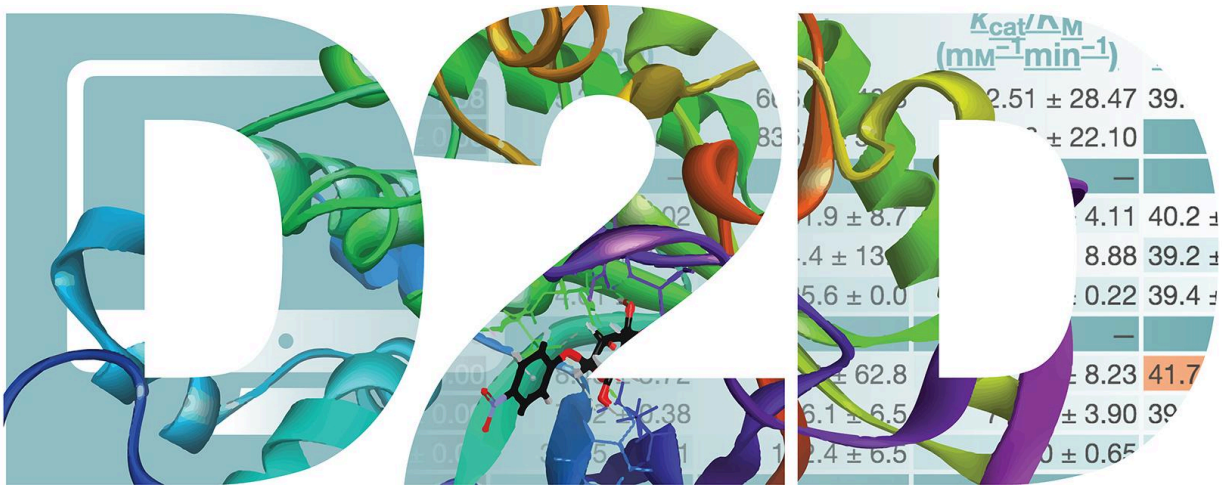
MB&B 251L/
MCDB 301L
Design2Data
with β-Glucosidase
Lab Manual



Version 0.1 (Jan 2024)

Adapted from Design2Data with β-Glucosidase Lab Manual & Workflow (Siegel lab, UC Davis, 2023) for Yale University MB&B 251L/MCDB 301L by Dr. Claire Laxton and Dr. Freya Wencker, Gerard Walker, Yara Mustafa and Dr. Katherine Schilling.

Special thanks to Dr Allen Esterly, Dr Najla Arshad, Matthew Spencer and Ying-Chun Tong, as well as the 2023/24 MB&B 251L/MCDB 301L students for their feedback, which has shaped and improved this manual for future classes.

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Design-to-Data Workflow Overview

The Design-to-Data workflow was developed in the Siegel Lab at UC Davis with the central research of improving the current predictive limitations of protein modeling software by functionally characterizing single amino acid mutants in a robust model system. This workflow is undergraduate-friendly, and students have an opportunity to practice protein design, Kunkel mutagenesis, and enzyme characterization assays. The workflow is intuitively organized through engineering's conceptual progression of design-build-test.

This version of the D2D lab manual was adapted by academics at Yale to meet the requirements for the MB&B 251L / MCDB 301L classes.

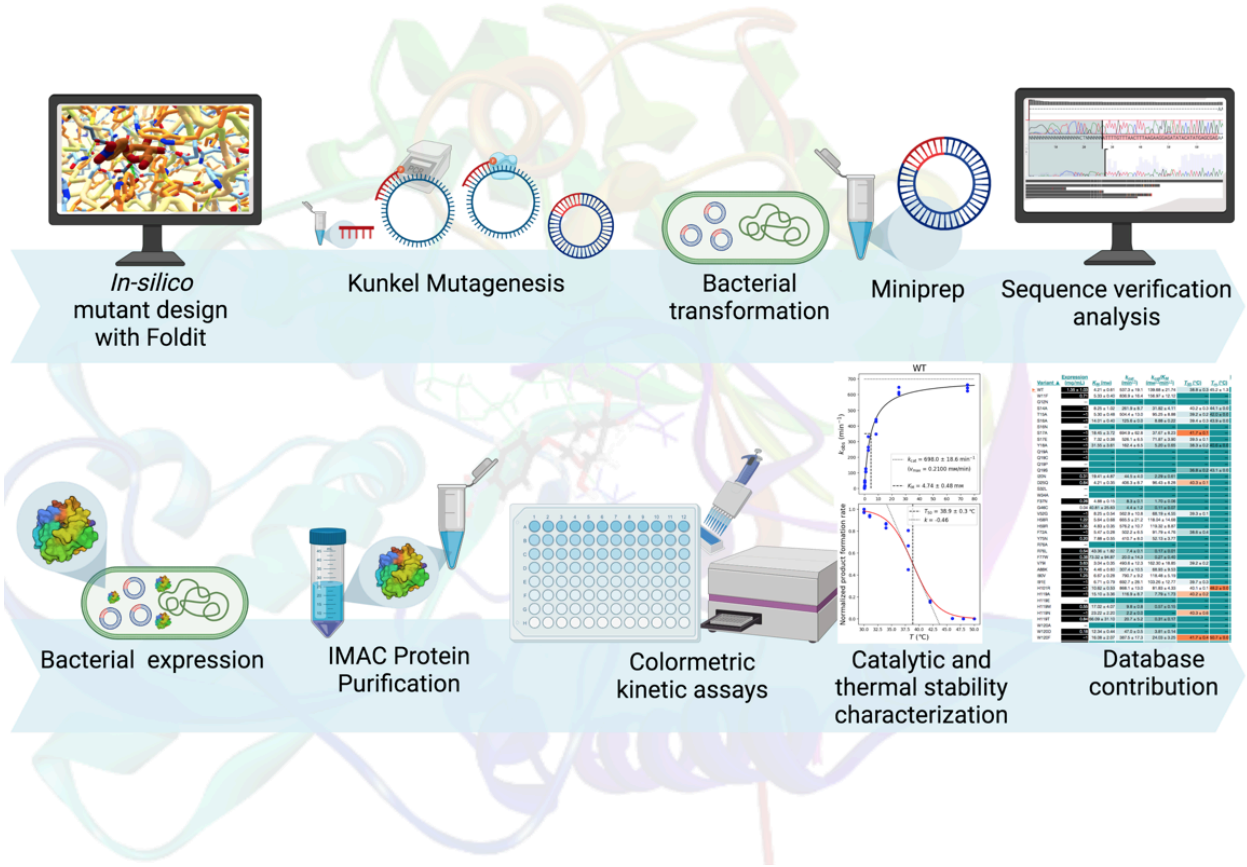


Figure 1. Design to Data: milestone features that comprise the D2D workflow progression.

D2D Progression Summary and learning objectives

Table 1. Design-to-Data Workflow Summary. *Design* activities are highlighted in blue, *Build* activities are highlighted in green, *Test* activities are highlighted in yellow, *Learn* activities are highlighted in red. *For all Test activities, instructors may demonstrate with the wild-type BglB, to produce WT protein for the kinetic assays.*

Time required in class	Activity	Research and Learning Goals, Activity Descriptions	Next Steps and Stop Point Notes
1 hour	Lab safety introduction	Learn about how to operate safely in the lab (may contribute toward credits for some students)	Delivered by Yale EHS team
2 hours	Pipetting 101	Utilize micropipettes with accuracy and precision. Evaluate spectrophotometer data to assess pipetting success. Orient to lab equipment. Establish teamwork, skills, and principles.	Must be completed before any wet lab research takes place.
30 min	Design-to-Data Project Intro	Articulate how protein structure informs function. Explain how increased data on enzyme structure and function contribute to innovative product development.	N/A. This is not currently a written section but is a key component of the workflow.
2.5 hours	Foldit Protein Design (computer lab)	Utilize protein modeling software to assess the effect of a mutation, Analyze hypothesized enzyme structure to predict functionality.	
2 hours	Oligo Phosphorylation	Apply a molecular process that facilitates oligo phosphorylation. Explain how this reaction fits into the Kunkel Mutagenesis unit.	Stop point. Phosphorylated oligos can be frozen indefinitely.
3 hours	Oligo/Plasmid Annealing and Polymerization	Apply molecular processes that facilitate oligo to plasmid annealing and polymerization. Explain how these reactions fit into the Kunkel Mutagenesis unit.	Stop point. Polymerized products can be frozen indefinitely. **Cannot stop between annealing and polymerization steps.**
3 hours	Plasmid Production Transformation	Apply technical processes that enable heat shock transformation and plasmid production. Explain how these processes fit into the Kunkel Mutagenesis unit.	Transformed cells must grow for 24 hours and then transfer to the fridge. Parafilmed plates can remain in the fridge for two weeks. *Instructors to store plates after 24-48 hr incubation*
1 hour	Overnights	Use sterile technique to transfer colonies to the media and grow overnight. Explain how this step is necessary for Miniprep.	Overnights can be spun down and stored in -20°C. *Instructors to perform this step the day before class.*
3 hours	Plasmid Extraction (Miniprep)	Extract plasmids from bacterial culture using a commercial spin column-based kit. Describe why sequencing at this point is essential to the workflow.	Extracted plasmid DNA can be frozen and remain that way indefinitely.

1 hour	A260 assay	Analyze A260 absorbance values to assess extraction quality. Describe how spectrophotometer can generate this concentration data.	N/A
1 hour	Sequencing plate/tube prep	Prepare tubes for plasmid sequencing to determine the mutant transformation success. Describe the reason sequencing must take place in both the forward and reverse directions.	Samples are typically sequenced in three business days. *Instructors to submit through Keck after class.*
2 hours	Plasmid Sequence Analysis (computer lab)	Organize, manipulate, and analyze plasmid sequence data to identify successful mutations to carry forward into test modules of the workflow.	N/A
2 hours	Protein Production Transformation	Conduct heat shock transformation using BLR *E. coli* to induce cells to uptake and produce protein. Explain how this process fits into the Protein Test Unit.	Transformed cells must grow 18 to 24 hours and then transfer to the fridge. Plates can remain in the fridge for two weeks. *Instructors to store plates after 24-48 hr incubation.*
1 hour	Protein Growth and Expression Cultures	Use sterile technique to grow transformed cells and induce protein production through gene expression manipulation. Explain how these processes fit into the Protein Test Unit.	This step requires two sequential overnight cultures. *Instructors set up overnights the day before class, and spin cells down to freeze pellets 24 hrs after class.*
4 hours	Protein Purification	Extract histidine-tagged protein from bacterial culture using bead-base chromatography. Describe the biochemical properties that facilitate this process.	Purified protein is stored in the fridge (4°C) until use (or snap frozen and stored at -80 ˚C for long term storage).
1 hour	A280 Assay	Analyze A280 absorbance values to assess extraction quality. Describe how a spectrophotometer can generate this concentration data.	N/A
2 hours	SDS Gel Set Up	Use gel electrophoresis to analyze protein purity. Describe the importance of purity when analyzing data from functional assays.	
1 hour	Bradford Assay set up	Prepare 96-well plate for Bradford assay using serial dilutions and biological replicates that are assessed by spectrophotometer plate reader. Explain the different merits of protein quantification by Bradford vs A280.	To be done while SDS-PAGE gels are running
3 hours	Enzyme Kinetic and Thermostability Assay	Prepare 96-well plate for colorimetric kinetic assay using serial dilutions and biological replicates that are assessed by spectrophotometer plate reader. Conduct the assay after incubation of samples at multiple temperatures to assess the thermal stability of the enzyme. Describe how Michaelis-Menten curves and associated values of interest are produced from this data.	N/A
3 hours	Characterization Data Analysis (computer lab)	Analyze spectrophotometer data to integrate results and make inferences about mutant functionality in the context of thermal stability.	N/A

	Lab report write up	Write a lab report describing the experiments undertaken, present the findings and discuss the impact of a mutation on enzyme kinetics and thermal stability.	N/A

Pipetting 101

This protocol is based on work created by Marc T. Facciotti, Andrew Yao, and Hyunsoo Kim. The protocol underwent a big revision in Spring 2022. You can see the original protocol in the UC Davis Google Doc History in versions prior to April 4th 2022.

Overview

Mastering the skill of accurately measuring and transferring both large (1 mL is about half of a tablespoon of liquid) and small (50µL is equivalent to one raindrop) volumes of liquids is critical to successful work in the biotechnology lab. This module will introduce you to several common fluid handling tools, how to achieve the most accurate results, the proper techniques associated with their use, and the chance to practice this skill with a variety of different solutions. In the molecular biology lab, two main tools are used for manually moving volumes of liquid in the milliliter (mL - 10^{-3} L) or microliter (µL - 10^{-6} L) scale:

Micropipettes - Micropipettes are useful for moving liquids in the 0.1 µL to 5 mL range. In our lab, we have pipettes capable of handling volumes from 0.5 µL to 1 mL. Each micropipette (pipette for short) is segregated to specific amounts of solution as shown in the pictures below:

Learning Objectives

1. Demonstrate basic principles about how pipettes work.
2. Demonstrate ability to accurately weigh materials using a laboratory scale.
3. Demonstrate how to select an appropriate pipette for a fluid transfer task.
4. Demonstrate how to properly operate a pipette (e.g. select volume, transfer fluid, select/dispose of tips, etc.).
5. Demonstrate the ability to accurately create specific solution mixtures using different pipettes.
6. Demonstrate the ability to create reproducible microliter-scale dilutions.
7. Learn how to use the plate-based spectrophotometer to quantitatively assess your dilutions.

Summary

The goal is to become familiar with using pipettes. By improving our technique, we improve accuracy and secure data integrity. To do this, you will practice suctioning up water, ethanol, and glycerol. At the end of this exercise, you will create a colorful pattern in a 96-well plate and

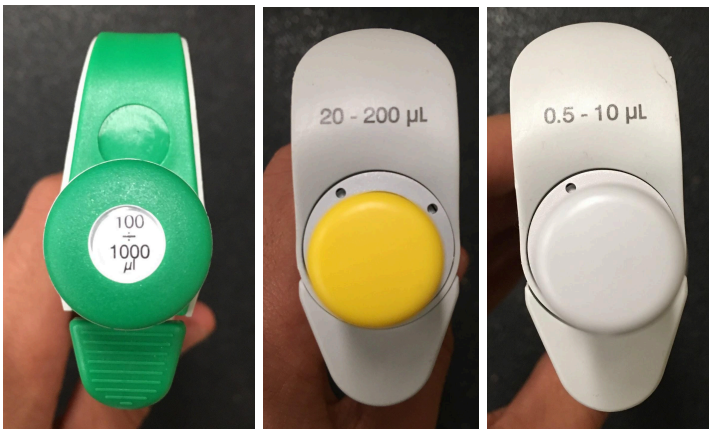
compare your answers to a reference plate to perform a statistical analysis of how accurate your results were.

Pipette Introduction

Safety and Trash Management

Chemicals and/or biologicals: *Food dye; water; ethanol; glycerol*
Materials: *Disposable pipette tips; plastic weigh boats; serological pipettes; plastic tubes*
Equipment: *Pipettes; pipette-aid; scale; Vortexer; centrifuge; Plate reader*
Required PPE: *Lab coat, gloves and eye glasses while the wet-work is happening*
Trash and disposal: *Pipette tips: Dispose of these in marked plastic lined collection bins on each lab bench*
1.5 Eppendorf tubes: discard leftover solutions in the sink, quickly rinse the tubes and place them in a marked collection box near the sink
Solutions: dump into sink when done
Hazards: *Ethanol is flammable - use small volumes and keep away from ignition source; Ensure that all tubes are fully closed before mixing or using Vortexer; Ensure that centrifuges are balanced*
Possible Nuisances: *The colored dye solutions are food grade but may stain clothing - use your PPE*

Note: While the volume ranges and button function is consistent, the appearance of the pipettes you will find in the lab will vary.



The pictures above illustrate how to differentiate which pipette to use depending on the amount of liquid needed to transfer.

1. The left hand photo has a minimum amount of 100µL and a maximum amount of 1mL (or 1000µL). These are used for ‘large amounts’ of liquids.
2. The middle image ranges from a minimum amount of 20µL to 200µL

3. The right hand photo ranges from .5µL to 10µL. This is a relatively small volume to be transferred.

How do we change the amount of liquid that the pipette will take up?



Notice the minus (-) to plus (+) rotation located on the pipette

- Rotate the knob right to left to either increase or decrease the amount of liquid to be suctioned.
- The pipette volume you set should only go as high as the maximum and as low as the minimum indicated by the pipette.

In theory, the knob can be turned to set volumes above the maximum and below the minimum, but this can damage the pipette and reduce the accuracy of future measurements.

What amount will I aspirate through my pipette?



The pipette has a numerical value indicated on the front

- Some pipettes (like the one in this image) have a line that indicates whole numbers and decimal values.
- In this image the top number is the tens place, the next is the ones, then the tenths and hundredths
- This system is common on small quantity pipettes, and the decimal places are omitted as size increases

How do I aspirate liquids?



A: Shows the neutral position

B: Illustrates the first stop, used to suction up liquid
C: Shows how to expel liquid.

1. To suction liquid start at a neutral position (**A**). There is no pressure on the pipette handle yet and it is already set to a designated amount indicated by the numbers on the front.
2. Have your media ready and press down on the pipette until you feel it start to resist (known as the first stop and indicated by picture **B**).
3. Place the pipette tip into the media and SLOWLY release pressure. You will notice the liquid begin to move up the pipette tip. You should return to position **A**.
4. Lastly, find the container you hope to place the recently suctioned media in, and press down to the second stop (indicated by picture **C**) to expel all of the liquid.

The impact of immersion depth and angle on precision and accuracy is very important! A vertical angle and immersion depth at about 1 cm guarantees the most accurate pipetting result as seen below.

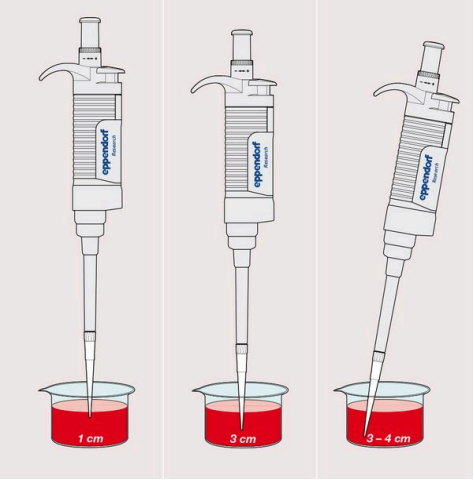


*Image from Eppendorf

If any reagents are missing or you are unable to find them, please do not hesitate to ask an Instructor, TA, or Learning Assistant

Ask questions if you are confused. The Instructor and TF will be present to give additional pointers on how to improve your individual technique and help you locate the materials needed.

Essential tips before getting started

- You do NOT need to watch your groupmate do everything or help hold their tubes. Do your own pipetting.

- Do not pipette “blind.” Be sure that you can *see* the liquid moving into and out of the pipette and where it lands in the tube.
- Work SLOWLY. Bubbles are the enemy.

