs can be available for questions throughout lab. This lab requires several check-ins with TAs, so be aware that you may have to wait. (This is a good simulation of the live version of the lab, where there is always a line to use the plate reader.)
- Complete the lab/partner/self evaluation Google form.
- Get a sharable link to your Google sheets planning document and submit it to Canvas.

Lab 11: DATA ANALYSIS

During data analysis you will calculate the following:

1. The K_D for binding of DNSA to WT HCAII
2. The K_D for binding of DNSA to your HCAII mutant
3. The K_D for binding of AZ to WT HCAII
4. The K_D for binding of AZ to your HCAII mutant

For these calculations, you will use Excel to determine values and then plot ligand concentration versus fraction bound in Prism.

K_D for binding of DNSA to HCAII

Step 1: Look at your data. You should have fluorescence emission data at 470 nm (F_{470}) at various concentrations of DNSA that has been added to a solution containing HCAII. You should also have the volume of solution in each well, and the total concentration of DNSA ($[DNSA]_{total}$) for each point in the titration. Identify any outliers in your data and determine whether they can be removed by applying the Dixon's Q test.

For help with statistics, see the Appendix (link opens in a new tab): Appendix 2: Statistical methods used in 551 data analyses

The following video will help you become oriented to your data and show you how to identify and remove outliers.

For all videos, you should be able to actually read the numbers being shown. You may need to view full-screen, and/or manually increase the quality using the button in the lower right corner of the video. You can also turn on closed captioning for all videos.
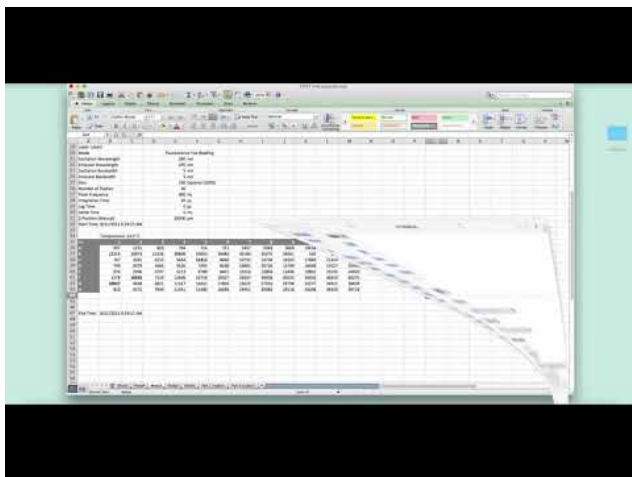
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Step 2: Correct the F_{470} data for dilution during the titration (i.e. determine $F_{adjusted}$). Use the following equation:

$$F_{adjusted} = \frac{(F_{470})(Total\ volume)}{(Initial\ volume)}$$

Step 3: Calculate the fraction saturation, r, at each point (i.e. normalize the data). Use the following equation:

$$r = \frac{F_{adjusted} - F_{min}}{F_{max} - F_{min}}$$

where F_{min} is the smallest value of $F_{adjusted}$ and F_{max} is the largest value of $F_{adjusted}$.

Here is an example wt data set that has been analyzed through step 3. Note that the r values range from ~0 (at low DNSA) to ~1 (at high DNSA).

WT

Column	[DNSA]	Final Volume	F raw	F adj		r	[DNSA]free
1	0	100	767	767	Fmin:	0.00111213	-0.00027803
	0	100	739	739	739	0	0
	0	100	974	974		0.00933392	-0.00233348
2	0.05	105	3181	3340.05	Fmax:	0.10331056	0.02417236
	0.05	105	3079	3232.95	25916	0.09905668	0.02523583
	0.05	105	2996	3145.8		0.09559519	0.0261012
3	0.1	101	3253	3285.53		0.10114509	0.07471373
	0.1	101	3466	3500.66		0.1096898	0.07257755
	0.1	101	3797	3834.97		0.12296819	0.06925795
4	0.25	102.5	5634	5774.85		0.20001787	0.19999553
	0.25	102.5	5526	5664.15		0.195621	0.20109475
	0.25	102.5	5213	5343.325		0.18287822	0.20428044
5	0.5	105	12212	12822.6		0.47994598	0.3800135
	0.5	105	7295	7659.75		0.27488382	0.43127904
	0.5	105	6788	7127.4		0.25373952	0.43656512
6	0.75	107.5	8460	9094.5		0.33187036	0.66703241
	0.75	107.5	8598	9242.85		0.33776264	0.66555934
	0.75	107.5	8401	9031.075		0.32935119	0.6676622
7	1	101	10732	10839.32		0.4011725	0.89970688
	1	101	10891	10999.91		0.40755094	0.89811227
	1	101	10316	10419.16		0.38448425	0.90387894
8	2.5	102.5	13758	14101.95		0.53076022	2.36730995
	2.5	102.5	20726	21244.15		0.81443977	2.29639006
	2.5	102.5	13804	14149.1		0.53263296	2.36684176
9	5	105	16103	16908.15		0.64221909	4.83944523
	5	105	15769	16557.45		0.62828971	4.84292757
	5	105	15496	16270.8		0.61690432	4.84577392
10	10	101	17869	18047.69		0.68748024	9.82812994
	10	101	18468	18652.68		0.71150971	9.82212257
	10	101	19805	20003.05		0.76514477	9.80871381
11	20	102	21324	21750.48		0.83455058	19.7913624
	20	102	19157	19540.14		0.74675855	19.8133104
	20	102	19105	19487.1		0.74465186	19.813837
12	40	104	24837	25830.48		0.99660325	39.7508492
	40	104	25430	26447.2		1.02109862	39.7447253
	40	104	24920	25916.8		1.00003178	39.7499921

Step 4: Calculate the concentration of free DNSA at each point (i.e. calculate [DNSA]$_{free}$). Use the following equation:

$$[DNSA]_{free} = [DNSA]_{total} - (r) \times [HCAII]_{total}$$

The concentration of [HCAII]$_{total}$ is probably 0.25 μM, unless you used something different.

Step 5: Use Prism to plot r as a function of [DNSA]$_{free}$. Note that even though you did the experiment in triplicate, each Y-point (r) has a different X ([DNSA]$_{free}$), and so you cannot enter them as replicates in Prism. You can still enter all the data into Prism at once, you will just enter one "replicate" each for wt and mutant that contains all the data.

When you open Prism, choose "Enter and plot a single Y value for each point" from the initial menu. Enter the [DNSA]$_{free}$ and r values for wt. Then add the mutant values below, always keeping [DNSA]$_{free}$ in the "X" column but using two separate columns for r wt and r mutant.