Instructions

Tools and disposables	***Equipment***	***Reagents and Colored Buffer Stocks***
1 - 100 µL Pipette or 200 µL Pipette (yellow top) 1 - box of 1000 µL Tips 1 - box of 200 µL Tips 6 - 1.5 mL conical tubes 1 - plastic weigh boat 1 - 96 well plate	Digital scale Centrifuge(s) Vortexer Plate reader	1 - Green Stock in 80% ethanol 1 - Yellow Stock in H_2O 1 - Red Stock in 70% glycerol 1 - tube of 70% glycerol 1 - tube of 80% ethanol 1 - tube of H2O

Comparing Weight and Volume

Follow the instructions to practice micropipetting, then respond to the question set. **Each person should do this independently from their partner.**

1. Place a plastic weigh boat onto the digital scale on your bench.
2. Press the Zero/Tare button on the scale.
3. Find a 100 µL or 200 µL Pipette and a box of matching tips.
4. Set your pipette to pipette 100 µL.
5. Open the cap to the 1.5 mL stock of H2O located at the weighing station.
6. Carefully open the tip box without touching the inside of the box or the lid.
7. Attach the pipette to a single tip by pressing the pipette shaft firmly onto a tip.
8. To begin pipetting, push down on the plunger to the FIRST stopping point.
9. Keeping the pipette vertical, place the pipette tip just under (~1 cm) the H2O surface.
10. SLOWLY release the plunger without letting go. *Never separate your thumb from the plunger while liquid is in the tip*
11. Pause for about 1 second after aspirating and before removing the tip from the liquid to allow the liquid to settle.
12. Visually use your eyes to inspect the fluid in the tip - are there any bubbles? How far up the tip does the liquid reach? Has the desired volume been aspirated?
13. Gently touch your tip to the bottom of the weighing boat and SLOWLY push the plunger to the FIRST stopping point. *It is important to always expel the liquid directly on the surface/bottom of the vessel or the side of the tube, or into the liquid already in the tube*
14. Before removing the pipette from the weight boat, press the plunger to the SECOND stopping point to ensure all the liquid is expelled and the liquid does not stick to the tip.

15. Expel the tip into the biotech collections bucket on your bench by pressing the button below the plunger.

When practicing, ensure you follow this technique for the most accuracy! Here you can see how your results are affected by not immersing the pipette at the correct angle or depth when aspirating.

How much does this volume of liquid weigh: _____ g

16. Repeat Steps 2-14 above for 70% glycerol (labeled: “70% Glycerol”) and 80% ethanol (labeled: “80% EtOH”):

70% glycerol weight: _____ g

80% ethanol weight: _____ g

Do these values make sense? Why?

What about pipetting the 70% glycerol and 80% ethanol solutions feel different than pipetting the water solution?

*****KEY DATA: Density of H_2O = 1g/ml; Density of 70% glycerol = 1.205 g/ml; Density 80% ethanol = 0.885 g/ml *****

17. Calculate the percentage difference between the mass you recorded, and the mass you should expect. The error should be less than 10%, record this in your lab book. If your error was higher than 10%, try the exercise again to gain additional pipetting practice.

Pipetting a 96-Well, multicolored solution plate

The goal of this exercise is to practice pipetting by recreating a colorful pattern in a 96-well plate; 6, 24, 96, or 384-well plates are standard tools in research labs. At the end of this exercise, you will compare your plate to our reference plate (prepared by our resident expert pipette), and group(s) should strive to most closely match the reference plate.

You will need these 3 solutions from the kit:

- 1 - Green Stock in 80% ethanol
- 1 - Yellow Stock in H_2O
- 1 - Red Stock in 70% glycerol

1. Make each of the following solutions in different six different microcentrifuge tubes:

   Solution 1: Add 300 uL of Green to 341 uL of 80% EtOH

   Solution 2: Add 320 uL of Yellow to 460 uL of Milli Q H_2O

   Solution 3: Add 236 uL of Red to 364 uL of 70% Glycerol

   Solution 4: Add 41 uL of Solution 1, 180 uL of Solution 2 into 379 uL of Milli Q H_2O

   Solution 5: Add 240 uL of 70% Glycerol to 260 uL of Milli Q H_2O

   Solution 6: Add 100 uL of Solution 5, 100 uL of Solution 4, 100 uL of Solution 3, 100 uL of Solution 2, 100 uL of Solution 1

Notes for successful completion: Be sure to change pipette tips every time you change the liquid you are pipetting. This will help avoid contamination. In addition, glycerol is much more viscous than water, so be sure to mix and pipette slowly to get more accurate results.

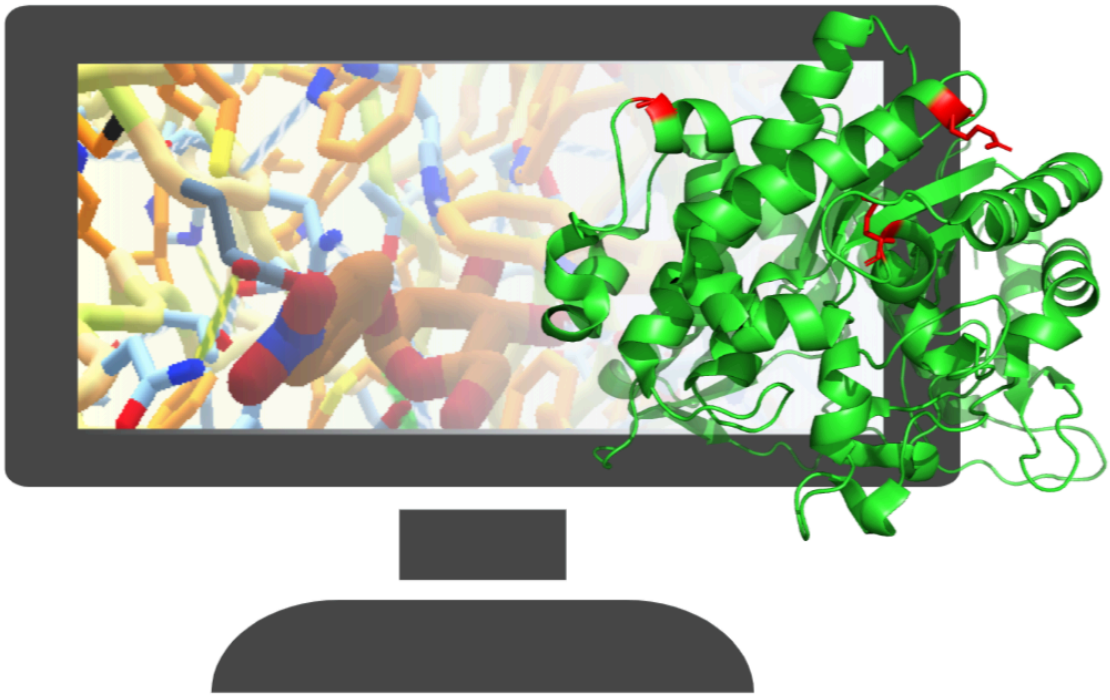
2. Mix solutions by either (1) inverting the tubes, (3) vortexing, and (3) pipetting up and down until uniform - try each of these methods, you'll be using all of them in the coming weeks. Briefly spin the tubes using a microcentrifuge or pulsing the samples in a centrifuge for ~5 seconds.
3. Pipette 150 µL into the wells highlighted in dark gray. Pipette 50 µL of each solution into the wells highlighted in light gray below. Avoid bubbles!

Table 4.1. The table below models the first 6 columns and first 2 rows of a 96-well plate; the outermost row and column names are indicated by the letters A-D and 1-12, respectively. Solutions should be deposited in the shaded wells/table cells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6						
B	6	5	4	3	2	1						
C												
D												

4. Use the BioRad iMark microplate reader to read absorbance at 415 nm in endpoint mode. Compare the values you get to the instructor's to see how accurate your pipetting is.
5. As before, calculate the percentage difference between your values and the instructors, and note these down in your lab book.

Module 1: Design



Designing β-Glucosidase Mutants: Foldit

You will be using Foldit, a graphic user interface (GUI) and online-game software, to examine the effect of a mutant on the β-glucosidase (BglB) enzyme from *Paenibacillus polymxa* that you will go on to characterize using the Design2Data workflow. Foldit runs using Rosetta protein modeling software. A good synopsis of the software and game can be found on Wikipedia: https://en.wikipedia.org/wiki/Foldit

More information describing the Foldit Standalone version that we'll be using can be found in the following paper: Kleffner, R., Flatten, J., Leaver-Fay, A., Baker, D., Siegel, J. B., Khatib, F., & Cooper, S. (2017). Foldit Standalone: a video game-derived protein structure manipulation interface using Rosetta. *Bioinformatics*, **33**(17), 2765–2767.

Learning objectives

- Use Foldit **Education mode** to learn about the effect of amino acid variation on protein structure and function
- Use Foldit **Standalone** to predict the effect of amino acid substitutions on protein stability.
- Use conceptual understanding of protein structure and data to predict the effect of amino-acid substitutions on protein stability and catalytic efficiency.

We will be conducting this section during the Foldit lab class in the computer room in the Marx Library (C27). However, if you wish to play with the software ahead of time, you can *optionally* install Foldit Standalone on your own device by following the installation instructions at the end of the manual.

Foldit Education mode games

These games will help you familiarize yourself with the Foldit software, as well as how the basic properties of amino acid residues affect protein folding.

1. Load **Foldit** (in education mode, NOT standalone) on your computers and complete the following games

(Click on the green 'eye' on the bottom left to try some different visualization options, try switching to 'Detailed' mode for this part so you can better see the sidechains).

- ☐ Intro to Foldit
- ☐ Swing it around
- ☐ Hide the hydrophobic

- ☐ Intro to Design
- ☐ Primary Structure

Using Foldit Standalone to model BglB mutations

Downloading BglB Data Files

1. Navigate to the Google Folder: Foldit Files.
2. Download all five *Foldit BglB data files* onto your desktop.

Alternatively, the files can be downloaded from http://www.d2dcure.com/resources/. You should see files that end in the following:

.pdb .cnstr .params
.puzzle_setup .conf.pdb

3. If you hit 'Download all' it will be extracted as a .zip file. Make sure to extract all files by right clicking and hitting 'extract' before importing.

Navigating Foldit

1. Open Foldit Standalone. You will be taken to a splash page with the options to either (1) "Import PDB/Fasta" or (2) "Load Session". **Select "Import PDB/Fasta".**
2. Select all the data files you just downloaded from the Google Folder.

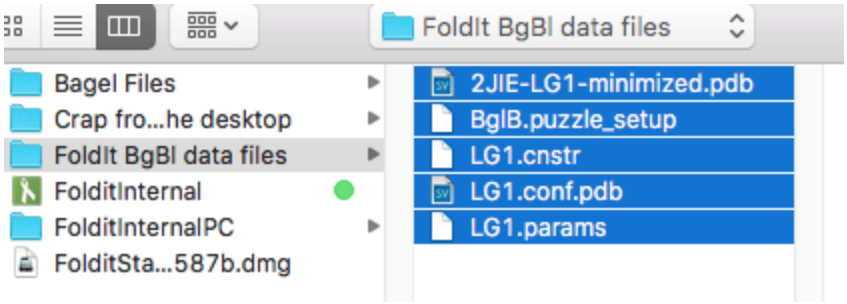


Figure 1.2. All five data files highlighted. These *must* be opened at the same time for the protein to be properly imported into Foldit.

It can take a bit of time for the protein to load—be patient. Once the protein is loaded, you should see it as illustrated in Figure 1.3.

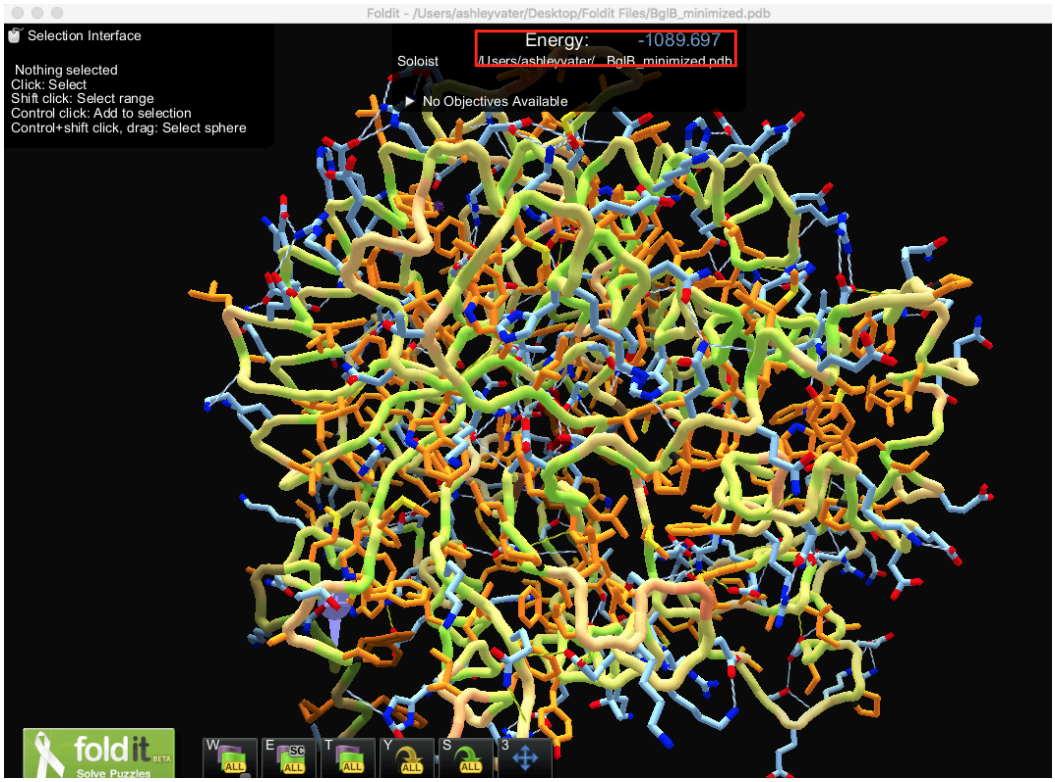


Figure 1.3. BglB wild type enzyme structure. The top left shows that we are in selection interface mode. As highlighted by a red box (top-middle), the energy/starting score is **−1089.697**. From this angle, we can just barely see the ligand (brown).

Changing Settings to Improve Enzyme Design Visualization

1. Observe the Foldit home buttons (lower left, green buttons on black banner) to bring up options and functions.



Figure 1.4. Options and functions for Foldit via the left-hand side icons.

2. Choose “View Options”, then “More view options”. This will bring up a dialog box. (You may have to drag the box up, into the window to see all the selection options.)

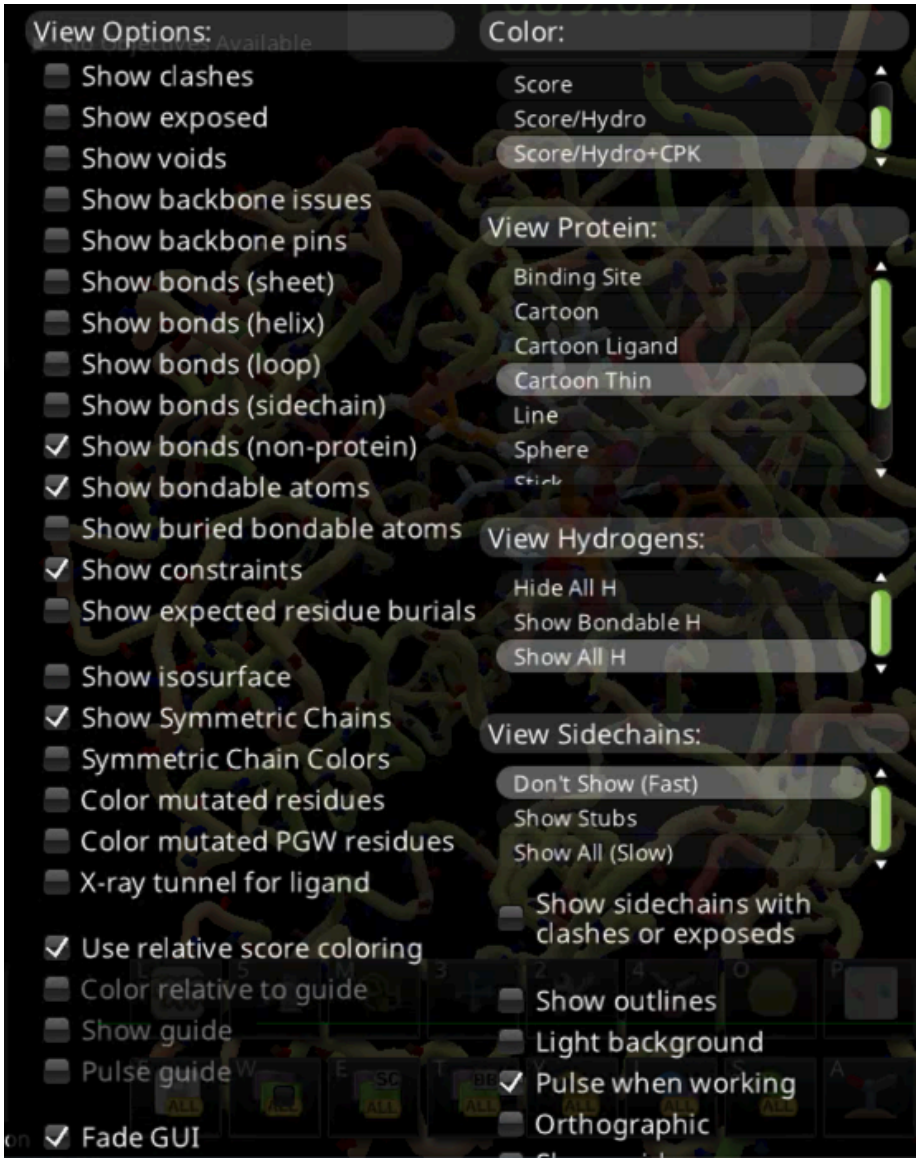


Figure 1.5. Best “View Options” for viewing and mutating BglB.

3. From the Color subsection, select “Score/Hydro+CPK”. Note that oxygen atoms appear red, and the nitrogen atoms are blue. Under “View Protein:” select “Cartoon Thin”. Use all other default options. Under “View side chains” select “Don’t Show (Fast).” Also untick “show voids” and “show clashes” for now.

If you experience color blindness, there is a Foldit setting that should make the visual information much more accessible. Follow this link for details.

Finding the Active Site

4. Click and drag the background to turn the protein around to better visualize the ligand and the active site. You can also zoom in and out to better see the areas of interest.

- The ligand is the large brown molecule, which can be seen in Figure 1.6.
- When orienting the protein by clicking and dragging, **DO NOT** click the protein itself because that can change the protein's conformation and affect your score.
- **If you think you may have accidentally tweaked your protein structure**, use "Control or "Command + Z" to return to the original score.

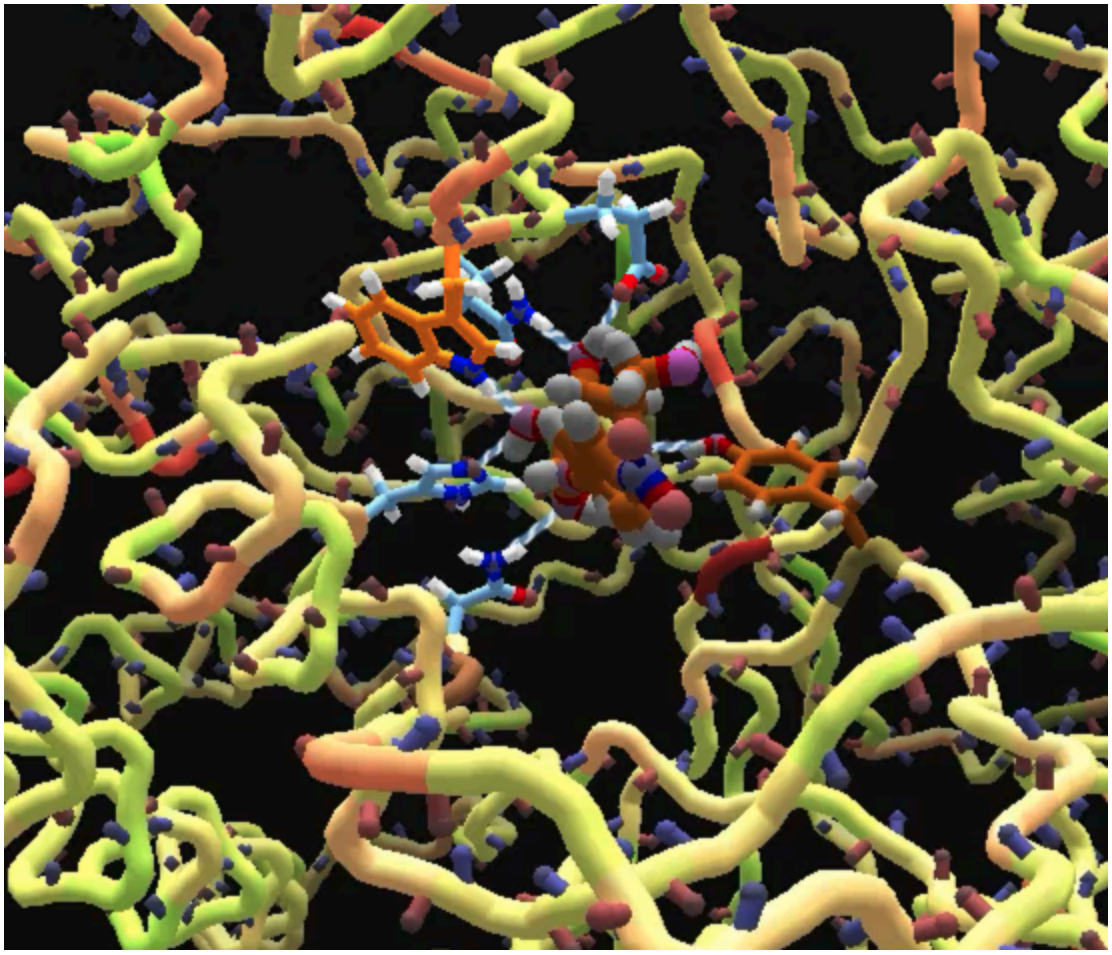


Figure 1.6. The ligand and area of the active site. Ligand shown in brown.

Mutating a residue

You will be assigned a mutant which you will model using Foldit, to assess its impact on protein structure, and folding energy (ΔG).

ΔG denotes the folding energy of the protein and thus the energy required to break the interactions which stabilize folding. If the ΔG is closer to zero, then less thermal energy is required for unfolding, therefore the structure is less thermally stable. Conversely, the more negative the ΔG is, the more stable the folding of the structure is.

1. Click on the background (black space) and use the “B” key to bring up the “Blueprint” panel. This brings up the primary amino acid sequence for the BglB protein. Use this panel to locate your specific residue, by scrolling along. When you click on an amino acid in the Alignment Panel, it will highlight (turn pink) in the protein.

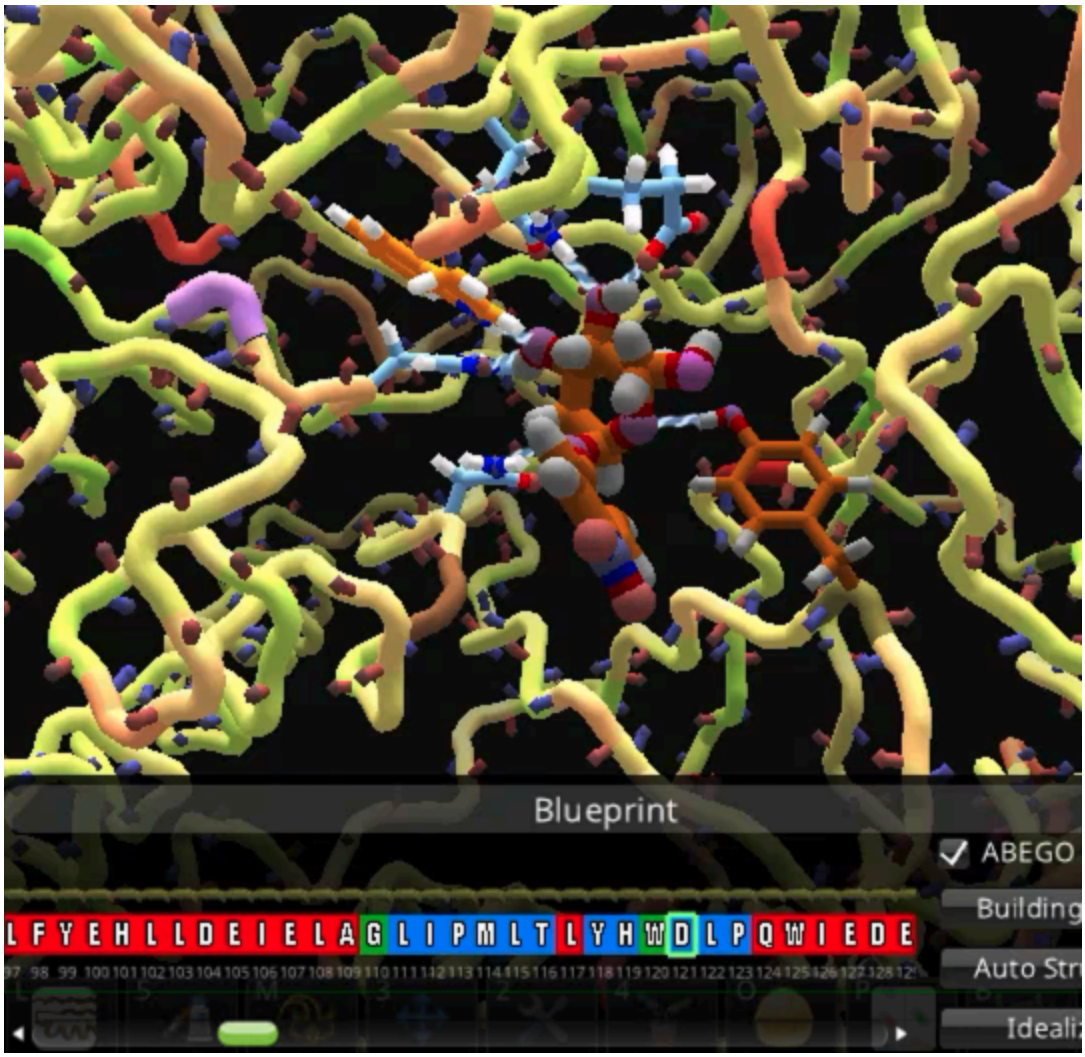


Figure 1.8. Example of the Alignment Panel in Blueprint mode in Foldit.

2. To zoom around and look at specific residues, hold your left mouse button down over the residue of interest and hit “shift” + “Q.” This will zoom your focus into just the residue you are interested in. If you mouse over or click on the background and hit “shift” + “Q,” you will zoom out, back to the whole enzyme.

3. While navigating around, ensure that your score is the same as the original you started with. If it's not or if you are unsure, use "Command" or "Control" + "Z."

Another way to confirm that you have not modified the score is to use the hotkey: "Alt+ U." or hit the green rewind arrow in the home menu. This will bring up a graph that shows the energy changes that you've made. You can click on different parts of the graph to go back.

4. Click on your residue of interest and then press the Tab key to bring up residue information (Segment Information).

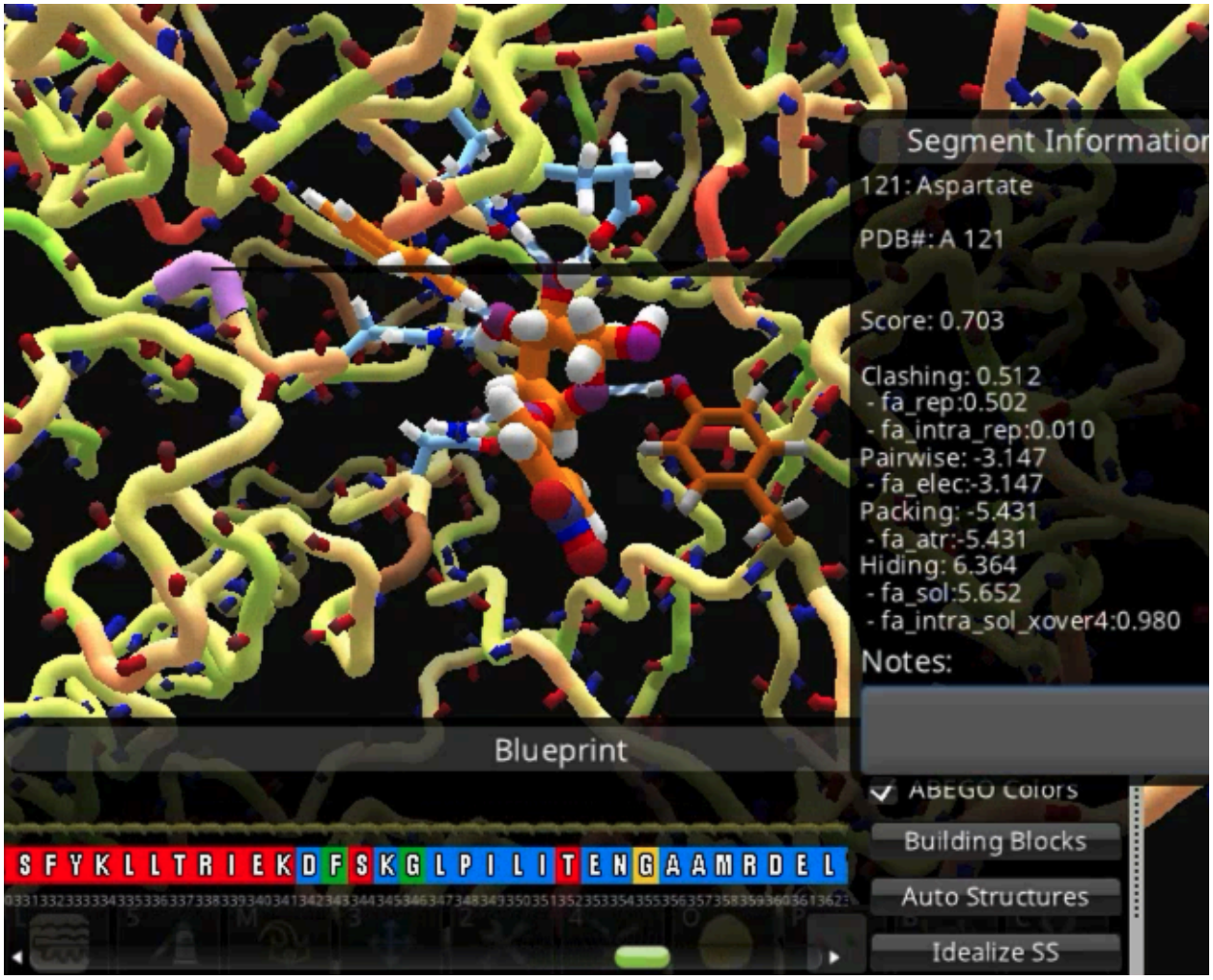


Figure 1.7. Segment Information revealed. Highlighted residue shown in pink is amino acid #409. This residue is tryptophan. The score listed in the "segment information" box is called the "Ligand Energy Score" (e.g. -3.036) and represents this particular residue's contribution to the overall score. Changes in this score may be interpreted as especially meaningful in comparison to overall energy score changes after making a mutation.

5. Before mutating, take a note of the ligand energy score of the wild type residue.

(Click on the window icon at the center bottom of your screen and search for the snipping tool, drag over the section you wish to capture and save the file to your drive).

6. Use the “M” key to bring up mutation options. Your residue will pulse between blue and pink.
7. Note down the new ligand energy score and take a screenshot of your mutant residue using the Windows Snipping Tool. Use the mutation tool to revert the selected residue back to the wild-type, and also take a screenshot of this.
8. Navigate to the in-class FoldIt assignment on Canvas and complete the assignment.

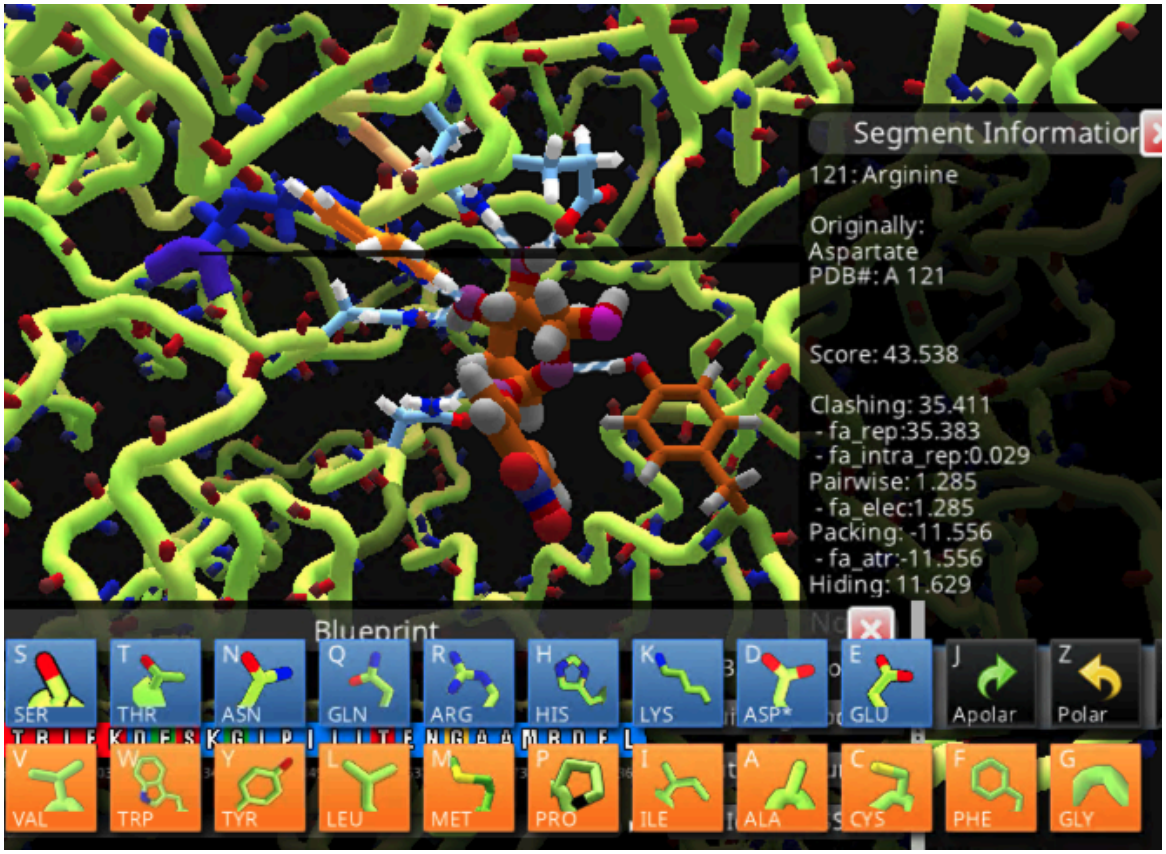


Figure 1.9. Amino acid options pulled up using the “M” key, and the resulting mutation following selection of Arginine from Aspartate.

You will need your screenshots for your lab report, so make sure to save them somewhere safe for later

Hypothesize the impact of your mutation

For the following exercise, you will need these definitions:

- Km = the binding affinity of the substrate to the enzyme
- Tm = the melting (i.e. unfolding) temperature of the enzyme
- T50 = the temperature that an enzyme lose 50% activity

- Kcat = the turnover number – the number of substrate molecule each enzyme site converts to product per unit time, and in which the enzyme is working at maximum efficiency
- Km/Kcat = the overall catalytic efficiency of the enzyme (the higher the better)

1. Search your mutation in the D2D database and compare the previously observed effects of the mutation as compared with the wild type.

Variant	Yield, c_E (mg/mL)	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($mM^{-1}min^{-1}$)	T_{50} (°C)	T_m (°C)	c
WT	1.40	3.92 ± 0.47	377.2 ± 11.4	96.32 ± 11.83	42.1 ± 0.4	—	
WT	0.90	2.07 ± 0.29	251.8 ± 8.2	121.44 ± 17.72	39.4 ± 0.3	—	
WT	1.70	1.63 ± 0.61	190.3 ± 16.4	116.93 ± 45.24	39.8 ± 0.4	—	
WT	1.70	2.36 ± 0.92	252.5 ± 23.1	106.78 ± 42.65	40.1 ± 0.5	—	
WT	1.50	2.32 ± 0.29	685.8 ± 20.3	295.82 ± 38.59	38.2 ± 0.4	—	
WT	4.09	6.62 ± 0.49	400.3 ± 8.5	60.45 ± 4.70	—	43.2 ± 0.0	
WT	2.10	5.64 ± 0.45	430.7 ± 9.5	76.31 ± 6.35	—	—	
WT	0.86	4.29 ± 0.80	723.3 ± 34.8	168.79 ± 32.36	41.0 ± 0.4	—	
WT	1.20	5.98 ± 1.75	1,423.4 ± 108.7	238.05 ± 71.98	40.9 ± 2.9	—	
WT	1.44	4.66 ± 0.68	707.1 ± 27.3	151.73 ± 22.98	40.7 ± 0.2	—	
WT	1.49	5.38 ± 0.79	846.3 ± 27.0	157.26 ± 23.73	40.3 ± 0.2	—	
W11F	0.71	5.33 ± 0.40	836.9 ± 16.4	156.97 ± 12.12	—	—	
G12N	—	—	—	—	—	—	
T13K	*	—	—	—	—	—	
T13S	1.51	2.95 ± 0.33	408.5 ± 11.1	138.60 ± 15.98	—	46.3 ± 0.6	

Figure 1.10. Screenshot from the D2D database of data from previous students, showing the effect of different mutations on enzyme yield (ie. amount of protein purified), catalytic efficiency (Kcat/Km), enzyme turnover (Kcat), binding affinity (Km) and thermal stability (Tm and T50).

2. Based on your observations from FoldIt, and the info in the D2D database for your mutation, fill in the below statement and submit to Canvas.

   I hypothesize that β-glucosidase mutant _____ will demonstrate increased/decreased/the same (indicate one) catalytic efficiency (k_{cat}/K_M), enzyme turnover rate (k_{cat}), estimated binding affinity (K_M), and/or thermal stability (T_m) in comparison to the wild type because its Foldit score suggests a _____, and previously published data on the enzyme kinetics of similar mutations _____, _____ support this hypothesis.

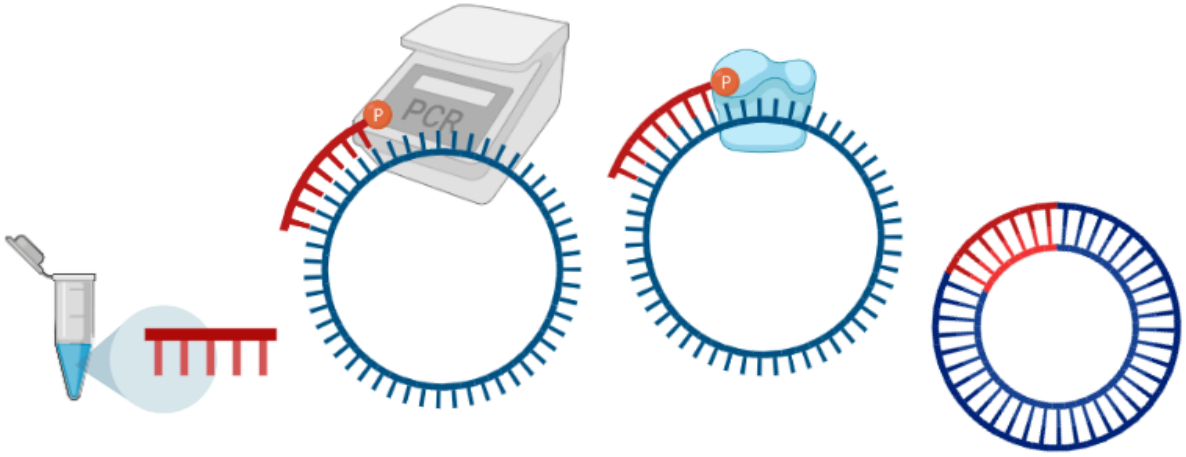
Notes and Helpful Tips

- Orange represents that the residue is hydrophobic.
- Cyan represents that the residue is hydrophilic.