See example:

Table format: XY		X	Group A	Group B
		[DHSA]free	r wt	r mut
	○	X	Y	Y
20	Title	0.69811221	0.45755094	
21	Title	0.80387894	0.38449425	
22	Title	2.36730994	0.53076003	
23	Title	2.29699006	0.51440977	
24	Title	2.36584170	0.53253296	
25	Title	4.83944523	0.54221909	
26	Title	4.84282727	0.52828971	
27	Title	4.84571392	0.51980433	
28	Title	9.62812994	0.68168024	
29	Title	9.62212297	0.71150971	
30	Title	9.80871381	0.76514477	
31	Title	19.79136240	0.53455058	
32	Title	19.81031040	0.74675855	
33	Title	19.81383700	0.74169186	
34	Title	39.76084800	0.99440329	
35	Title	39.74473800	1.02109962	
36	Title	39.74699210	1.08003178	
37	Title	-0.00289530		0.01158139
38	Title	0.00080080		0.00000000
39	Title	0.02932284		0.06608860
40	Title	0.02994086		0.09036732
41	Title	0.06066870		0.15804619
42	Title	0.06247031		0.16011678
43	Title	0.06545762		0.17625193
44	Title	0.17533243		0.23890708
45	Title	0.18306011		0.27115868
46	Title	0.18303705		0.26705168
47	Title	0.39663235		0.41347047
48	Title	0.41146700		0.35417202
49	Title	0.40465725		0.38137108
50	Title	0.62673767		0.49304931
51	Title	0.63684580		0.45282001
52	Title	0.64478819		0.42320723

The plot of your data should look hyperbolic, as is expected for ligand binding behavior.

See example plot:
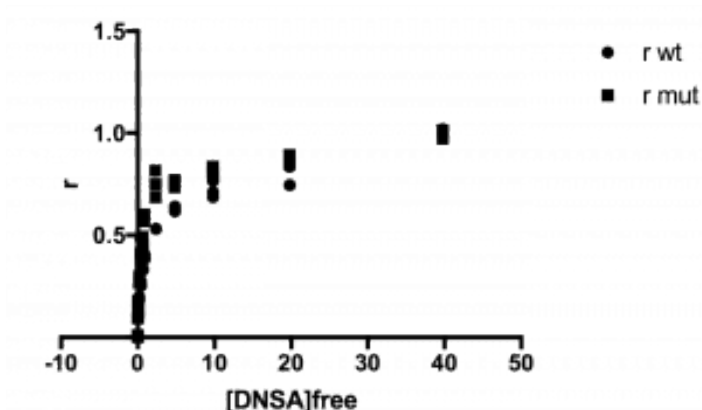
A note about data analysis: Before continuing to step 6 take a look at your data. Does it look like a binding curve? If your protein doesn't bind ligand, or if your data is of poor quality, you can still force Prism to calculate a K_D, but that value would have no physical meaning.

Step 6: Fit the data to the Langmuir isotherm to determine the K_D.

$$r = \frac{[DNSA]_{free}}{K_D + [DNSA]_{free}}$$

Fitting the equation should give you the K_D. You can also use Prism to determine whether there is statistical difference between wt and mutant. The following video demonstrates how to do the fit in Prism, which requires you to create your own fitting equation.

Note: the video uses an older version of Prism. In order to get error measurements in Prism 8 (and beyond), you need to follow the additional step: After choosing that you will compare Kd values, go to the "Confidence" tab. Select "Symmetrical (asymptotic) approximate CI" and then select "Show SE of parameters."
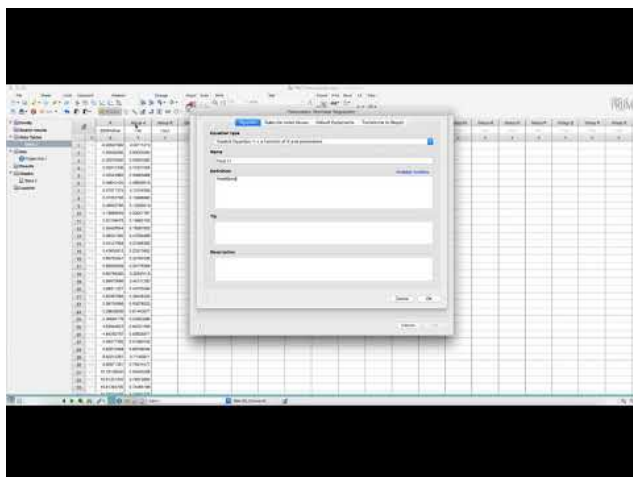
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K_D for binding of AZ to HCAII

Much of this data processing is similar to what you just did for DNSA.

Step 1: As before, calculate the total volume of solution in each well after addition of ligand, as well as the total concentration of AZ ($[AZ]_{total}$) for each point in the titration.

Step 2: Calculate $F_{adjusted}$ as before, by identifying F_{max} and F_{min} and using the equation:

$$F_{adjusted} = \frac{(F_{470})(Total\ volume)}{(Initial\ volume)}$$

**Again, do this separately for wt and mutant (i.e. use different values for F_{max} and F_{min}).

Next calculate fraction saturation (*r*) as before using the equation:

$$r = \frac{F_{adjusted} - F_{min}}{F_{max} - F_{min}}$$

Step 3: Calculate the concentration of AZ-bound HCAII (i.e. [AZ*HCAII]) at each point. This should be a new column in your Excel sheet.

Since there is a negligible amount of protein not bound to either ligand, we approximate [HCAII]$_{free}$≈ 0. Therefore, we can treat all non-fluorescing HCAII as AZ-bound, and thus use the equation:

$$[HCAII * AZ] = ([HCAII]_{total}) \times (1 - r)$$

Step 4: Calculate the concentration of free AZ at each point (i.e. calculate [AZ]$_{free}$). Use the equation:

$$[AZ]_{free} = [AZ]_{total} - [HCAII * AZ]$$

If you calculate any negative values for [AZ]$_{free}$, change them to 0.

Here is a sample data set that has been analyzed through step 4. Note that r values range from ~0 to ~1. Note also that a number of the [AZ]$_{free}$ values, at the three lowest [AZ] tested, were actually negative and so were set to 0.

WT

Column	[AZ]	Final Volume	F raw	F adj			r		HCAII*AZ	[AZ]free
1	0	100	28038	28038			0.96763393		0.00809152	0
	0	100	27950	27950	Fmin:		0.96336374		0.00915906	0
	0	100	27501	27501		8097	0.94157609		0.01460598	0
2	0.01	101	28421	28705.21			0.99996167		9.5832E-06	0.00999042
	0.01	101	26747	27014.47	Fmax:		0.91796729		0.02050818	0
	0.01	101	26345	26608.45		28705	0.89826524		0.02543369	0
3	0.025	102.5	19062	19538.55			0.55519944		0.11120014	0
	0.025	102.5	17550	17988.75			0.47999563		0.13000109	0
	0.025	102.5	17466	17902.65			0.47581764		0.13104559	0
4	0.4	104	16589	17252.56			0.44427213		0.13893197	0.26106803
	0.4	104	16287	16938.48			0.42903144		0.14274214	0.25725786
	0.4	104	16622	17286.88			0.4459375		0.13851563	0.26148438
5	0.6	106	15954	16911.24			0.42770963		0.14307259	0.45692741
	0.6	106	14879	15771.74			0.37241557		0.15689611	0.44310389
	0.6	106	15169	16079.14			0.3873321		0.15316697	0.44683303
6	0.8	100.8	12727	12828.816			0.22961064		0.19259734	0.60740266
	0.8	100.8	12223	12320.784			0.20495846		0.19876038	0.60123962
	0.8	100.8	11818	11912.544			0.18514868		0.20371283	0.59628717
7	1	101	12068	12188.68			0.19854814		0.20036297	0.79963703
	1	101	11785	11902.85			0.18467828		0.20383043	0.79616957
	1	101	12084	12204.84			0.1993323		0.20016693	0.79983307
8	2	102	11031	11251.62			0.15307745		0.21173064	1.78826936
	2	102	10092	10293.84			0.10660132		0.22334967	1.77665033
	2	102	10398	10605.96			0.12174689		0.21956328	1.78043672
9	5	105	9870	10363.5			0.10998156		0.22250461	4.77749539
	5	105	10673	11206.65			0.15089528		0.21227618	4.78772382
	5	105	11133	11689.65			0.17433278		0.2064168	4.7935832
10	10	101	8450	8534.5			0.02122962		0.2446926	9.7553074
	10	101	8925	9014.25			0.04450941		0.23887265	9.76112735
	10	101	8798	8885.98			0.03828513		0.24042872	9.75957128
11	15	101.5	7978	8097.67			3.2512E-05		0.24999187	14.7500081
	15	101.5	8339	8464.085			0.01781274		0.24554681	14.7544532
	15	101.5	8746	8877.19			0.0378586		0.24053535	14.7594646
12	20	102	8828	9004.56			0.04403921		0.2389902	19.7610098
	20	102	9602	9794.04			0.0823486		0.22941285	19.7705872
	20	102	10907	11125.14			0.14694002		0.21326499	19.786735

Step 5: Fit the data in Prism.

Open a new Prism file and, as before, choose "Enter and plot a

single Y value for each point." Copy your $[AZ]_{free}$ values into the X column and *r* into the Y column – using separate columns for wt and mutant, just as before.

When you input your data into the data table, make the title of the column the K_D for DNSA that you measured in Part 1 above. So for example, instead of titling the column "wt" you might title it "1.568". This looks a little weird, but will allow Prism to take the K_D for DNSA into account when you fit this data in the next steps.
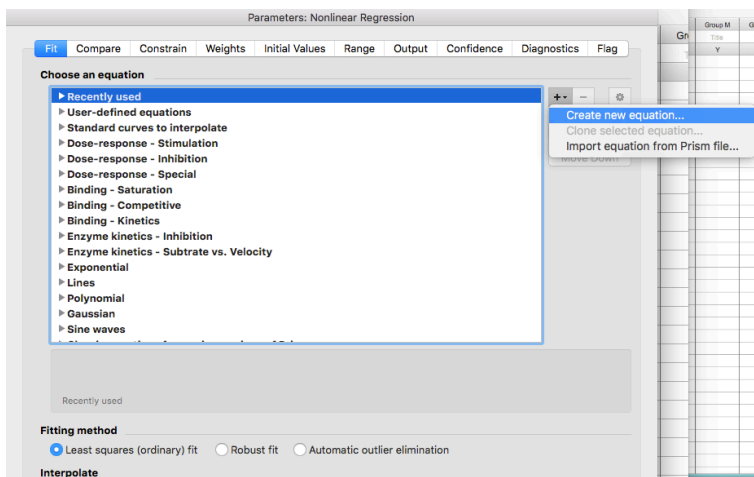
Click on "Analyze"

Select "XY Analyses" then "Nonlinear regression (curve fit)"

Press ok
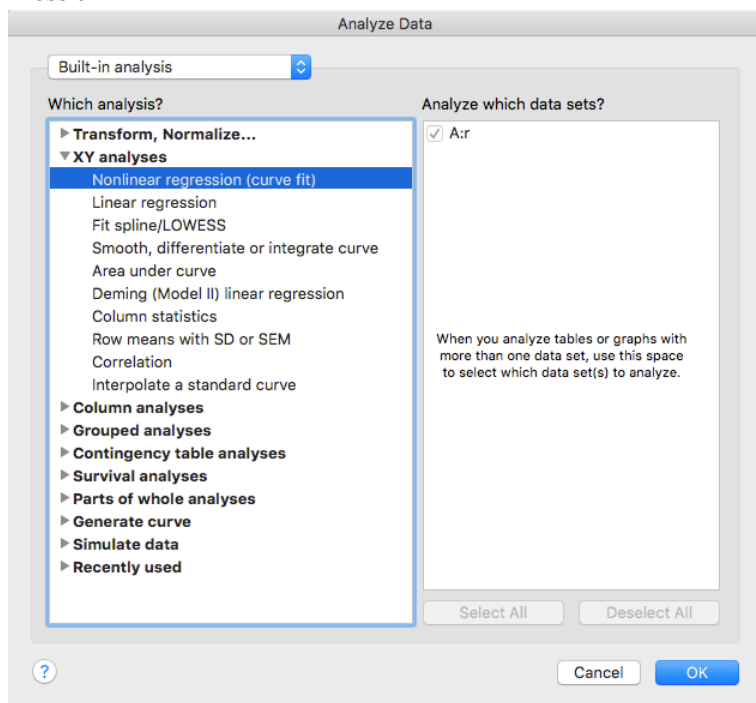
From the "+"drop down menu on the right side, choose "Create new equation"

(Note that if you are using Prism on a PC instead of a Mac this may look slightly different)

Give your equation a name

Type in the competitive langmuir isotherm equation:

$$Y=1/(1+(Kd/DNSA)(1+(x/(KdAZ))))$$

Note that in this equation "Kd" is the K_D for DNSA, which you determined in the first part of this analysis. "DNSA" is the concentration of DNSA that was used in this AZ experiment, probably 20 uM. "KdAZ" is the K_D for AZ, which you are going to have Prism find for you.

Now click on the "Rules for Initial Values" tab across the top. Remember that KdAZ is what we are having Prism solve for. In the drop down menu to the right of KdAZ, select *(Value of X at YMID)

Leave the other values alone.

Now click on the next tab over, "Default Constraints"

Remember that DNSA is a constant. Change the dropdown menu next to DNSA to "Constant equal to"

Kd is the K_D for DNSA that you measured in part 1, and that you made the column title in this data set. Change the dropdown menu to "Data set constant (from column title)"

Press "ok"

You will get a dialogue box yelling at you for not defining rules. Just click "Continue"

Now your equation should show up under "User-defined equations"

With your equation highlighted, press ok

You will be taken to the "Constrain" tab, where you need to enter the concentration of DNSA that was used in your AZ experiment. Be sure you are consistent with your units here – I would use μM.

Next click over to the "Compare" tab. Select "Do the best-fit values of selected unshared parameters differ between data sets?"

Then select "KdAZ".

Next click over to the "Confidence" tab and select the option to "Show SE of parameters."

Finally, click "ok"!