- Hydrogen bonds are shown as blue or green candy-cane-looking linkages between atoms. Confusingly, the green linkages do not represent disulfide bonds but instead indicate that a hydrogen bond is “unhappy”. The blue hydrogen bonds are “happy”.
- Enzyme variants are named by standard convention, e.g., H243W. This translates as: "at amino acid #243, the wild-type histidine is changed to tryptophan.”
- Link to Amino Acid Codes: https://www.compoundchem.com/2014/09/16/aminoacids/

Module 2: Build

Kunkel Mutagenesis



Module Build Overview

Learning Objectives

- Describe the role of various components of recombinant DNA technology in producing recombinant proteins in bacterial cells
- Understand the Kunkel mutagenesis approach we use to produce BglB mutants
- Explain the purpose of the Kunkel mutagenesis steps, including oligo phosphorylation, plasmid annealing and phosphorylation

Introduction

We will use bacteria *E. coli* to produce BglB mutant proteins. Here is a quick and very general video introducing the concept of recombinant proteins with an insulin example.

Plasmids: Scientists use plasmids as natural vectors that exist in bacterial cells and "trick" bacterial cells into producing proteins that come from other organisms. Since the genetic code and DNA structure is essentially identical across the living world, scientists can combine DNA fragments from various organisms together, and bacterial cells can "read" genes from different organisms, thus producing proteins coded by these genes. Plasmids are short (usually, 3,000 - 10,000 bp) circular DNA molecules that contain their own *origin of replication* that functions independently from bacterial DNA. Plasmids contain *antibiotic resistant genes* that are naturally present in various organisms and scientists use these genes and media containing antibiotics to ensure that bacterial cells continue to produce plasmid DNA. Scientists insert *genes encoding the protein of interest* under control of a strong *promoter*, a DNA sequence that signals the start of RNA synthesis. All of these parts are essential for successful protein production. Here is our Plasmid: pET29b+ - BglB.

Kunkel mutagenesis: Kunkel mutagenesis approach was designed by Thomas Kunkel in 1985 and remains (with many modifications) one of the most robust approaches introducing mutations in protein-coding sequences. This approach utilizes *a 33 bp - long oligonucleotide* that is identical to the region of the gene that needs to be mutated. It is built to anneal (connect) to gene DNA but its three middle nucleotides are changed into a new sequence representing a mutation that needs to be introduced. This oligonucleotide is combined with *an unusual single stranded plasmid DNA* **(annealing)** to build a

complementary DNA strand that contains a desired mutation (**polymerization**). When such hybrid plasmid is introduced into *bacterial cells* **(transformation)**, the cells break down the original DNA strand, building a double stranded mutated version of the plasmid. This newly constructed plasmid can now be used to produce a mutant protein. Since plasmids are circular DNA molecules, the 5′ of the original oligonucleotide eventually is connected to the 3′ end of the second DNA strand **(ligation)**. This requires the 5′ end of the oligo to contain a phosphate group, so it needs to be **phosphorylated**.

Oligo Phosphorylation/Kinase Reaction

The kinase reaction, or phosphorylation reaction, adds a phosphate group to the 5′ end of the mutant oligo. The second DNA strand will elongate from the mutated oligonucleotide (a short piece of DNA that encodes for your mutation; 'oligo' for short) using a single-stranded plasmid DNA as a template, and eventually will be circularized; its 5′ and 3′ ends will be connected, or ligated, together. DNA ligation can only connect a phosphorylated 5′ DNA end to a 3′ DNA -OH group. The oligonucleotides we order do not contain the 5′ phosphate group and thus need to be phosphorylated prior to the next steps. DNA kinase is the enzyme that transfers a phosphate group from ATP to the Oligo.

Preparation

Reagents

Material	Amount per reaction (µL)	Storage (°C)
DNA Oligos	7	-20
Ligase Buffer with 10 mM ATP	4	-20
T4 Polynucleotide Kinase (T4 PNK)	1	-20
MilliQ Water	18	RT

Equipment*

Thermal Cycler, Incubator, or Water Bath

**Single channel pipettes, pipette tips, 1.5mL tubes, PCR tubes, ice-buckets, and serological pipettes, waste beakers, graduated cylinders are not listed in this lab manual; they are considered normal equipment and consumables.*

Preparation Notes

- *All benchwork can be performed at room temperature (e.g. centrifugation)*
- ***Keep reagents on ice as much as possible.***

Instructions

In this part, you will be preparing a 'Master Mix.' Often, we can simplify procedures by preparing a cocktail of reagents called a Master Mix, much like you would make a multi-component batter for baking. Master Mixes reduce repetition and improve accuracy.

Prepare Master Mix

1. Calculate the quantities of the reagents below based on the number of samples you have, plus one for your blank, plus *one extra* sample to compensate for any pipetting error. When making the Master Mix, add in order (below) and check off as you go.

2. Pipette up-and-down when the Master Mix has been completed.
   - ☐ _____ µL Nuclease free H_2O
   - ☐ _____ µL Ligase buffer with 10 mM ATP
   - ☐ _____ µL T4 Polynucleotide Kinase (add this last)

Combine Master Mix with Oligos

1. Aliquot 23 µL of Kinase Master Mix in **LABELED** (e.g., C167N plus initials) PCR tubes, one for each mutant, and one for the blank. Make sure all liquid is at the bottom by tapping or briefly spinning.
2. Add 7 µL of the respective 100 µM Oligo (or 7 µL water for the blank) to the BOTTOM of each tube.
3. Pipette up and down to mix.

Note: if your sample is not on the bottom of the tube or there is a chance that the master mix and oligo are stuck to the sides of the tube in different places, you can gently centrifuge the tubes to ensure the sample is mixed.

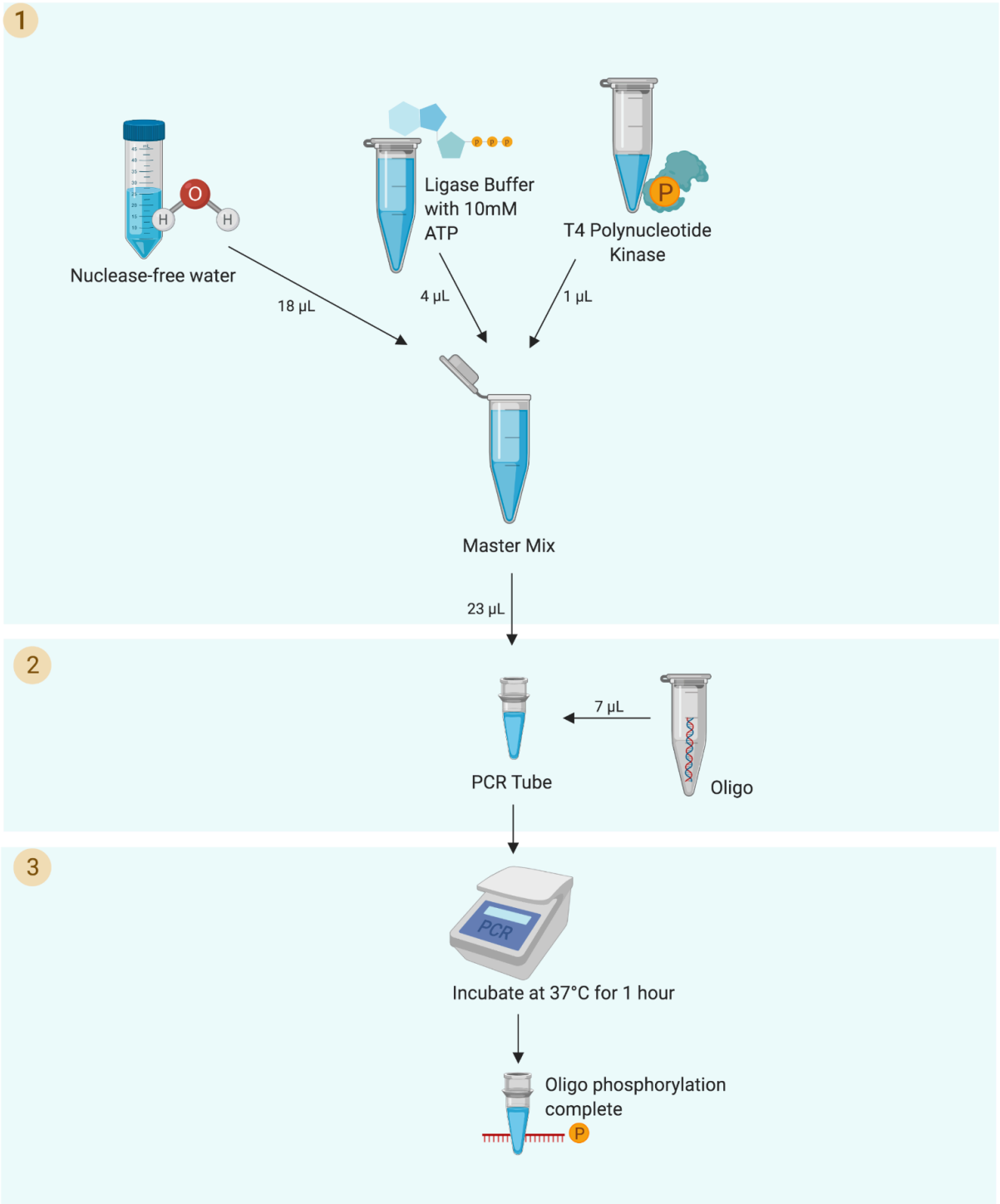
1. Incubate Mixture

☐ Seal the PCR tube and incubate at 37°C in a water bath, PCR machine/thermal cycler, or incubator for **one hour.**

☐ Store on ice for short-term storage or at -20°C for long-term storage. These can be re-used in the future so no need to throw them away after next steps.



Kinase Reaction, Oligo Phosphorylation



Annealing and Polymerization Reactions

The Annealing Reaction

In the annealing process, you will combine your oligo with the single-stranded DNA (ssDNA) plasmid; the ssDNA plasmid serves as the “base” or “backbone” for the Oligo to attach to, and contains other useful genes for late steps. At high temperature, the two strands of a DNA molecule separate from each other due to a breakdown in hydrogen bonds between bases. As the temperature decreases, however, complementary regions of DNA molecules “find” each other and reform hydrogen bonds. In our reactions, oligonucleotides will anneal / connect to the complementary BglB region of single stranded plasmid DNA. Remember that the oligonucleotides we use contain a 3 nucleotide mismatch in the middle (for introducing a mutation); however, the 15 nucleotides on each side of this mismatch provide sufficient homology for successful annealing.

Here is a resource for more information about annealing reactions.
Here is a link that will take you info about the BglB plasmid.

Preparation

Reagents

Material	Amount per reaction (µL)	Storage (°C)
Nuclease-free H2O	200	RT
Phosphorylated oligos	2	-20
T4 DNA Ligase Buffer (w/ATP okay)	0.2	-20
ssDNA (single-stranded DNA)	2	-20

Equipment

- Thermal Cycler

Instructions

Dilute Phosphorylated Oligo

1. Label a 1.5 mL microcentrifuge tubes with d+oligo name (for dilution).
2. Pipette 2 µL of phosphorylated oligo (or blank) into separate tubes of 200 µLnuclease-free water in the 1.5 mL microcentrifuge tube.
3. **Mix together VERY well by pipetting up and down. This is critical!**

Anneal Diluted Oligo and ssDNA

1. To prepare the Master Mix (in a PCR tube), combine 0.2 µL of T4 DNA Ligase Buffer and 2 µL of ssDNA per reaction in a microcentrifuge tube labeled 'MM.' When preparing your Master Mix, make enough for: (a) your mutant, (b) the "blank"*
   **To conserve on ssDNA, you may omit running a blank alongside your samples. Inquire with your instructor about this (the instructor may just run one blank for the whole class). If so, then it's recommended that if each student is only running one sample, they group together with multiple students to make a larger mastermix to share.*

   Note: This "blank" sample will tell us the approximate ratio of successfully transformed cells with mutagenized plasmids vs. transformed cells with WT plasmids, which is an undesirable carryover byproduct of the ssDNA preparation. See link for schematic.

2. Aliquot 2 µL of the Master Mix you just made into fresh PCR tubes. Ensure liquid is at the bottom of the tube by spinning it or tapping it down.
   Note: Be sure to save the excess Master Mix, in case of any accidents. But once you're done for the day, toss the remaining master mix.
3. For the mutant samples: Add 2 µL of the *diluted* Oligo into the PCR tubes now containing the Master Mix solution. Mix by pipetting up and down.
4. For the Blank: Combine 2 µL of nuclease-free water (in lieu of the oligo) into the PCR tubes now containing the Master Mix solution. Mix by pipetting up and down.
5. Cap and Anneal in Thermal Cycler using a ***"Fast Anneal"*** *protocol*
   Alternative Fast Anneal Protocols:
   - Start at 95°C for 2 minutes, then 50°C for 2 minutes, hold at 18°C .
   - Start at 95°C for 2 minutes, then 50°C for 2 minutes, then 25°C for 2 minutes, hold at 4°C.

   Use a heated lid when annealing. Thermal cyclers might have different adaptors for different PCR lids; ensure that you have the correct one before beginning.
6. Transfer the sample to ice when complete

Must move on to Polymerization, this is NOT a STOP step.

The Polymerization Reaction

Polymerization consists of a series of biochemical reactions that build a complementary strand of DNA from a single-stranded template, resulting in a double-stranded product. First, DNA polymerase builds the second DNA strand using the single stranded plasmid DNA from the previous step as a template and an annealed oligo as a start point / primer. Next, DNA ligase connects a phosphorylated 5' end and a 3' end of this newly built second DNA strand to make it circular.

This reaction can be achieved *in vitro* by creating a Master Mix with Ligase Buffer, ATP, free nucleotides (dNTPs), DNA polymerase, and DNA ligase. The ligase buffer controls pH, which is essential for maintaining the integrity of the enzymes, ATP, and dNTPs. The dNTPs act as building blocks for new DNA strands, the ATP provides energy for the reaction, the polymerase synthesizes the new DNA strand, and the ligase connects the ends of the polymerized product to circularize the plasmid. DNA polymerase works efficiently in the buffer designed for DNA ligase; this is why these reactions can proceed in the same buffer and do not require a two-step setup. This mixture of reagents will be added to annealed oligo-ssDNA from the previous step for plasmid polymerization. The polymerized product can then be used in *E. coli* transformation. We generate a background (blank) in the annealing step to evaluate latent potential wild type levels in our transformation (the ssDNA contains some amount of double stranded plasmid carrying the wild type BglB gene, and cells transformed with these will survive transformation).

Preparation

Reagents

Material	Amount per reaction (µL)	Storage (°C)
Annealed oligo/ssDNA solution from previous step	4	RT
T4 DNA Ligase Buffer 10X with 10 mM ATP	1.0	-20
dNTPs 10 mM	0.8	-20
T7 Polymerase	0.4	-20
T4 Ligase	0.4	-20

Equipment

- NA

Instructions

Preparing the Master Mix

Prepare a Master Mix that combines ingredients needed for polymerization reaction.

1. Label a PCR tube with MM (for Master Mix).
2. Calculate the quantities of the reagents you need for your master mix based on the number of mutant samples you have, the blank, and one more to hedge against for pipetting error.

Reagent	**Quantity for one reaction**	**Quantity for master mix**
10x T4 ligase buffer with 10 mM ATP	1.0 µL	
10 mM dNTPs	0.8 µL	
T7 polymerase	0.4 µL	
T4 ligase	0.4 µL	

3. In a 1.5 mL microcentrifuge tube, combine the reagents starting with the ligase buffer and adding the remaining reagents in any order. Mix well by pipetting up-and-down

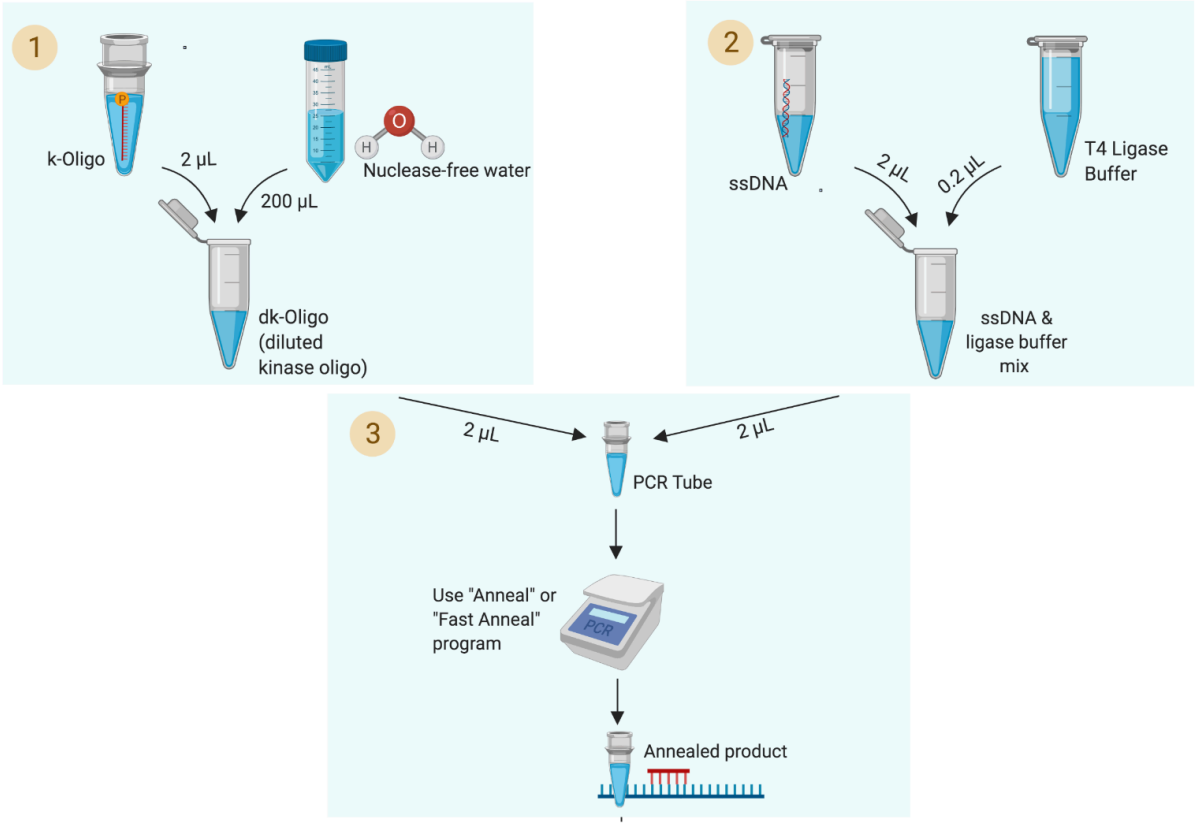
Combining Master Mix and the Annealed Product

4. Add 2 µL of the polymerization Master Mix into each PCR tube containing the annealed product (annealed oligos + ssDNA). Gently mix the PCR tubes—do not vortex.
5. Label your tube with your initials and mutant, ‘Kunkel’ and the date.