Your data has been analyzed! It should look something like this sample data:
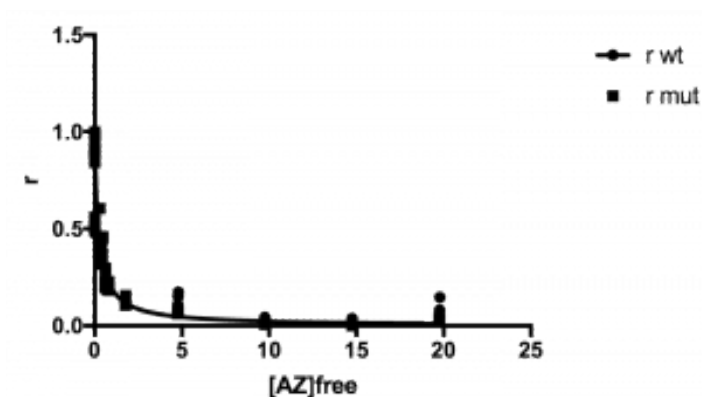
Nonlin fit		A	B	C	D
		1.528	0.6942	Global (shared)	Title
		Y	Y	Y	Y
1	Comparison of Fits				
2	Null hypothesis			KdAZ same for all data sets	
3	Alternative hypothesis			KdAZ different for each data set	
4	P value			0.0080	
5	Conclusion (alpha = 0.05)			Reject null hypothesis	
6	Preferred model			KdAZ different for each data set	
7	F (DFn, DFd)			7.464 (1, 69)	
8					
9	KdAZ different for each data set				
10	Best-fit values				
11	Kd	= 1.528	= 0.6942		
12	DNSA	= 20	= 20		
13	KdAZ	0.0178	0.008534		
14	Std. Error				
15	KdAZ	0.003321	0.001584		
16	95% CI (profile likelihood)				
17	KdAZ	0.01186 to 0.02558	0.005701 to 0.01221		
18	Goodness of Fit				
19	Degrees of Freedom	35	34		
20	R square	0.8137	0.8068		
21	Absolute Sum of Squares	0.6528	0.681		
22	Sy.x	0.1366	0.1415		
23	Constraints				
24	Kd	Kd = 1.528	Kd = 0.6942		
25	DNSA	DNSA = 20	DNSA = 20		
26					

Lab 11: FINAL LAB REPORT

FINAL LAB REPORT

There is no Mini Lab Report for Lab 11. What follows are some suggestions for what to include from this lab in your final report.

Methods

You again need to write a detailed methods section. Include:

- Final concentration of HCAII, DNSA, AZ
- Equations for calculating for K_D for both DNSA and AZ and make sure to define the values for F_{max} and F_{min}.

Results

- A graph of the fraction saturation (r) vs $[DNSA]_{free}$, and graph of r vs $[AZ]_{free}$
- Describe the results of your plots in the text including relevant values
- Be sure to include measurements of error and p-values, where appropriate

Discussion

- For which ligand, Dansyl amide or acetazolamide, does HCAII have a tighter affinity? By how much? Based upon the structure of DNSA and AZ, which would you expect to have higher affinity and why?
- How do your K_D values compare to that of the literature? What are some reasons why your values could differ from the published results?
  - Here is one useful reference on this topic (pdf opens in a new tab): Structural Basis of Inhibitor Affinity to Variants of Human Carbonic Anhydrase II
- How does the HCAII mutation you studied affect ligand

binding? Discuss the relative affinity of DNSA and AZ to wt HCAII and the mutant. Your answer should include information about the chemistry of the amino acid in the wild type protein and the mutation you made as well as a statement about how the mutation affected the structure of the protein and thus its ability to bind the ligands. Use your results to support your conclusions.

APPENDICES

Appendix 1: Information on HCAII

*HCAII*Gene Sequence:

5'ATGGCCCATCACTGGGGGTACGGCAAACACAACGGACCTGAGC
ACTGGCATAAGGACTTCCCCATTGCCAAGGGAGAGCGCCAGTCCC
CTGTTGACATCGACACTCATACAGCCAAGTATGACCCTTCCCTGAA
GCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCCTGAGGATCCTC
AACAATGGTCATGCTTTCAACGTGGAGTTTGATGACTCTCAGGACA
AAGCAGTGCTCAAGGGAGGACCCCTGGATGGCACTTACAGATTGAT
TCAGTTTCACTTTCACTGGGGTTCACTTGATGGACAAGGTTCAGAG
CATACTGTGGATAAAAAGAAATATGCTGCAGAACTTCACTTGGTTC
ACTGGAACACCAAATATGGGGATTTTGGGAAAGCTGTGCAGCAACC
TGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTGGCAGCGCT
AAACCGGGCCTTCAGAAAGTTGTTGATGTGCTGGATTCCATTAAAA
CAAAGGGCAAGAGTGCTGACTTCACTAACTTCGATCCTCGTGGCCT
CCTTCCTGAATCCCTGGATTACTGGACCTACCCAGGCTCACTGACC
ACCCCTCCTCTTCTGGAATGTGTGACCTGGATTGTGCTCAAGGAA
CCCATCAGCGTCAGCAGCGAGCAGGTGTTGAAATTCCGTAAACTTA
ACTTCAATGGGGAGGGTGAACCCGAAGAACTGATGGTGGACAACT
GGCGCCCAGCTCAGCCACTGAAGAACAGGCAAATCAAAGCTTCCTT
CAAATAA

HCAII Protein Sequence:

MAHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSL
KPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGPLDGTYRLIQ
FHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQP
DGLAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFDPRGLLPE
SLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGE
PEELMVDNWRPAQPLKNRQIKASFK

HCAII size: 260 amino acids, 29.25 kDa

The extinction co-efficient for HCAII is 50,420 cm-1M-1.

Appendix 2: Statistical methods used in 551 data analyses

Statistical methods used in 551 data analyses

Please note: this appendix is not intended to be an exhaustive survey of biostatistical methods. For those interested in statistics, we encourage you to enroll in a statistics course. However, this appendix should provide enough information to allow you to make statistical analyses of your 551 data.

Error within samples: Standard Deviation

Standard deviation is a measure that quantifies the amount of variation in a data set. Whenever you collect multiple data sets, i.e. multiple replicates, you **must** report the result as the mean +standard deviation. This includes graphical representations of data, where the mean should be plotted as the data point, with error bars representing the standard deviation. It is also important to define in the figure legend that the plot shows the mean with error bars representing standard deviation.

Standard deviations are easily calculated within Excel or other mathematical software.

Outliers: Q test

The Q test is used to identify and discard outliers, assuming a normal distribution. It is only used to discard outliers within a replicate. The Q test should be used sparingly and not more than

once within the same replicate. It won't rescue poor quality data, but it can clean up your results if you have a single point that is far off from others within the same replicate.

The Q test is defined as:

$$Q = \frac{gap}{range}$$

where *gap* is the difference between the value in question and the closest number to it, and *range* is the difference between the highest and lowest values in the set.

For example, consider the following triplicate data set:

561 590 10541

Calculate Q, hypothesizing that 10541 is an outlier:

$$Q = \frac{gap}{range} = \frac{10541-590}{10541-561} = \frac{9951}{9981} = 0.997$$

With three data points, at 95% confidence, Q must be greater than 0.970 to discard the point as an outlier (see table below). As our calculation satisfied this requirement, we can conclude that 10541 is an outlier and discard it from the data set. If you do this, you **must** state in the methods section that a Q test was used to discard outliers at 95% confidence.

Table A.1: Limit values for Q. Limit values are provided for different sample numbers at different confidence levels. If your calculated Q value is above the value appropriate value in the table, you can discard the value in question as an outlier.

Number of replicates:	3	4	5	6	7	8	9	10
90% confidence:	.941	.765	.642	.560	.507	.468	.437	.412
95% confidence:	.970	.829	.710	.625	.568	.526	.493	.466
99% confidence:	.994	.926	.821	.740	.680	.634	.598	.568

Significant Differences: Student's t test

The t test is a statistical hypothesis test that can be used to determine if two data sets are significantly different from each

other. The test either accepts or rejects the null hypothesis: that there is no difference between two sets of data.