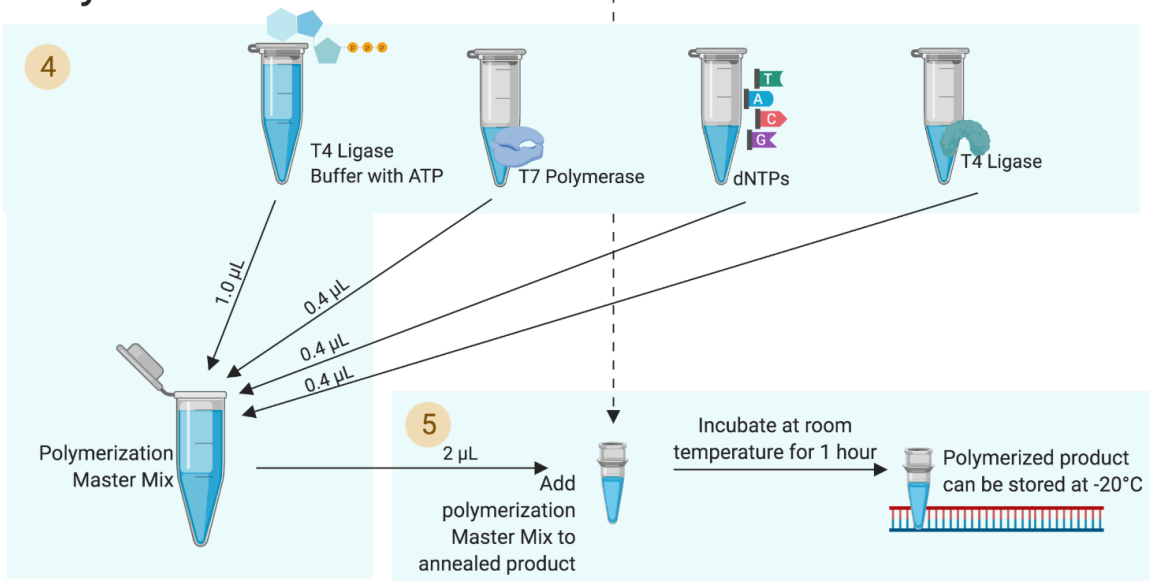
Incubating the Solution

6. Incubate the polymerization reactions for 1 hour at room temperature (~22°C). After incubation, you can put the product in the -20°C. **This is your Kunkel Product.**

Annealing Reaction



Polymerization Reaction



DH5α Transformations

In this step, you will be transforming a strain (DH5α) of bacteria (*E. coli*) that is very good at replicating and fixing damaged DNA. Transformation is a process in which cells uptake foreign DNA from their environment; in this case, the foreign environment is the solution they are suspended in. We will induce DH5α *E. coli* cells to uptake the polymerized product that we made in the previous steps. The polymerized product is not yet a perfect DNA plasmid because (1) it likely contains some misjoined fragments and (2) our initial plasmid backbone contains many uracil bases where there should be thymine bases, which makes the *E. coli's* job more difficult. For these reasons, we are using this relatively robust strain of *E. coli*. The end goal for this transformation step is to use the *E. coli* to replicate our plasmid. In doing so, they will create perfect—fully formed, completely uracil-absent—plasmids for our downstream reactions.

Sterile Technique I

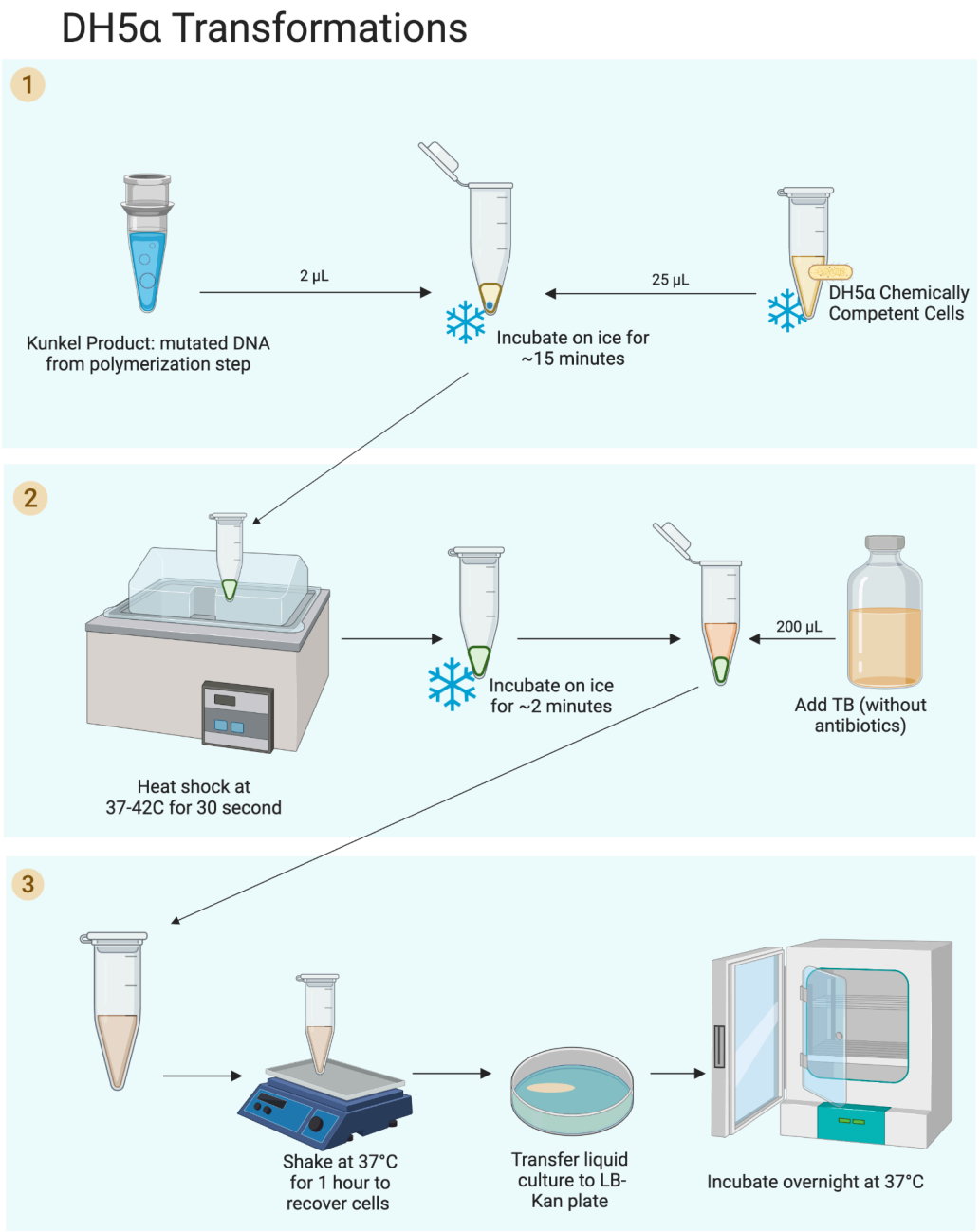
At this point in the workflow, you will be starting to work with bacteria. Any time we are working with bacterial cultures, you must use sterile technique to prevent contamination. The growth media we use—both liquid and solid—contain the perfect mixture of ingredients to feed bacteria and other microbes. As such, they are very easy to contaminate. To prevent other microbes from getting into and taking over our cultures, it is essential to do the following:

- Do: use a 70% ethanol solution to wipe down your countertop and any tools you will be using (e.g., pipettes).
- Do: use sterile tips that have been autoclaved.
- Do: consider working under flame. Bunsen burners create a ~50 cm zone of protection around them as their heat creates an updraft which pushes the air, and any microbe/particulates in it, up and away from the working area.The flame is the most dangerous hazard in our lab—**use with extreme caution**.
    - Ensure you have set up your equipment such that you are not reaching over or around the flame.
    - **Do NOT**: lean close to the flame.

    - **Do NOT**: work above the flame or directly under it, work in a foot radius of the flame on your bench.
    - Do: Ask for help with this when getting started.
- **Do Not**: breathe on your plates or tubes.
- Do: Keep your media containers closed as much as possible. Keeping the lid gently covering your media container (tube or plate) is sufficient for keeping contaminants from falling into your culture. It does not need to be sealed.

Heat-Shock Transformation Protocol for Chemically Competent DH5α

Overview



Preparation

Reagents

Material	Storage Notes (°C)	Amount per reaction
Chemically-competent *Escherichia coli* DH5α (e.g. Invitrogen 18265017)	-80	15 µL
Kunkel Product*	-20	2 µL
SOC media	RT	200 µL
LB agar plates with 50 µg/mL kanamycin	4	1

Equipment

- -80C Freezer for cell storage, water bath, shaking incubator, incubator

Instructions

Combining ingredients on ice

7. Set up the water bath to 42ºC
8. Thaw DH5α chemically competent cells on ice. While you wait for the cells to thaw, start on the next steps.
9. Label sterile 1.5-mL tubes, one with your mutant names and one ‘blank’ and place them on ice to chill.
10. Pipette 2 µL of Kunkel Product into the mutant tube, and 2 µL sterile water into the blank tube, in a visible location at the bottom of the tube. Put the tubes back on the ice to further chill.
11. Pipette the cells *on top of* the droplet of Kunkel Product/water. Ensure that you can see the cells and Kunkel Product solutions have come into contact with each other. Work to keep the cells cold and minimize their exposure to the warm air or your (hot!) fingers; place them back in the ice whenever you are not actively pipetting them. **Cells must always be kept on ice. They will lose competency if not on ice!**
12. Incubate the cells and Kunkel Product/water on ice for 15 minutes.

Heat shock & Recovery

1. Heat-shock cells **30 seconds** in a 42°C water bath; do not shake. Then *immediately*, place the cells on ice for at least two minutes.
2. Add 200 µL room temperature SOC medium *without antibiotics*

Recovery incubation & plate preparation

1. Shake at 225 rpm (37°C) for 1 hour to recover transformed cells.
2. Clearly label a LB kanamycin plate for each of the mutants you are working with. The label should include the mutant name, your initials, '*E. coli* DH5α', and the date of transformation. Don't forget to label a plate for the blank/control if you have one.

Plating for overnight growth

1. Pipette 200 µL of culture on an individual kanamycin LB agar plate, use an L-shaped spreader to spread the cell suspension as evenly as possible.
2. Allow the plate to "dry" for a few minutes
3. Turn plates upside down and incubate at 37°C overnight.

The next step will be carried out by your instructors, however you will need to prepare the tubes for them, so ensure you have read the Overnight Cultures protocol, and left labeled tubes on your bench before leaving class.

Overnight Cultures

This step will be completed 'behind the scenes' by your instructors, however it is important that you understand what has been done. In this step, we will be growing up, or multiplying, the bacteria that we transformed. We need large numbers of bacteria to produce a sufficient amount of plasmid DNA for downstream use. To do this, use individual colonies from each plate to prepare two separate overnight cultures per plate (two colonies per plate). You will be working in duplicate because we expect some of the colonies to be "blanks." In other words, not all the colonies have the mutation encoded in the plasmid; working with more replicates increases the likelihood that we successfully isolate a mutant colony.

Preparation

Material	Storage Notes (°C)	Amount per mutant
Plates with transformed colonies (from previous step)	37 or 4	
50 mL round-bottom culture tube (2 per sample/plate)	RT	2 tubes
LB	RT	2 mL
1000X kanamycin stock solution (recipe in "Deep Prep")	-20	1

Preparation notes: Use sterile technique, see details in the introduction section of DH5α Transformations protocol. Make a master solution of LB with kanamycin.

Equipment

- shaking incubator

Instructions

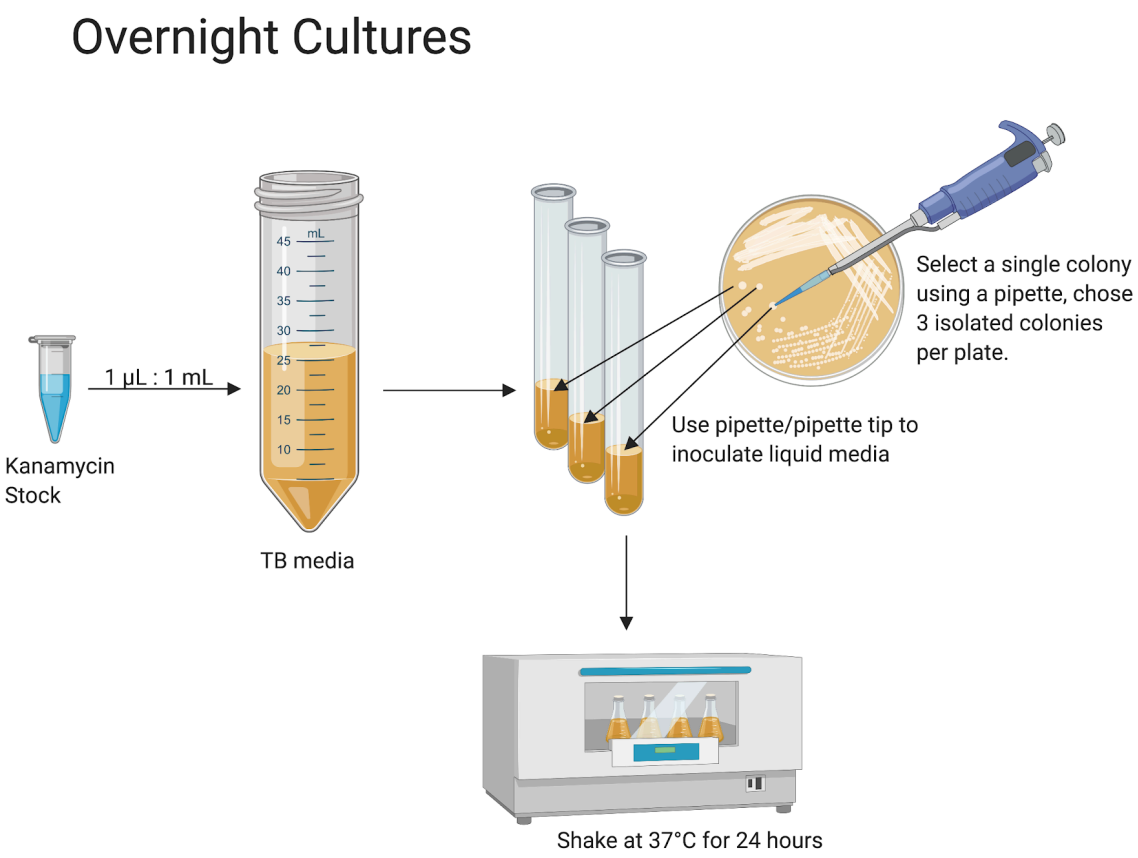
Day 1

1. You will prepare and sequence two minipreps per mutant, to increase the chances of success. Therefore, label two tubes, 1 and 2, each with your mutant name, your initials, '*E. coli* DH5α', and the date of the overnight culture (the date of the *day before* your next class).
2. (Done by instructors from this point on) Prepare a LB + kanamycin growth media solution: add 1 µL of Kan Stock for every mL of media needed; This is a 1000X dilution. *For example, if you need 20 mL of media, you should add 20 µL of kanamycin Stock to 20 mL of LB.* Add 5 mL to each overnight 50 mL *Falcon tube.

"Falcon tube" is like "kleenex": the commercial product name that everyone uses interchangeably with the item (i.e. kleenex = tissue). Falcon tubes are ~14-15 mL round bottom culture tubes.

3. From each of the plates containing your mutants, pick **two** different, individual colonies and inoculate the tubes you prepared with those colonies.

If your tubes are screw-type/threaded (screws on tightly, forming a seal), consider using tape seals (allows air in, enabling aerobic cell growth). Incubate tubes at 37°C in the shaker overnight.



Miniprep: Plasmid Extraction and sequencing prep

The day after growing bacterial cells in liquid culture, you will be extracting plasmid DNA for (a) downstream sequencing to verify that the Kunkel mutagenesis process was successful and (b) transforming BL21 (DE3) strain *E. coli* for protein production with the plasmids extracted in this step. We will be using the NEB Monarch kit, however there are a lot of different 'column-based' kits available.

Preparation

Material	Amount per Reaction	Storage Notes (°C)
Monarch Plasmid Resuspension Buffer (B1) (pink)	200 µL	RT
Monarch Plasmid Lysis Buffer (B2) (blue/green)	200 µL	RT
Monarch Plasmid Neutralization Buffer (B3) (yellow)	400 µL	4°C*
Monarch Plasmid Wash Buffer 1	200 µL	RT
Monarch Plasmid Wash Buffer 2	400 µL	RT
Monarch DNA Elution Buffer	30 µL	RT
Spin Columns	1	RT

*Buffer B3 contains RNase A.

Equipment

- microcentrifuge with max speed greater than or equal to 13000 rpm
- Beaker for waste (to be bleached at end of protocol

Instructions

Pelleting cells & cell lysis

1. Pellet overnight cultures by spinning 50 mL Falcon tubes at 4,700 RPM (3043 xg) for 10 minutes.
2. Carefully remove the supernatant (liquid at the top layer) by pouring into bleach bucket, blot tube mouth on paper towel.
3. Resuspend pellet in 200 µL of Plasmid Resuspension Buffer (B1) (pink).

The bacterial pellet should be suspended completely by carefully pipetting up and down.
Keep the resuspension buffer on ice.

DNA shearing & adhering DNA to spin column membrane

1. Add 200 µL of Plasmid Lysis Buffer (B2) (blue/green) into the microcentrifuge tube with your resuspended mixture.
2. Mix by thoroughly inverting (~6-10) times until solution color changes to dark pink and the solution is clear and viscous and incubate at room temperature for 1 min. Do not vortex, as this will result in shearing of genomic DNA. Do not allow the lysis reaction to proceed for more than one minute. After use, the bottle containing Buffer B2 should be closed immediately to avoid acidification from CO_2 in the air.
3. Pipet 400 µL of Plasmid Neutralization Buffer (B3) (yellow) and gently invert until color is uniformly yellow and a fluffy, white precipitate forms. **Do not vortex!** Incubate for 2 minutes. The precipitated material contains genomic DNA, proteins, and cell debris. The lysate should be mixed thoroughly!
4. Mix sample again by inversion, then centrifuge for ten minutes at 13,000 RPM. After centrifugation, the supernatant should be clear. If the supernatant is not clear, a second, shorter centrifugation should be carried out to avoid collecting any suspended or particulate material.
5. Carefully transfer the supernatant (lysate) onto the membrane of the spin column using a P1000 pipette without touching the membrane and carrying over precipitate. **DISCARD** tube with the precipitate once transfered.

Note: Lysate is a more specific term for supernatant that refers to the liquid above the pellet after the cells are broken up (lysed).

6. Centrifuge the spin column for 30 to 60 seconds and discard the solution washed through at the bottom, which we call the *flow-through*.

Washing contaminants off plasmids

1. Wash the silica membrane by pipetting 200 µL of Plasmid Wash Buffer 1 into the spin column.
2. Centrifuge for another 30 to 60 seconds. Discard flow-through.
3. Wash the silica membrane by pipetting 400 µL Plasmid Wash Buffer 2 into the spin column.
4. Centrifuge for 30-60 seconds. Discard flow-through.
5. Centrifuge spin column for 60 seconds to ensure all wash buffer is pulled out of the membrane and to the bottom of the collection tube.

   Note: While the second spin may seem redundant, residual wash buffer can remain after the first spin and it is essential to remove all of it for pure and high-yield sample.