There are a number of different versions of the t test, depending on specific parameters of the data sets in question. For the purposes of 551 lab, we will use a two-tailed t test for two unpaired samples and assume equal variance. Below is a list of tools that can be used to perform a t test, followed by the interpretation of the t test results.

- Vassarstats: http://vassarstats.net, click on *t-Tests & Procedures*, choose **Independent Samples**, and then type in your data.
- GraphPad QuickCalcs: http://www.graphpad.com/quickcalcs/ttest1.cfm
- Microsoft Excel can be used to perform a t test using the command:
  - T.TEST(array1, array2, 2, 2)
  - "2,2" in the parenthesis above indicate the independents samples, and equal variance of samples.
  - You can search excel help for "t test" to get more information on this command.
- Prism can be used to perform a t test when you run a curve fit on two different data sets (i.e. wild type vs mutant). When running a curve fit, look for the tab "Compare" and select the option to compare best fit values of selected parameters between data sets.

The important output of a t-test is a p-value, which in our case is essentially a measure of the probability that there is no statistical difference between the two samples. It is generally accepted that a p value < 0.05 indicates a statistically significant difference. It is important to note that this cutoff is arbitrary, so a p-value of 0.05001 is not very different from 0.04999.

For example, consider the following Km values (in mM) from three experiments with HCAII and PNPA:

wt: 4.4, 4.1, 4.7

mutant: 2.9, 3.3, 2.9

Using this data and the T.TEST command, Excel calculates a p-value of 0.0038. As this is lower than 0.05, we can conclude that wt and mutant HCAII have a statistically significant difference in Km.

A p-value is well-correlated with the spread of your data. The less spread the values are in each group (smaller standard deviation) and the more different they are from the other group, the smaller the p-value is going to be, meaning there's a significant difference between the two groups.

For the same reason, a big difference between two group means doesn't necessarily mean a significant difference. For example, if the Km values for wt are 4.2, 4.3, 6.3, and mutant are 2, 3.1, 3.9. We have a big, but non-significant, difference between the groups (1.9 difference and p-value = 0.089). On the other if the Km values for wt are 4.1, 4.2, 4.3, and for the mutant are: 4,4,4, we'll have a small, but statistically significant, difference between the two groups (difference= 0.2 and p-value =0.025). This last case would be a time to consider whether *statistical*significance is necessarily the same as *biological* significance.

Propagation of error

When measurements that contain error are used in calculations, that error must be carried through in a way that allows you to estimate the error of the final value. This concept is called the propagation of error, and you can find information online for how to do this under a wide range of circumstances. For 551, we will make the assumption that all error values are indeterminate, which means that values have a symmetric distribution around their mean, and errors are unbiased with respect to sign.

There are some basic rules when carrying indeterminate error through mathematical calculations. First, you calculate the fractional error (to get the error as a percent of the value so that

for largely different numbers your error is on the same scale). Then, when manipulating values with error, add the fractional error. Finally, once you have finished the calculation, you turn the fractional error from a percent back into absolute error.

For example, consider a calculation for K_{cat} for WT HCAII:

$$k_{cat} = \frac{V_{max} \pm error}{[enzyme] \pm error}$$

$$V_{max} = 80.30 \pm 8.987$$

$$fractional\ error = \frac{8.987}{80.30} = 0.11$$

you didn't measure the error of enzyme concentration added so we'll just assume it's close to 0

$$k_{cat} = \frac{80.30}{0.2} = 401.5$$

$$final\ fractional\ error = 0.11 + 0 = 0.11$$

$$Absolute\ error = 401.5 \times 0.11 = 44.17$$

Therefore, K_{cat} = 401.5 $\pm$44.17

Appendix 3: Additional laboratory details

Lab 3: Agarose Gel Electrophoresis

Agarose is a highly purified form of agar that is extracted from seaweed. The density of the gel is determined by the weight percentage concentration of agarose in the solution. Different concentrations of agarose are used in gels depending on the relative sizes of the fragments expected or the separation desired. Acrylamide gels can be used when small pieces of DNA are to be resolved.

The molecular weight of DNA determines its rate of migration. Typically, the smaller the molecule, the faster it will migrate through the gel. However, DNA of the same size can have differing mobilities depending upon the shape: linear, circular relaxed, or circular supercoiled. Circular supercoiled DNA migrates faster than either linear or circular relaxed DNA, while linear DNA typically migrates faster than circular relaxed DNA.

When the voltage is low, migration of DNA is proportional to the strength of the electric field, regardless of the DNA size. At higher field strengths, larger molecules move disproportionately faster so that the resolution is decreased.

The buffer system can also affect mobility. If you forget to add the running buffer to the agarose gel, electrical conductance will be decreased and the DNA will migrate very slowly. Alternatively, if you use 10x or 50x buffer to make the gel, the ionic strength will be too high, and consequently the amount of heat produced will be excessive.

The sample is loaded after the gel is just covered with buffer. It is important to add just enough buffer to cover the gel, but not a lot more. Excess buffer allows a substantial conduction pathway on

top of the gel instead of the desired pathway through the gel, so migration of DNA through the gel will be slowed.

Samples are mixed with a solution containing bromphenol blue and xylene cyanol as tracking dyes; the bromphenol blue migrates about the same rate as a 0.3-0.5 kb fragment and the xylene cyanol migrates similarly to a 4-5 kb fragment. This loading dye can also contain SDS, EDTA, and sucrose. The sucrose makes the sample denser than the buffer so the sample will sink to the bottom of the well when it is loaded. Generally 1-2 µL of loading dye is added per 10 µL of sample.

Ethidium Bromide Staining

Since DNA cannot be detected in a gel, ethidium bromide, a fluorescent dye, is added to the DNA. The planar, polycyclic ethidium bromide intercalates between the bases of double-stranded DNA and RNA. Ethidium absorbs UV light at 302 nm and produces a reddish-orange fluorescence at 590 nm; the amount of this fluorescence is proportional to the mass of the DNA, assuming there is enough ethidium present to saturate all binding sites on the double stranded nucleic acid. The fluorescence of RNA is less than that of an equal mass of DNA, because most of the RNA is single-stranded and less susceptible to ethidium intercalation.

Ethidium bromide can be added to the DNA in the gel in any of three ways:

1) Ethidium bromide may be added to the gel when the hot agarose is poured onto the slide.

2) The ethidium may be added to the running buffer.

3) Following electrophoresis, the gel can be placed in a staining tray containing the dye, incubated for a short time, and then rinsed to remove any unbound ethidium bromide.

Lab 4: Transformation

When bacterial cells take up DNA they are said to undergo "transformation." The exact mechanism of this process is unknown, but host bacterial cells must be in a state called "competence."

Healthy, mid-log phase cells are treated with a solution of ice-cold calcium chloride and then, in the presence of the recombinant DNA to be transferred, subjected to a brief heat shock. One hypothesis is that treatment of the cells at 0 ˚C crystallizes the cell membrane, stabilizing charged phosphate distribution. Then the cations in the treatment solution could complex with exposed phosphate groups to shield negative charges and a plasmid molecule might pass through "pores" in the cell's wall/ membrane called "adhesion zones." It is hypothesized that heat shock complements this process, possibly by creating a thermal imbalance on either side of the membrane that will move the transforming DNA through the adhesion zone into the competent host cell.