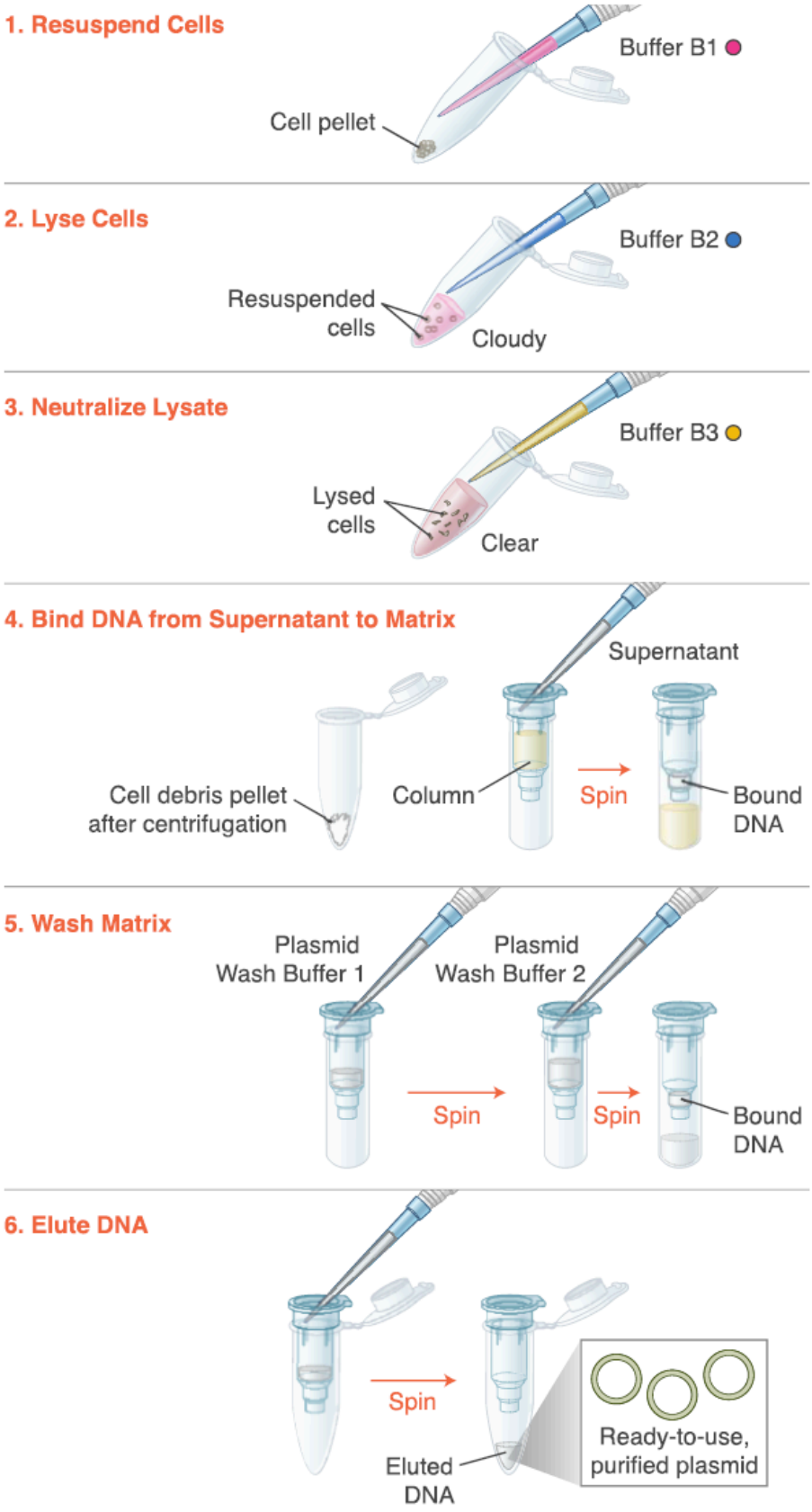
6. Throw out the collection tube taking care to not splash the remnant Buffer caught in the bottom of the collection tube from the last spin.

   Note: if you are worried that some of the flow-through buffer did touch the membrane, spin again.

7. Transfer column to a clean, **labeled(!)** 1.5 ml microfuge tube. (labeled with mutant on the cap and initial, mutant and date on the side)

Eluting Plasmids from the Membrane

1. Elute by pipetting 40 µL of DNA Elution Buffer on the membrane.
2. Allow the solution to settle for at least **one minute**, and then centrifuge for a final 60 seconds.
3. **KEEP** the solution that passes through the spin column, which is now resting at the bottom of the 1.5mL microcentrifuge tube. Discard the spin column; this may feel counterintuitive because up until this point you've been doing the opposite.
4. Eluted DNA should be stored at -20°C


1. Resuspend Cells
Buffer B1
Cell pellet
2. Lyse Cells
Buffer B2
Resuspended cells
Cloudy
3. Neutralize Lysate
Buffer B3
Lysed cells
Clear
4. Bind DNA from Supernatant to Matrix
Supernatant
Cell debris pellet after centrifugation
Column
Spin
Bound DNA
5. Wash Matrix
Plasmid Wash Buffer 1
Plasmid Wash Buffer 2
Spin
Spin
Bound DNA
6. Elute DNA
Spin
Eluted DNA
Ready-to-use, purified plasmid

DNA Concentration Assay (A260)

For successful sequencing, you will need to determine the concentration of DNA from your Miniprep product. This assay will tell you if your Miniprep was successful in eluting a (relatively) pure sample of DNA. DNA absorbs the light at a wavelength of 260 nm. We will use a spectrum of light from approximately 200-300 nm. Protein absorbs light at 280 nm. Significant absorbance at 280 nm in this assay suggests sample contamination. Depending on the yielded concentration of your Miniprep DNA, you may need to dilute your sample before sending it for sequencing.

Equipment

- Nanodrop

Instructions

Preparing the Nanodrop

1. Select the dsDNA program on the nanodrop
2. Open the arm and lean the plate by adding 1 µL deionised water to the sample input plate. Close the arm *(gently!)*, initialize the instrument, open the arm and use a kimtech wipe to blot it dry on the top and bottom contact. See figure 2.1 for demonstration.

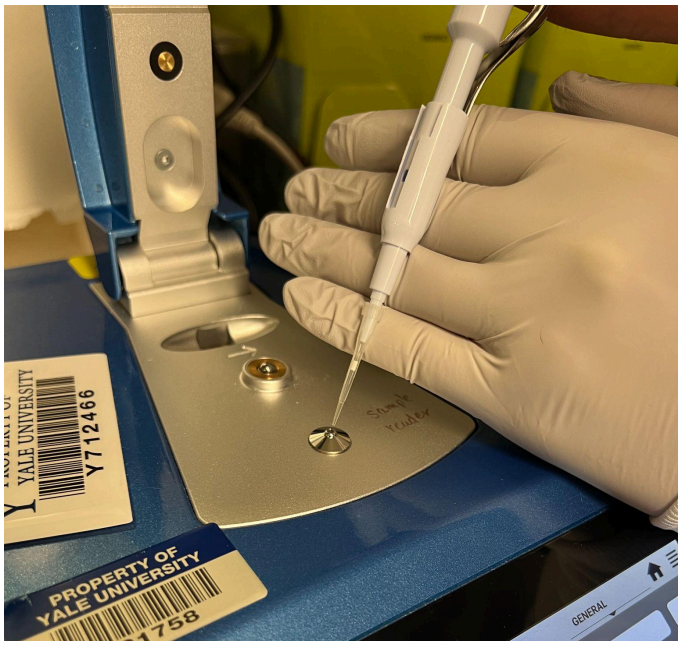


Fig 2.1: demonstration of how to add a sample to the nanodrop- ensure the sample is added to the front sample plate, in a single intact drop with no bubbles. Steady the pipette with your other hand to help.

3. Add 1 µL miniprep elution buffer to the plate, taking care not to pipette out any bubbles, close the arm and hit Blank. Clean again with a kimtech wipe.
4. Add 1 µL of plasmid prep sample to the plate, close the arm and press Measure.

Reviewing A260 Data

The data will be reported in ng/µL. **Record your results into your lab book** using these units

Note: Any samples that fall between 20-50 ng/µL might cause low sequence quality reads. However, you should still proceed with these lower concentrations. For anything that is less than ~20 ng/µL, you should regrow the samples/re-miniprep.

Sequencing tube preparation

To determine if the mutation was successfully completed during the Kunkel reaction step, we will be sequencing the BglB portion of the plasmid, and comparing it to the wild type sequence. We will be doing this using Sanger Sequencing, a technique that can very cheaply sequence DNA fragments ~1000 bp long. Sanger sequencing requires amplification of the target DNA using a special PCR called chain-termination PCR. We will need to prepare tubes containing the correct mix of template DNA and primers to read both forward and backward across the gene, to ensure we get proper coverage of the whole gene in our sequencing.

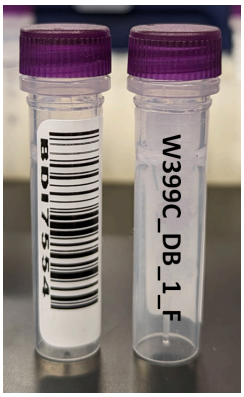
Reagents

Material	Storage Notes (°C)	Amount per reaction
Isolated plasmid DNA	-20	≥ 1 µg
10 µM ‘T7 forward’ OR ‘T7 reverse’	-20	0.8 µL
Nuclease-free H2O	RT	ad vol 18 µL

Materials

- Barcode labels
- 0.5 mL conical base screw cap microcentrifuge tubes (purple lid)

Instructions



1. Place the sequencing barcode VERTICALLY on the tube as shown:
2. Label the tubes EXACTLY as described below:
    a. Your mutant code (e.g., W399C)
    b. Your initials
    c. 1 or 2 (for plasmid DNA from colony 1 or 2)
    d. F or R (for T7 forward or T7 reverse primer)

Example: W399C_DB_1_F

Ensure your writing is totally legible, if you don't feel confident with this, ask your colleague to assist.

3. Calculate the volume of extracted plasmid from the miniprep required to have a total of 1 **µg**. *Take care with your units and check with an instructor if you are unsure.*
4. Calculate the volume of water to be added for a final volume of 18 µL per reaction.

Use the equation: Water µL = 18 µL - 0.8 µL (primer) - [1 µg plasmid DNA] µL

Reagent	Seq 1	Seq 2	Seq 3	Seq 4
Isolated plasmid DNA (**≥** 1 **µg**)				
10 µM primer (F or R)	0.8 µL (F)	0.8 µL (R)	0.8 µL (F)	0.8 µL (R)
Nuclease-free H2O				
Total volume	**18 µL**	**18 µL**	**18 µL**	**18 µL**

5. Add the respective volumes of water, primer and plasmid DNA solution to each tube.
6. Tightly screw caps and place them in the rack at the front of the class.
7. Input your sample information into the class spreadsheet.

Plasmid Sequence analysis

Sequencing Results: Data Analysis

In this step, you will be analyzing your mutant sequence data files. We'll be looking at the sequence file type: "ab1." These files hold the string of ATGCs that make up the BglB gene sequence, but they also contain information that tells us about the nucleotide reads, which we view through a chromatogram. From this data, we can observe contamination, sequence quality, and approximate likelihood that a base-call is correct. Our sequence analysis will tell us which plasmids have our mutation; we will move forward with these plasmids and discard the ones that remained wild type.

Thermo produced a great video explaining the sanger sequencing tech and how the chromatograms that you'll be looking at in this step are generated.

Equipment

- Laptop or desktop computer.

Finding and Downloading Your Sequences

Your sequence data will be deposited* in the group Benchling folder. So you will need to make an account **using your Yale email.** The instructors will then add you to the Benchling group.
**note for instructors: Instructors will need to set up a Yale Keck sequencing account, and all reads will be deposited into their Box folder. These should be retrieved and deposited into a new Benchling folder which students can be added to.*

Opening Benchling

Open Benchling in your web browser. Benchling is a web-based application that requires you to create an account. It has sequence analysis software that is free and easy to use.

Creating Sequence Alignment

Navigate to “New Alignment.”

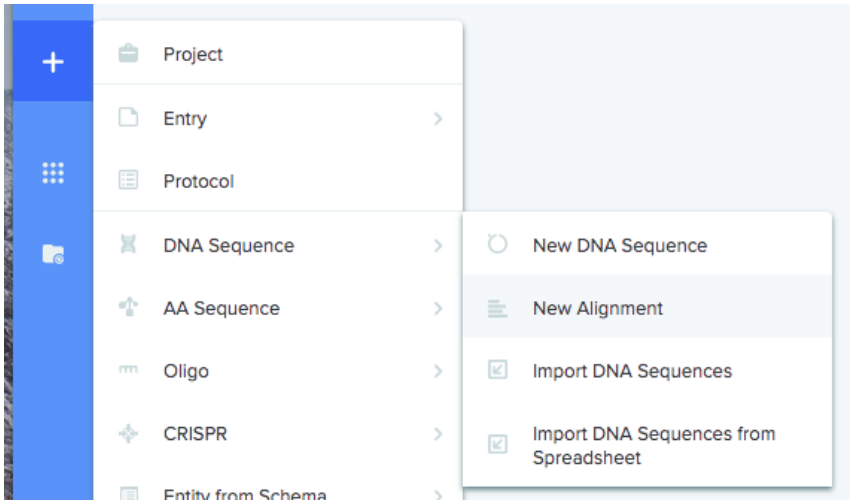


Figure 12.1. Benching navigation to New Alignment option

1. In the “Alignment” pop-up window, use the search bar to select:

- The ab1 files from one of your two colonies (C1/C2) (**both** the forward and the reverse files)
- Bagel_B fasta file (‘pET29b+ Plasmid + bglB gene’ vector map)

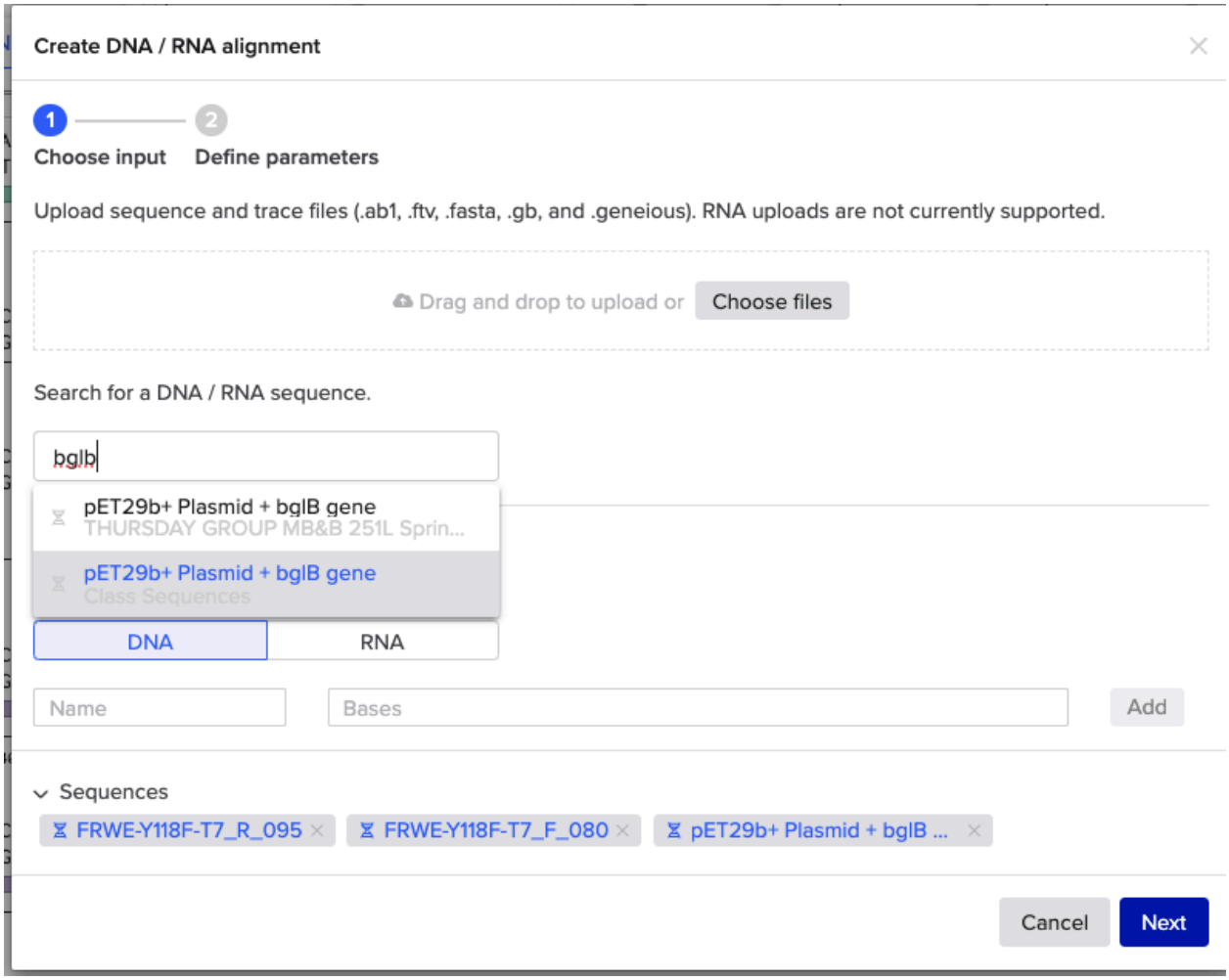


Figure 12.2. Use the search feature to search for your ab1 read files and the Bagel_B fasta file in the MB&B 251L folder. Then you are ready to hit “Next”.

2. In the “Define Parameter” options, set the “Template” to the Bagel_B fasta and use “Show Parameter” to adjust direction to “Enabled, accurately”

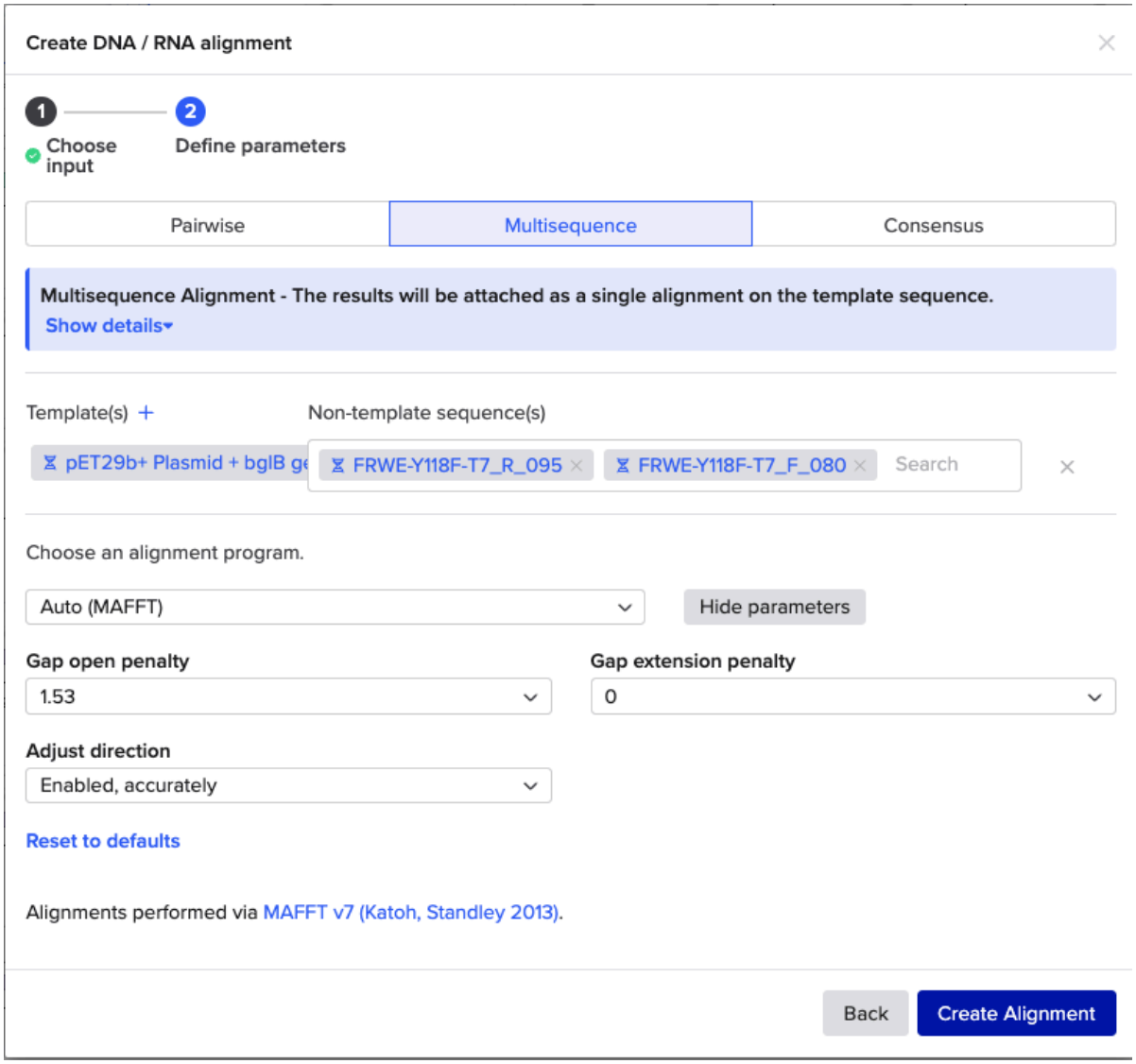


Figure 12.3. Select your template and hit Show parameters to set Adjust direction to ‘Enabled, accurately’.