Competent cells are very fragile and must be treated with care – kept on ice, mixed very gently. The timing of the heat shock step should also be precise – if kept too long at the high temperature, the cells will die. Furthermore, careful, aseptic technique must be used throughout, because any contaminating bacterial cell will have the opportunity to grow and compete with desired cells.

After the recombinant DNA is transferred to the competent host cells, these cells are incubated in rich medium to allow them to grow and replicate the newly transferred DNA. Then aliquots of the culture are plated on a selective medium, so that only clones that received the plasmid will grow. The colonies are then screened to locate those that have the desired characteristics. Please note the difference between "selection" and "screening." The former allows only the clones with a desired trait (antibiotic resistance in this case) to grow. The latter allows both desired and undesired to grow, but provides a way for us to differentiate among colonies with different traits.

Lab 5: Mini-Preps of Plasmids

Although all miniprep kits have slightly different procedures, the general steps are the same. SDS and NaOH are used to lyse the cells, solubilize and denature cellular constituents, begin to degrade RNA, and denature (separate the strands of) DNA. Then, in alkaline

conditions, the small plasmid strands remain linked to their complementary strands. Hydrogen bonds can reform between these strands, which are close to each other when the lysate is neutralized. Proper base pairing can then occur and an intact double-stranded plasmid is reformed. Since genomic DNA will not be linked to a complementary strand and thus, not necessarily remain nearby, a rather large interlinked mass of DNA strands will result after neutralization. The potassium acetate added to neutralize the alkaline solution also elevates the salt concentration, so that the SDS and protein form a flocculent precipitate to trap the genomic DNA mass for removal by centrifugation.

Each commercial kit generally includes a proprietary resin (specially-treated glass) that is mixed with the plasmid DNA/RNA solution. The plasmid DNA sticks to the resin in a small column. The column is washed with a solution that contains ethanol, so the plasmid remains on the resin. The plasmid DNA is then eluted from the glass resin with water or TE buffer. Sometimes the DNA is stuck to the resin with high salt and eluted with low salt (water or TE). This resin replaces the alcohol precipitation steps done in other procedures and in the purification of genomic DNA.

The miniprep kit we will be using is made by Qiagen. All of the buffer formulations are proprietary, so Qiagen refers them to as P1, P2, N3 and PE.

In general the buffers do the following:

P1 – bacterial re-suspension buffer, contains RNase A to remove RNA.

P2 – alkaline lysis buffer containing NaOH-SDS. SDS causes lysis of the cell membrane. NaOH denatures chromosomal DNA, plasmid DNA, and proteins. The lysis time is optimized to release the plasmid DNA from the cell and minimize its exposure to denaturing conditions.

N3 – neutralization buffer containing acidic potassium acetate. High salt in the buffer precipitates the detergent and traps proteins and chromosomal DNA in salt-detergent complexes. The small, covalently closed plasmid DNA renatures and remains in solution.

This high salt buffer also allows the plasmid DNA to bind to the resin in the spin columns.

PE – wash buffer that removes salts from the column-bound DNA.

Lab 7: Gel Electrophoresis

Gel electrophoresis is a powerful separation technique used for analyzing many biologically important molecules, including both proteins and nucleic acids. Since most biological polymers carry a net charge in solution, they will migrate in an electric field. The molecular migration is stabilized by polyacrylamide, agarose, or some other inert, solid support.

Electrophoretic mobility can then be used to distinguish molecules that differ only slightly in net charge or shape, to detect amino acid residue changes in proteins, to determine protein molecular masses, or the size of polynucleotide fragments. This laboratory exercise focuses on protein electrophoresis.

The function of sodium dodecyl sulfate (SDS) and β-mercaptoethanol (BME) in denaturing gel electrophoresis

An important application of protein electrophoresis requires the use of the denaturing detergent sodium dodecyl sulfate (SDS) and a reducing agent. At neutral pH in 1% SDS and 0.1 M β-mercaptoethanol (BME), most protein molecules unfold, and inter- and intra-chain disulfide linkages are reduced. All higher order protein structure is therefore lost, and multi-subunit proteins separate into their constituent subunits. Each protein chain is coated with a layer of SDS molecules such that negatively charged sulfate groups of the detergent, which usually far outnumber the charged groups belonging to the protein side chains, are exposed to the aqueous medium.

Hence, the presence of SDS confers a large negative charge to the protein-detergent complex. Moreover, SDS is bound to *most* proteins in a nearly **constant ratio (about 1.4 g SDS per g protein)**, which provides a nearly **constant charge to mass ratio**. Consequently, proteins treated with SDS and β-mercaptoethanol and then subjected to electrophoresis migrate toward the anode

at a rate that is determined by the mass of the polypeptide, the porosity of the gel, and the electric field potential. In general, a small molecular mass polypeptide will have a faster rate of migration than a larger one. This observation forms the basis for using SDS polyacrylamide gels to characterize mixtures of proteins based on molecular mass.

Gels of a given porosity (achieved by adjusting the acrylamide and crosslinker concentrations) are calibrated with standard proteins of known molecular masses. The molecular mass of an unknown polypeptide can then be estimated from a standard curve for the given gel system.

Discontinuous gel electrophoresis

An important refinement of electrophoresis is the method of discontinuous gel electrophoresis. A discontinuous gel system consists of two acrylamide gels; one gel is cast directly on top of the other. The lower gel (which is poured first and allowed to polymerize) is called the resolving gel. The resolving gel is so called because resolution takes place in this gel on the basis of size (due to sieving action of the polyacrylamide). The resolving gel contains an acrylamide concentration ranging from 5-30%, depending on the range of protein molecular masses to be separated. The higher the percentage of acrylamide used, the lower the molecular weight range that will be resolved.

The upper gel, called a stacking gel, contains a much lower percentage of acrylamide than the resolving gel (usually about 2-3%), and offers little separation based on molecular mass. Instead, the stacking gel compresses the protein sample into a narrow band before it enters the resolving gel and thereby improves the resolution of the resolving gel. The stacking phenomenon is provided by differences in the pH and ionic strength of the buffers in the stacking gel, the resolving gel, and the buffer reservoir. The larger the volume of the sample to be loaded, the more useful a stacking gel becomes.

The stacking phenomenon

The protein bands are stacked as follows: At the pH of the stacking

gel (6.8), the glycine amino group is predominantly in the protonated form, so that the amino acid is an electronically neutral zwitterion. In contrast, the protein coated with SDS has a net negative charge. In this state, protein has an electrophoretic mobility between that of the glycine zwitterion (not migrating) and Cl-(the fast-moving counter-ion in the discontinuous gel buffer). The Cl-ion migrates rapidly primarily because it is small and is followed by SDS-coated protein (having a large size, but also a large net charge), followed by glycine (which has low net charge due to the pH equilibrium). As the Cl-ion moves ahead of the glycine, a drop in conductivity occurs at the interface between the Cl-ions and the glycine ions. This decrease in conductivity gives rise to a voltage gradient that accelerates the slower moving protein and glycine. Ions ahead of the glycine-chloride interface experience are not exposed to a voltage gradient because the conductivity is not affected in this region: therefore, they are not accelerated. By continued acceleration into a zone of high conductivity, the protein band, which sits between the chloride-glycine interface, is stacked into a sharp band. The formation of a sharp band greatly improves the resolution that can be attained in the resolving gel.