Then go ahead and hit “CREATE ALIGNMENT.”

Selecting Reading Frame Settings

Once your alignment has been created and opened successfully, ensure all settings are selected as follows:

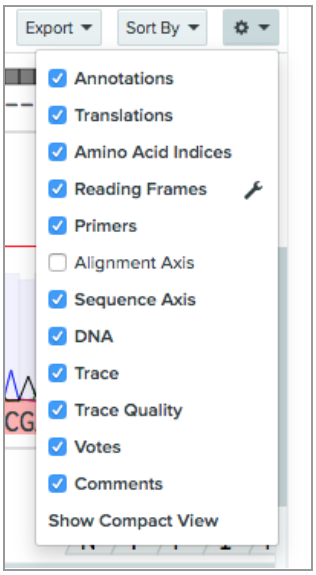


Figure 12.4. Correct selection of “Alignment” viewing settings.

The wrench icon to the right of “Reading Frames” will take you to the reading frame setup. Select “Show frame FWD1.”

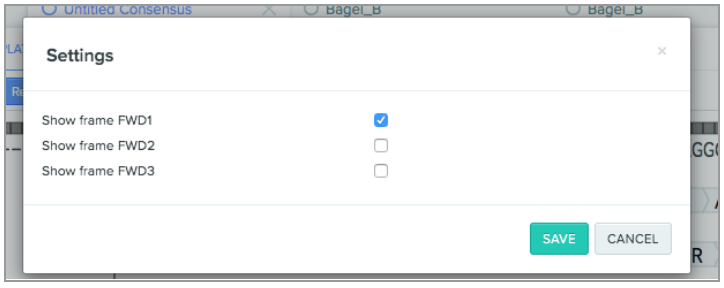


Figure 12.5. Expanded dialog box showing Reading Frame Settings.

Orienting and Navigating in Alignment

Finding Your Mutant Location and Looking for Changes

Multiply your mutant number (amino acid number) by 3 to find the nucleotide sequence number; e.g., H285A will be found at base-pair sequence number 855. Then, use the numbers generated by the sequence axis to find your mutation’s spot.



Figure 12.6. Alignment orientation components: (a.) WT / Template Bagel residue; (b.) Mutant residue with change from D to E highlighted in red; (c.) sequence summary navigation pane with mutations highlighted in red; (d.) Mutant nucleotide number/count *should have boxed “End 105”, which divided by 3 (3 nucleotides per codon) = amino acid residue 35; (e.) sequence file name checks out, indeed D35E has “taken”, Kunkel worked!

Red flags and common issues

Contamination

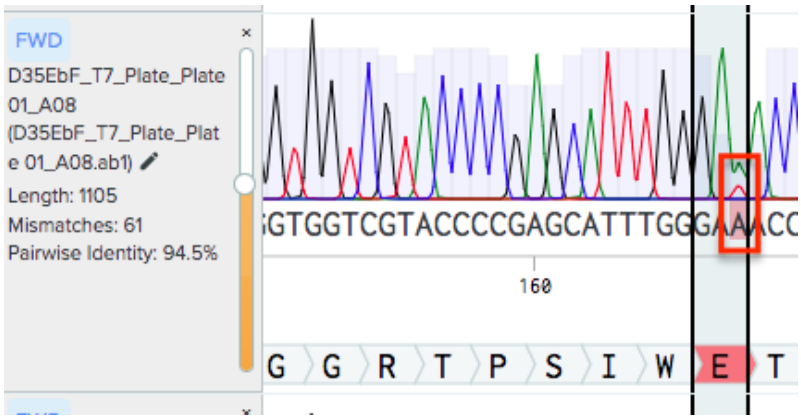


Figure 12.7. Contamination “double peak”. Here we see a tiny but present second peak underneath the mutant. It appears there is a little WT DNA in the sample. This sample triplicate must be thrown out.



Figure 12.8 End-of-read garbage. These double peaks are just artifacts of the read “going bad” after ~800 base-pair. The reactions run out of reagents and the capillaries don’t work properly and we expect this kind of data at the very beginning of and ends of the reads.

Chemically Competent BL21(DE3) Transformation

In this step we will be transforming your mutant β-glucosidase B, WT, and GFP plasmids into BL21(DE) chemically competent *E. coli*; this strain of *E. coli* is particularly good at producing protein. You will be using the heat shock method again to make the cells' membranes porous enough to uptake the plasmids from the solution they are suspended in. For this and the following procedures through protein purification, typical yields are 1.0 ± 0.4 mg/mL for the wild type protein.

Preparation

Reagents

Material	Storage Notes (°C)	Amount per reaction
Chemically-competent *Escherichia coli* Chem BL21(DE3)* (e.g. Thermo Scientific™ EC0114)	-80	15 µL
Mutant plasmid/ GFP-plasmid (neg control)	-20	~10-50 ng
SOC media	RT	200 µL
LB agar plates with 50 µg/mL kanamycin	4	1

* BL21(DE3) genotype: F– *ompT* $hsdS_B$ (r_B–, m_B–) *gal dcm* (DE3)
https://www.thermofisher.com/order/catalog/product/EC0114

Equipment

- -80C Freezer for cell storage, water bath, shaking incubator, incubator

Instructions

Combining ingredients on ice

1. Set up the water bath to 42ºC
2. Thaw BL21(DE) chemically competent cells on ice. While you wait for the cells to thaw, start on the next steps.
3. Label sterile 1.5-mL tubes, one with your mutant name and one 'GFP' and place them on ice to chill.
4. Pipette the volume required for a total of **10** - **50 ng** plasmid DNA of your mutant plasmid (verified by sequencing) into the mutant tube, and 1 µL GFP plasmid solution into the 'GFP' tube, in a visible location at the bottom of the tube. Put the tubes back on the ice to further chill.

Plasmid DNA solution	Conc. [ng/µl]	Volume [µl] plasmid DNA solution for 10-50 ng total
Example-plasmid	62	0.8 µl (= 50 ng)
Your mutant plasmid verified by sequencing		

Keep in mind that pipetting too small volumes (<0.4 µl) is not advisable. What can you do to avoid that when you have higher plasmid concentrations?

5. Pipette the cells *on top of* the droplet of plasmid DNA. Ensure that you can see the cells and plasmid solutions have come into contact with each other. Work to keep the cells cold and minimize their exposure to the warm air or your (hot!) fingers; place them back in the ice whenever you are not actively pipetting them. **Cells must always be kept on ice. They will lose competency if not on ice!**
6. Very briefly flick the tubes with your finger to mix the plasmid solution with the competent cells, make a wrist-flick to collect all liquid at the bottom of the tube
7. Incubate the cells with the plasmid on ice for 15 minutes.

Heat shock & Recovery

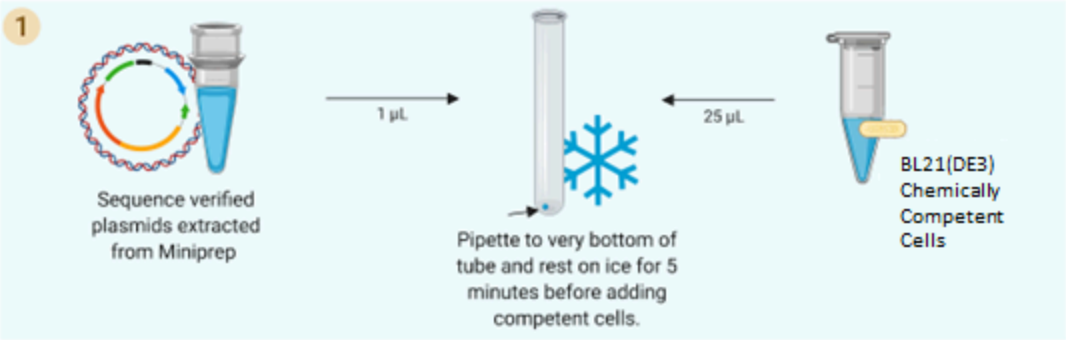
3. Heat-shock cells **30 seconds** in a 42°C water bath; do not shake. Then *immediately*, place the cells on ice for at least two minutes.
4. Add 200 µL room temperature SOC medium *without antibiotics*

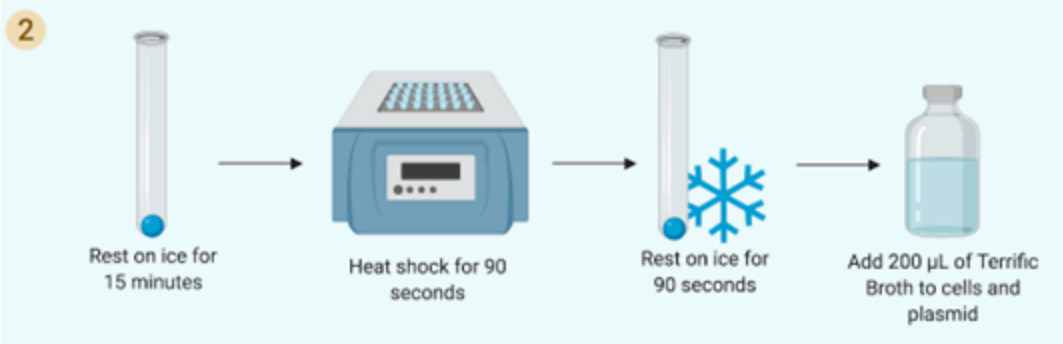
Recovery incubation & plate preparation

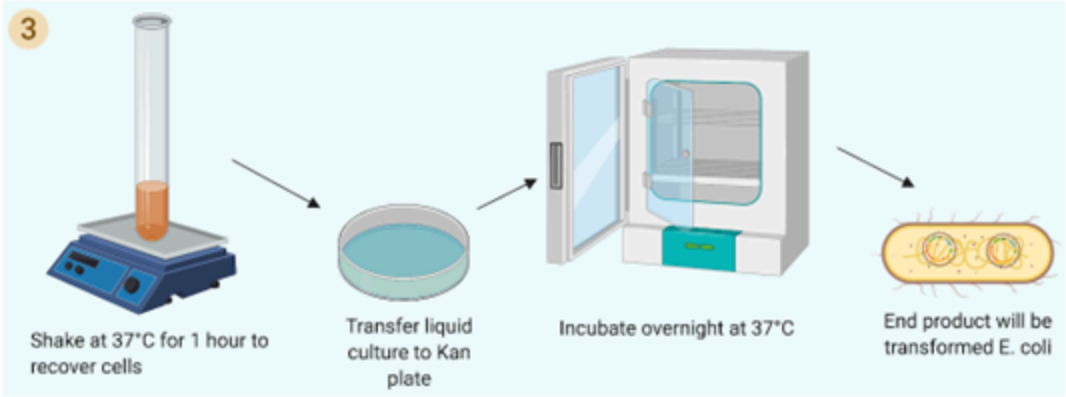
3. Shake at 225 rpm (37°C) for 1 hour to recover transformed cells.
4. Clearly label a LB kanamycin plate for your mutant and one for the 'GFP' plasmid. The label should include the mutant/plasmid name, your initials, '*E. coli* BL21(DE)', and the date of transformation.
5. Plating for overnight growth
4. Pipette 200 µL of culture on an individual kanamycin LB agar plate, use an L-shaped spreader to spread the cell suspension as evenly as possible.
5. Allow the plate to "dry" for a few minutes
6. Turn plates upside down and incubate at 37°C overnight.

The next step will be carried out by your instructors, however you will need to prepare the tubes for them, so ensure you have read the Overnight Cultures protocol on page 66-67, and left labeled tubes on your bench before leaving class.

BL21(DE3) Transformations







Plasmid long-term storage

Once your plasmids are sequence verified, congratulations! You have completed a major milestone. You will now be consolidating your successful samples, which have clean (contaminate-free) mutation-present reads. And, you will be discarding any samples without the mutation or that have contamination present. We will ‘re-transform’ plasmid DNA for each BglB mutant into *E. coli* DH5α cells for long-term storage of the plasmids. Storing the plasmid DNA in the bacteria allows it to easily isolate more plasmid DNA in the future. Glycerol stocks can be used to grow larger amounts of the bacteria again to do a plasmid miniprep.

Your instructor will tell you if your plasmid DNA will be used. If your plasmid DNA will not be used, don’t be disappointed, we only need each mutant (we have six in total) plasmid once for both sections. If your plasmid DNA has been selected, you will need to transform 10-50 ng of the DNA into competent *E. coli* DH5α cells IN ADDITION to transforming into the competent *E. coli* BL21(DE3).

Behind the scenes:
A single colony from the transformation plate is used to inoculate an overnight culture as described on p. 46. The next morning 500 µL of sterile glycerol (50% (v/v)) is filled into a properly labeled (see below) cryotube and an equal volume of the overnight culture is added and mixed well by pipetting up and down. Cryotubes are stored at -80°C.
**Cryotube labels should include the following information:*

1. *Bacterial strain name*
2. *Sample Name*
3. *Enzyme gene abbreviation (BglB)*
4. *vector name (pET)*
5. *the date the sample will be stored (day after overnight set up)*

e.g. E. coli DH5α E406D BglB pET 09/29/2023

Protein Production: Growth and Expression Overnight Cultures

In this step of the workflow, we will (a) grow, i.e., multiply/expand, our transformed BL21(DE3) cells and (b) induce protein expression of our BglB mutant enzyme with IPTG. These links will take you to additional materials focused on the science of how we get the *E. coli* to ramp up the production of our protein of interest. This article explains in more detail, and this video gives a good visualization of lac repression..

Equipment

- Shaking incubator, centrifuge that can hold 50 mL tubes and spin with 4,700 RPM (3043 xg), optimally at 4˚C

Overnight Cultures

This step will be completed 'behind the scenes' by your instructors, however it is important that you understand what has been done. In this step, we will be growing up, or multiplying, the bacteria that we transformed. We need large numbers of bacteria expressing the mutant BglB protein to obtain a sufficient amount of protein for downstream use. To do this, we use individual colonies from your mutant transformation plate to prepare an overnight culture. Refer back to the Overnight Culture instructions (page 46) for more information.

Note that we will be using an increased volume of LB medium in this reaction in comparison to the overnight cultures we made for downstream Miniprep.

Material	Amount per sample/mutant
Transformation plate from Session 6 (pp. 62)	1
Luria Broth	5 mL
1000X kanamycin stock solution (50 mg/mL)	5 µL
50 mL Falcon tube	1

Instructions

1. Prepare a LB + kanamycin growth media solution: add 1 µL of kanamycin stock per mL of media; this is a 1000X dilution. *For example, if you need 10 mL of media, you should add 10 µL of kanamycin stock to 10 mL of LB.*
2. Aliquot 5 mL of growth media to each 50-mL Falcon tube.
3. Using the tip of a P20 pipette, or sterilized toothpick, carefully touch a single colony from the plate and dip the tip in the growth media in the Falcon tube.

Note: If you have colonies with an uneven surface and rough edges, they may be contaminants and not E. coli. Do NOT inoculate with one of these colonies; choose a small, round, shiny colony.

4. Partly screw Falcon tube cap back onto the tube and secure with a piece of tape.
5. Incubate with shaking at 37°C overnight (~16 hours)

Expression cultures

In this step, you will exchange the spent growth media in your cultures with fresh growth media supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG induces expression of your target protein using the engineered pET expression system.

Preparation

Material	Amount per reaction
growth cultures from day 2	1
Luria Broth (LB)	50 mL
IPTG solution (1 M)	
kanamycin solution (50 mg/mL)	

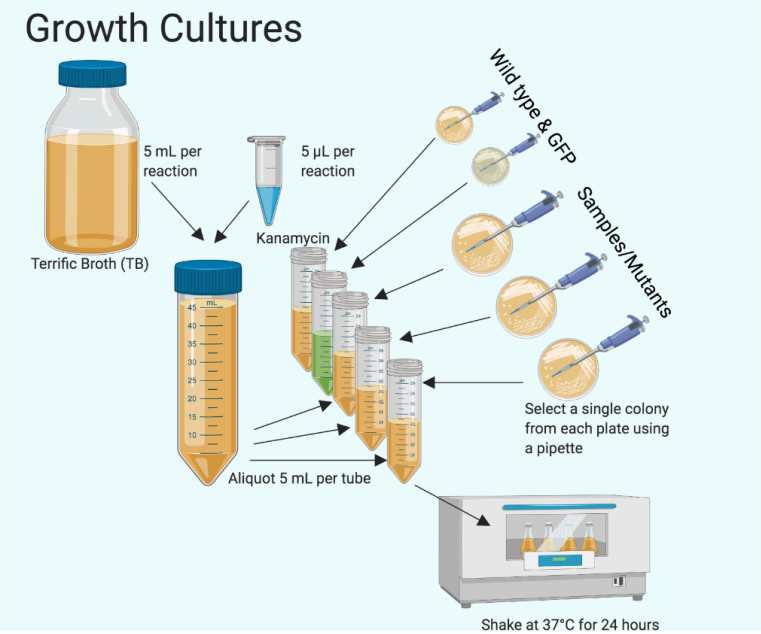
Instructions

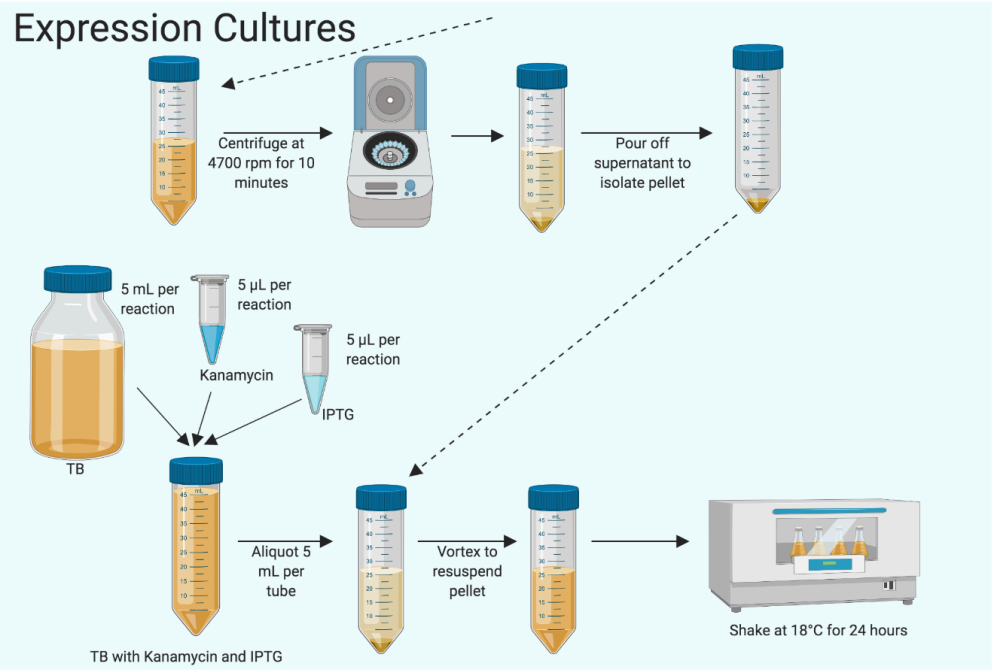
1. Fill two cuvettes with media, one with 1 mL LB (this is you blank) and one with 900 µL.
2. Take out 100 µL of your overnight culture and add to a cuvette containing 900 µL LB
3. Read the OD600nm using a spectrophotometer and note down the optical density your cells have reached.
4. Centrifuge the rest of your overnight cultures at 4,700 RPM (3,043 xg) for 10 minutes.
5. While you are waiting for the spin to complete, use a serological pipette to add 50 mL LB media to a 250 mL conical flask. Add the correct volume of 50 mg/mL kanamycin and 1 M IPTG such that you have a final concentration of 50 µg/mL kanamycin and 100 µM IPTG in your 50 mL of LB.
6. After the spin, carefully pour the supernatant from the tube into a bleach waste beaker.
7. Use a serological pipette to carefully resuspend the pellet in 10 mL of the expression medium you have set up in step 5 by pipetting up and down, or vortex the tube until the pellet is fully resuspended.
8. Add the whole volume from the 50 mL tube (10 mL media with the resuspended pellet (will be more than 10 mL now total) to your 250 mL conical flask with the remaining 40 mL expression medium, to make the final volume ~50 mL. This is now your expression culture. Carefully replace foil seal to flask. If your foil is broken, ask your instructor for more.
9. Incubate with shaking at 18°C for 24 hours.