Appendix 4: Buffer formulations

1. **COMMONLY-USED STOCK SOLUTIONS**

The following stock solutions are commonly used in the preparation of many reagents used in molecular biological procedures. Remember to use aseptic technique with sterile stocks. Use deionized or glass-distilled water to make reagents. Decrease volumes made as appropriate. It is also useful to maintain a store of steriledeionized water.

ß-mercaptoethanol (BME)
BME is purchased as a liquid at a concentration of 14.4 M. Store at 4 ˚C in a dark bottle. Do not autoclave BME or buffers containing it. Open BME in a hood.

1.0 M Calcium Chloride
Dissolve 14.7 g of $CaCl_2 \cdot 2H_2O$ in 80 mL of deionized water. Adjust the volume to 100 mL with deionized water. This may be stored at room temperature indefinitely.

100 mM Calcium Chloride(for preparation of competent cells)
Add 50.0 mL of 1.0 M $CaCl_2$to 450 mL of deionized water and mix. Sterilize by autoclaving. Store at 4 ˚C.

0.5 M EDTA, pH 8.0
Dissolve 186.1 g of disodium ethylenediaminetetraacetate•$2H_2O$ in 800 mL of deionized water that is being mixed on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (about 20 g of pellets). The EDTA will not completely dissolve until the pH has reached 8.0. Add the NaOH slowly so as not to adjust the pH above 8.0. Adjust to 1000 mL with deionized water. Dispense into appropriate containers and sterilize by autoclaving. Store at room temperature.

10 mg/ mL Ethidium Bromide
Dissolve 0.25 g of ethidium bromide in 25.0 mL of deionized water.

Stir at room temperature for several hours to ensure that the dye is completely dissolved. Store in a foil-wrapped or brown glass bottle at room temperature.

> NOTE: Ethidium bromide is a mutagen and a suspected carcinogen. Be sure to handle with gloves and wear a mask when weighing. Be careful to avoid spills and immediately clean up any spilled dye or solutions containing ethidium bromide.

3.0 M Sodium Acetate (pH 5.2) (anhydrous MW = 82.03)

Add 24.6 g of sodium acetate (anhydrous) to 70 mL of deionized water and mix to dissolve. Adjust the pH to 5.2 with glacial acetic acid. Make to 100 mL with deionized water. Sterilize by autoclaving and store at room temperature.

5.0 M Sodium Chloride

Dissolve 292.2 g of NaCl in 700 mL of deionized water. Heat may be necessary to completely dissolve the NaCl. Adjust to 1000 mL with deionized water and sterilize by autoclaving. Store at room temperature.

20% SDS

Add 200 g of molecular biology grade sodium dodecyl sulfate (often called sodium lauryl sulfate) to 800 mL of deionized water. Dissolve with heating on a hot plate. Adjust the pH to 7.2 with a few drops of 6N HCl. Adjust to 1000 mL with deionized water. Do not autoclave this stock. Store at room temperature.

> **NOTE:** Wear a mask when weighing out SDS powder.

4.0 N Sodium Hydroxide

Add 160 g of NaOH pellets to 800 mL of deionized water. Dissolve on a stir plate as the solution will get hot from an exothermic reaction. After all pellets are dissolved, allow the solution to cool and adjust to 1000 mL with deionized water. Store at room temperature in a polypropylene bottle (NaOH should not be stored in glass bottles).

1.0 M Tris, pH 8.0

Dissolve 121.1 g of Tris base in 800 mL of deionized water. To avoid inhalation, wear a mask when weighing out the dry powder. Adjust the pH to 8.0 with concentrated HCl (about 42 ml). Cool the solution to room temperature and adjust to 1000 mL with deionized water. Dispense into appropriate containers and sterilize by autoclaving. Store at room temperature.

NOTE: The pH of Tris buffers is temperature-dependent. Make all measurements at room temperature. The pH will decrease approximately 0.03 pH units for each degree Celsius that the temperature increases.

NOTE: Many pH electrodes do not accurately measure the hydrogen ion concentration of Tris buffers. Be sure that the electrode you are using is capable of measuring Tris.

50x TAE
   242 g Tris base
   57.1 mL glacial acetic acid
   100 ml of 0.5 M EDTA (pH 8.0)

1. **DNA AND ENZYME SOLUTIONS**

 DNA solutions (plasmids, lambda, etc.) are generally supplied at a known concentration in TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA). Dilutions of DNA stocks should be made into TE buffer. Wear gloves when handling DNA stocks and exercise caution to avoid nuclease

contamination. Store DNA solutions at 4 °C if you are using them frequently, or at -20 °C for long-term storage.

Restriction enzymes, ligase, and many other DNA modifying enzymes are prepared in buffers containing 50% glycerol and must be stored at -20 °C. The concentrations of commercially available enzymes varies from 5-20 units/ μL, but it is best not to dilute them for use. Dilution often results in rapid loss of activity and is time-consuming. Addition of an excess amount of enzyme makes reactions proceed faster. Some enzymes are purchased as dry powders and are prepared as follows:

Lysozyme (10 mg/ mL in 10 mM Tris, pH 8.0)

Make lysozyme solution immediately (1-4 hours) before use. To 11.9 mL deionized water add, 0.12 mL of 1.0 M Tris, pH 8.0 and 0.12 g lysozyme. Dissolve the lysozyme and keep the solution on ice.

Proteinase K (10 mg/ ml)

Dissolve 25 mg of proteinase K in 2.5 mL of sterile, deionized water. Dispense into sterile microcentrifuge tubes in 500 μL aliquots and store at -20 °C.

RNase A (10 mg/ mL in 10 mM Tris, pH 7.6, 15 mM NaCl)

Add 50.0 μL of 1.0 M Tris, pH 7.6 , 15.0 μL of 5 M NaCl, and 50 mg of RNase A to a total volume of 5.0 mL of deionized water:

Dissolve the RNase A and place in boiling water bath for 10 minutes to inactivated any DNase that is a contaminant. Transfer to sterile microcentrifuge tubes in 500 μL aliquots and store at -20 °C.

Gibson cloning recipes

Gibson Master Mix Isothermal Reaction Buffer:

Makes approximately 9 mL. Store in 350 μL aliquots at -20C.

Component	Amount
1M Tris-HCl pH 7.5	3 mL
1M MgCl2	300 µL
100 mM dNTP	60 µL
PEG-8000	1.5 g
NAD	19.9 mg
1M DTT	300 µL
Water	2.22 mL

Gibson Master Mix:

Mix up the reagents well. The solution is rather viscous, so pipette slowly.

Make 100 uL aliquots and store at -20C.

Component	Amount
Isothermal Reaction Buffer	320 µL
T5 exonuclease (10U/µL)	0.64 µL
Phusion DNA Polymerase (2 U/µL)	20 µL
Taq DNA Ligase (40 U/µL)	160 µL
Water	700 µL

III. MEDIA
LB (Luria-Bertani) Medium
Add to 1000 mL deionized water:

10.0 g tryptone

5.0 g yeast extract

10.0 g NaCl

Dissolve all ingredients and adjust the pH to 7.2 with NaOH (about 1 mL of 4 N NaOH). Sterilize by autoclaving. Prepare agar plates by adding 15 g/L agar prior to sterilization.

The following sterile stocks should be aseptically added to autoclaved and cooled (to 55 C) media. For cold storage, aliquot stocks into small volumes to avoid repeated freezing and thawing.

100 mg/mL IPTG

Dissolve 1.0 g of isopropylthio-ß-D-galactoside (IPTG) in 8.0 mL of deionized water. Adjust the volume to 10.0 mL with deionized water. Sterilize through a 0.2 µm membrane filter and dispense in 2-3 mL aliquots in sterile vials or tubes. Store at -20 ˚C. Add 1.0 mL per liter ofsterilizedmedium that has cooled to 55 ˚C.

NOTE: IPTG can be left out of media used for alpha-complementation if pUC or pGEM-type vectors are being used. These are such high copy number vectors that there is enough expression of the a-fragment of ß-galactosidase to obtain activity (*i.e.*blue colonies).

40 mg/mL X-gal

Dissolve 0.4 g of 5-bromo-4-chloro-3-indoyl-ß-D-galactoside (X-gal) in 10 mL of dimethlyformamide (DMF) in a glass or polypropylene tube. DMF WILL DISSOLVE POLYCARBONATE. Store in 2-3 mL aliquots in foil-wrapped vials at -20 ˚C. Do not sterilize. Add 1.0 mL per liter of sterilizedmedium that has cooled to 55 ˚C, giving a final concentration of 40 µg/mL.

50 mg/ mL Kanamycin

Dissolve 0.5 g of Kanamycin (sodium salt) in 10 mL of deionized water. Sterilize through a 0.2 µm membrane filter. Dispense in 2-3 mL aliquots in sterile 1-mL vials or sterile tubes. Store at -20 ˚C. Add 1.0 mL per liter of sterilizedmedium that has cooled to 55 ˚C, giving a final concentration of 50 µg/ mL. Adjust the volume added accordingly for different concentrations.

Kanamycin is stable for about a month as a stock or in agar plates stored in the refrigerator.

Buffers Needed for SDS-PAGE

For the SDS-polyacrylamide gels you poured, we provided the following reagents:

- 10% SDS Solution: 10 g of SDS dissolved in a final volume of 100

mL of deionized water;

- 40% Acrylamide Solution: This solution is 37.5:1 Acrylamide: Bis-acrylamide.
- 10% Ammonium Persulfate Solution: 0.1 g of ammonium persulfate diluted to a final volume of 1 mL with deionized water;
- TEMED: use as a neat solution;
- 4x Resolving Gel Buffer: Combine 45.42 g Tris Base and1 g SDS then add deionized water to a final volume that is almost 250 mL ,carefully adjust the pH to 8.8 with HCl, and then add enough water to bring the final volume to 250 mL.
- 4x Stacking Gel Buffer: Combine 15.14 g Tris Base and 1g SDS then add deionized water to a final volume that is almost 250 mL, carefully adjust the pH to 6.8 with HCl, and then add enough water to bring the final volume to 250mL.
- 4x SDS sample buffer: For 10 mL: Combine 2 mL 1M Tris pH 8, 0.8g SDS, 4 mL 100% glycerol, 1 mL 0.5M EDTA, and 8 mg of Bromophenol Blue and then add deionized water to final volume. Add a denaturing agent if required, such as 5% BME.
- 5X Laemmli Running Buffer:Combine 152 g Tris base, 720 g glycine, and 50 g SDS add water to a final volume of 10L. This buffer should be pH 8.5, and normally should not be adjusted;
- Staining solution: Combine 450 mL dH2O, 450 mL methanol and 100 mL glacial acetic acid. Add 1.0 g of Coomassie Blue R250. The stain can be re-used a few times but the gel may need to stay in used stain a little longer than in fresh.
- Destaining solution: Combine 875 mL dH2O, 50 mL methanol and 75 mL glacial acetic.

Buffers used for the Ni column (Lab 7)

Resuspension buffer:

50 mM NaH2PO4

300 mM NaCl

Adjust pH to 8.0 with NaOH

You are responsible for deciding on appropriate equilibration,

wash and elution buffers for use on the column, so those recipes are not provided here.

Protein Buffer:

50 mM Tris, pH 7.8

0.1 M K2SO4

Appendix 5: Operation of the Shimadzu spectrophotometers

Precautions:

- Clean up spills in the sample compartment immediately.
- Keep spectrophotometer vents (on sides of instrument) free of any obstructions.
- Use clean, dry hands on the keypads.
- Do not place anything on top of the spectrophotometer.

Spectrophotometer setup

- Remove dust cover from instrument and place in a clean, out of the way area.
- Remove any cuvettes from the sample and reference beams.
- Turn the POWER to ON (left side of instrument). Allow 15 min. for warm-up.
- After initialization, the MAIN MENU will be displayed.

Fixed wavelength measurements

- Press [1] from MAIN MENU to enter Photometric mode
- Select ABS or %T by pressing the [F1] key
- Press the [GOTO WL] key, enter the desired wavelength, and press [ENTER]
- Insert a sample blank into both the front and back slots and press the [AUTO ZERO] key. Be sure to wait until the machine beeps – it takes a minute.
- Insert an unknown sample into the front slot and press [START] to read the sample absorbance (%transmittance) on

the digital display.

Single Scan

- Set up the spectrophotometer as described in 1 above. Then:
- Enter the Spectrum mode from the MAIN MENU.
- Select the MODE (ABS, %T) by pressing [1].
- Select the scan speed:

  ◦ Press [4]
  ◦ Choose a speed (1-5)
  ◦ Press [ENTER]
- Enter wavelength range:

  ◦ Press [2]
  ◦ Enter upper limit
  ◦ Press [ENTER]
  ◦ Enter lower limit
  ◦ Press [ENTER]
- Set the (ABS, %T) range:

  ◦ Press [3]
  ◦ Enter upper limit
  ◦ Press [ENTER]
  ◦ Enter lower limit
  ◦ Press [ENTER]
- Perform a background correction

  ◦ Place the appropriate blank and reference samples in position.
  ◦ Press the [F1] key to perform a baseline correction. This may take a few minutes depending upon settings.
  ◦ Set the sample and reference cells in place.
  ◦ Press the [START] key to start scan.

Time Drive

- Set up the spectrophotometer as described in 1 above. Then:

- Enter the Time Scan mode from the MAIN MENU
- Select the MODE (ABS, %T) by pressing [1].
- Enter the desired wavelength by pressing the [GOTO WL] key, entering the wavelength, and pressing [ENTER].
- Set the (ABS, %T) range:
    - Press [3]
    - Enter upper limit
    - Press [ENTER]
    - Enter lower limit
    - Press [ENTER]
- Set the time scale (sec, min) by pressing [4]
- Set the measurement time by pressing [2]. The measurement interval will automatically be set relative to the length of measurement chosen.
- Press the [START] key to start measurement.

Shutting down

- Turn the spectrophotometer POWER to OFF.
- Replace dust cover on spectrophotometer and make sure all electronics and wires are covered.

This is where you can add appendices or other back matter.