You can STOP here. After pelleting the cells, they can be frozen in the -20°C freezer. Your instructors will pellet the cells for you and freeze the pellet.



Protein Production: Growth and Expression Overnight Cultures





Note: the illustration shows 5-mL cultures, but we will use 50-mL cultures in a 250 mL conical flask.

Protein Purification

In this step, you will be purifying your mutant enzymes using a technique called *immobilized metal affinity chromatography*. The goal of this process is to isolate the BglB enzyme (produced in the culturing steps) from all the other cell debris. Our BglB enzyme has been engineered to have a poly-histidine tail. Histidine's nitrogen-carbon aromatic ring interacts strongly with the positive charge of the nickel beads used for chromatography. We elute the protein with an EDTA solution (elution buffer), which has a strong negative charge and outcompetes the protein for charged "spots" on the beads.

Watch a short **tutorial** video on Protein Purification. These videos are in development. You can share feedback on the video anonymously: https://forms.gle/ZwP1iUzEtTQa1ff88

Preparation

Material	Storage	Amount per reaction
Expression culture from day 3	Incubator or -20℃	45 mL
Wash Buffer	Room temp	~ 30 mL
Lysis buffer	-20℃	4 mL lysis buffer (2 mL B-PER, 2 mL wash buffer, lysozyme (1.6 mg/mL), DNAse (0.2 mg/mL)
Elution Buffer*	Room temp	600 µL
Ni-NTA resin slurry	4℃	200 µL

Equipment

- Rocker/shaker, centrifuge that can hold 50 and 15 mL cultures and spin with 11,000 xg or greater, optimally at 4°C.

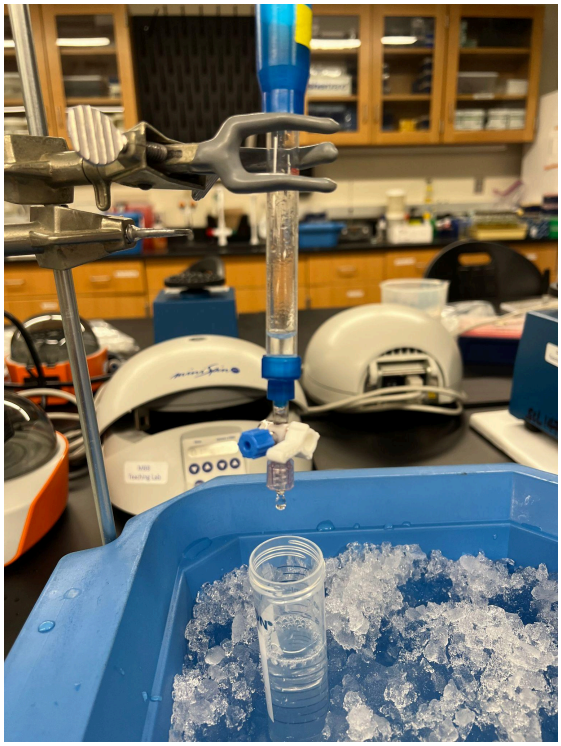
Instructions

Cell Lysis and Clarification

1. Carefully pour 45 mL of the expression culture into a 50 mL Falcon tube. Balance your tube with a partner, ensuring that the tubes are within 0.1 g of each other. *You can match the weights by adding fresh LB to the tube that is lighter, 1 mL of LB weigh approx 1 g.*
2. Centrifuge the expression cultures at 4˚C at 3000 xg for 10 minutes.
3. Pour away the supernatant into the waste bucket.
4. Use a serological pipette to add 4 mL of Wash Buffer to the pellet and resuspend by pipetting up and down repeatedly. (This may take a while, but make sure that the whole pellet is completely resuspended.
5. Add 4 mL of lysis buffer.
6. Rock the tubes at 70 RPM for 30 minutes at room temperature to lyse the cells.
7. Centrifuge the tubes at 12,000 xg for 30 minutes at 4℃ to clarify the lysate. While centrifuging, prepare your columns in the next step.

Gravity flow column Purification

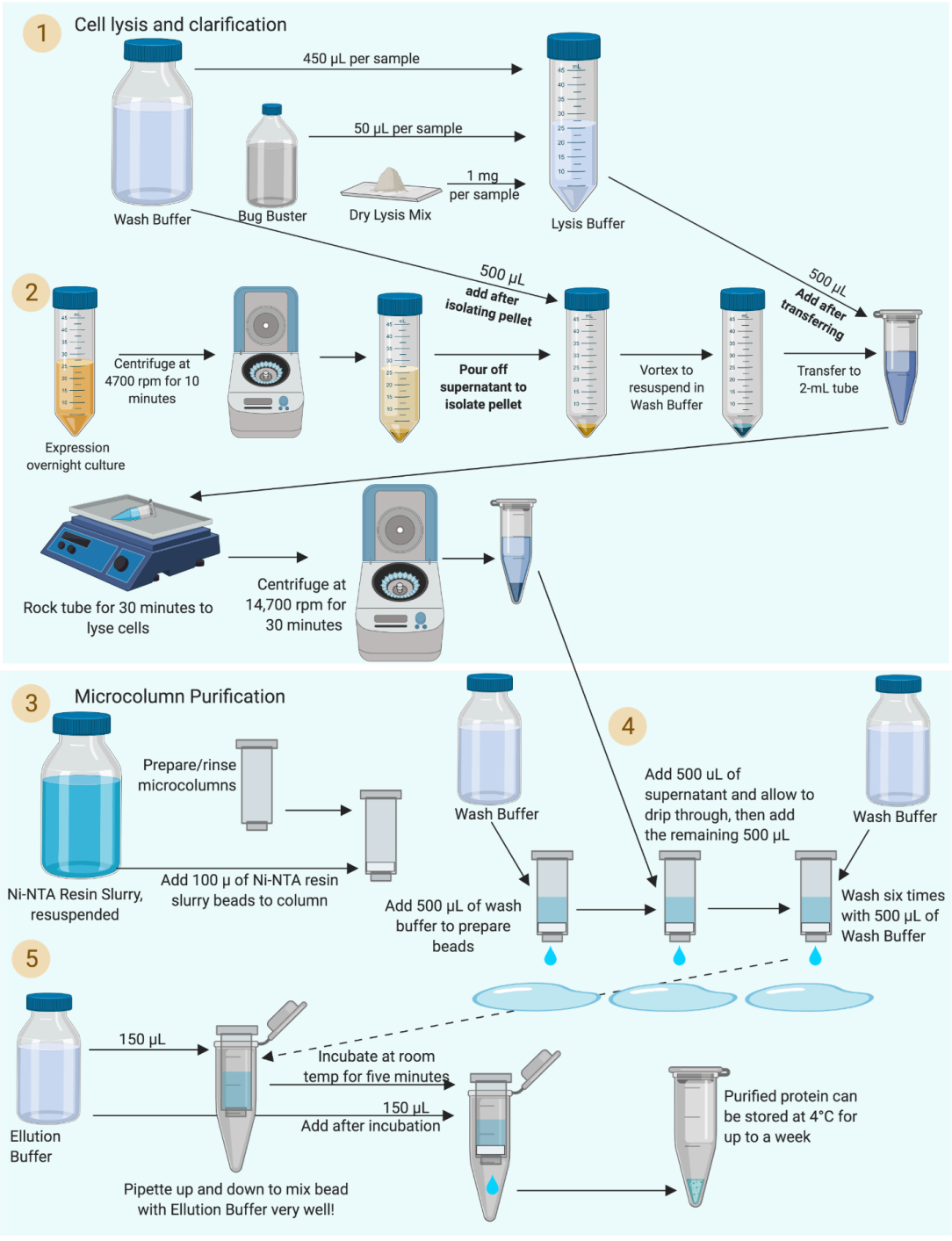
1. Place a ‘waste’ Falcon tube under your column and fill the column with 10 mL deionised water to ensure the column is flowing and the stopper tap is working.



Column set-up with 50 mL falcon placed beneath on ice to collect e.g. the flow through.

2. **Vigorously shake the bottle of the Ni-NTA resin slurry (aka Beads) to ensure the beads are completely resuspended in solution**.
3. Add 200 µL of Ni-NTA resin slurry to the column bed. **Use the P1000 pipette.**
   Note: P200 tips are too small and do not properly transfer the beads in the Ni-NTA resin slurry.
4. Add 5 mL of Wash Buffer and flow through to equilibrate the beads. Allow the Wash Buffer to drip through to clear off the buffer the beads are suspended in. Once washed, **close the stopper tap.**
5. Once the lysed cultures have finished spinning, carefully take up the clarified supernatant using a serological pipette and add this directly to the column. ***Take care to avoid touching or disrupting the pellet***
6. Add a fresh tube labeled 'Flow through' under the column, on ice, open the stopper tap and allow the supernatant to drip through. Once complete, close the stopper tap and seal the flow through tube and keep it on ice. Transfer 50 µl of the flow through into a 1.5 mL tube labeled 'flow through + your initials'- you will need this aliquot for the SDS-PAGE gel **in the next session.**
7. Place a fresh 50 mL Falcon tube labeled 'wash' under the column and wash the bead-enzyme complex at the bottom of the column **six** times with 5 mL of Wash Buffer. Add the wash buffer carefully to the top of the column and allow the Wash Buffer to drip through each time.
8. **Ensure the stopper tap is closed.** Add 300 µL of elution buffer directly to the beads and flick the column to mix. The solution will appear cloudy. Incubate at room temperature for five minutes.
   Note: this step is essential. If you don't resuspend by flicking the tube you will not get good yield.
9. Label a 1.5 mL tube 'elution 1 + your initials' and place it into a 15 mL Falcon tube and place this **in ice**, underneath the column. Open the stopper tap and collect all the flow through. **Close the stopper tap and place the 1.5 mL tube into the ice.**
   Repeat step 8 and 9 using a fresh 1.5 mL tube labeled 'elution 2 + your initials'.
10. Bring your flow through, and both elution 1 and 2 tubes to the instructors (ice bucket) ***These elution tubes contain the purified protein solution you worked so hard to create!***
11. Wash the beads out of the column by filling with deionised water and tipping out into the waste buckets. Run one final wash of deionised water through the columns and leave the stopper taps open.

Protein Purification



Note: we are using larger columns and use larger volumes of buffers to purify larger amounts of protein. This schematic is just supposed to give you an overview of the workflow.

Quantification of Protein Yield by A280

In this procedure, you will be quantifying the protein concentration of the BglB enzyme you produced and purified in the previous steps. Typical yields are 0.2-1.0 mg/mL for the wild type protein. To obtain concentration data, we use a spectrophotometer that shoots light through the sample. We expect to observe an absorbance spike at 280 nm. This is the wavelength of light absorbed by the protein. An A260/A280 ratio greater than one indicates that the sample is contaminated. We will also test for protein contamination by running an SDS-Page gel after completing our assays. More information about A280 assays can be found following the link below:
[Nucleic Acid Purity Assessment Using A260/A280 Ratios]

Equipment

- Nanodrop, Instructions

12. Set up nanodrop for protein A280 reading
13. Clean the sample reader with DI water. Then, dry with a Kimwipe.
14. Pipette 2 µL of elution buffer onto the reader, press ‘Blank’ it and then clean the plate
15. Pipette 2 µL of your sample to the plate and read.
16. Record the A280 value in your lab book.

Running an SDS-PAGE gel

This document contains instructions for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE determines protein yield and protein purity. **Typically you will run a gel after you have performed your assays** because this is the least time-sensitive of the post-purification steps.

Watch a short **tutorial** video: https://youtu.be/VYnIh-PSjG8:cc=on

Anonymous Feedback: https://forms.gle/LfxEMB5HBRh54bEa8

Preparation

Material	Storage notes	Amount per sample
Purified protein	-80°C or 4℃ if <2 weeks.	12 µL
4x loading dye (Lamelli sample buffer without added β-mercaptoethanol)	RT	4 µL
Protein gel (BioRad mini-Protean 4-15% 15-well gel)	4℃	1
Marker: BioRad Precision Plus Dual color standard (Cat: 1610374)	-20℃	6 µL
20x Bis-Tris running buffer	RT	25 mL
Coomassie Blue protein gel stain	RT	10 mL

Equipment

- Gel box for SDS-Page, gel box power source, microwave, gel imager.

Instructions

1. Select the elution (1 or 2) from the last session with the highest protein concentration, based on the A280 collected from the nanodrop. *Note down your choice and keep the other elution stored at 4˚C as a backup in case anything goes wrong!*
2. Prepare the following samples by pipetting 4 µL of 4x concentrate loading buffer in each labeled PCR tube followed by 12 µL each sample:
    a. Either elution 1 or elution 2, whichever had the highest A280 concentration
    b. Flow through aliquot from last session
3. Slowly pipette up and down to mix the contents well. Heat to 95°C for five minutes in the thermocycler. (Setting: "SDS-PAGE')
4. Prepare the protein gel and electrophoresis apparatus.
    a. Carefully remove the tape from the bottom.
    b. Place the gel into the apparatus with the long plate facing away from you and the short plate facing you.
    c. Lock in the gel by clamping the electrode cassette frame toward you.
    d. Carefully remove the comb from the well on top of the gel and
5. Pour 1X MES Buffer Solution into the gel electrophoresis apparatus to cover the gel. **Make sure both the front and back of the gel are submerged or you will get an error message.**
6. Load the protein standard (ladder) and samples:
    a. Load 6 µL of BioRad Kaleidoscope protein standard (ladder) into one well (typically the center-most well unless your instructor recommends otherwise).
    b. Load 12 µL of the heated, dyed protein into the gel wells. Make sure to note which protein is in each well in your lab book.
7. Place the tank cover with the electrodes inserting into the appropriate places in the corner and middle of the apparatus. (Electrodes: red to red, black to black).
8. Running the gel.
    a. Set the machine for 150V for 10 minutes to allow samples to run through the stacking gel. Once running, ensure tiny bubbles come off the metal plate in the front of the gel.
    b. Increase to 200 V and run for a further 45 min.
9. Check the gel every 20 minutes to ensure that the samples are not running off the gel. Once sample dye has migrated to the bottom, stop the run unplug from power pack.

Notes

- You should observe many small bubbles streaming from the electrode—this indicates that the gel is running properly.
- If you are running more than one gel, it is a good idea to load the ladder into different positions in each gel to easily differentiate between them

Staining the gel

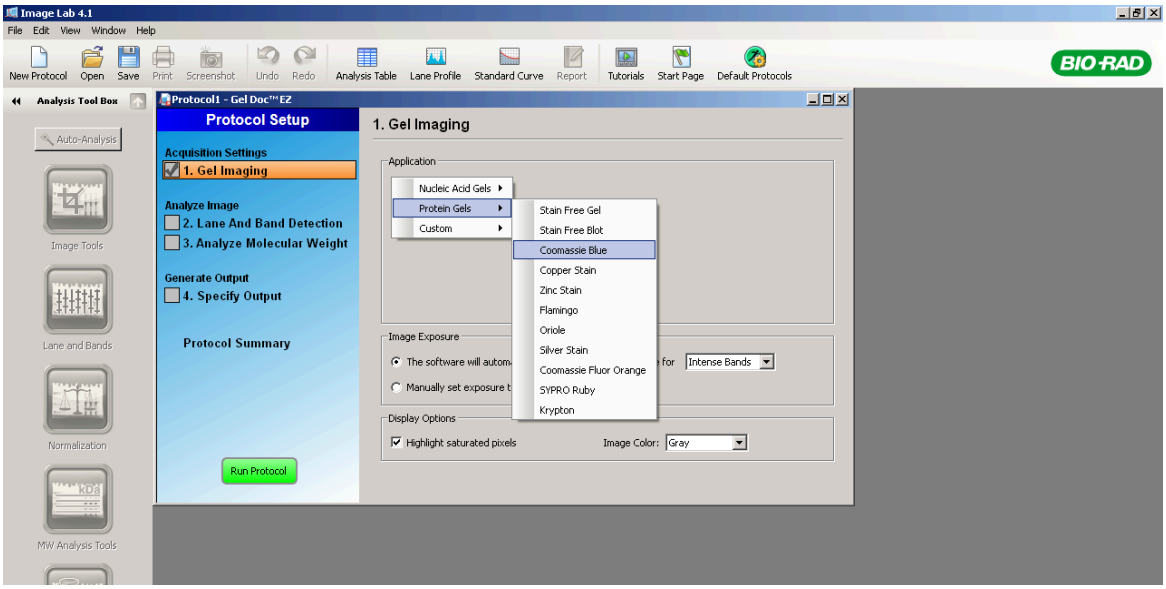
1. Carefully crack open the plastic around the gel with a spatula.
    a. Tip: carefully pry open the corners before slowly prying off the short plate. Fill a Tupperware container with enough water to cover the bottom, flip the plate so the open gel surface is facing down, and press the spatula into the long skinny opening at the bottom of the plate. The gel should easily fall off the plate and into the water.
2. Rinse with DI water. Be careful not to roughly hit the gel with water or it will break.
3. Pour away all the water and pour in around 10 mL Coomassie Blue gel stain to barely submerge the gel.
4. There are two techniques that you can use to stain and destain the gel: (a) the overnight method and (b) the quick microwave method.
    (a) The overnight method
        (i) To stain: Place the container holding the gel with the stain added on the shaker for 1-24 hours.
        (ii) To de-stain the next day: Pour off the stain and wash once or twice with DI water until the rinse water runs clear. Refill the container with just enough DI water to submerge the gel.
        (iii) Place the container holding the gel and water on the shaker for 12-24 hours.
        (iv) Pour off water and wash once or twice with DI water until the rinse water runs clear. Gently transfer the gel from the container to the gel-imager tray. (The tray will have an opaque white box in the center.)
    (b) The microwave method
        (i) Microwave the container holding the gel and stain on high for 30 to 45 seconds total. Check the gel every 10 to 15 seconds, making sure it is not curling. Gently shake the gel to evenly distribute the heated solution.
        (ii) Pour off the stain and wash once or twice with DI water until the rinse water runs clear. Refill the container with just enough DI water to submerge the gel.

(iii) Microwave the container holding the gel and water on high for 30 to 45 seconds, checking every 10 to 15 seconds to ensure no curling. Gently shake the gel to evenly distribute the heated solution. The water will turn blue and the gel will decolorize.

(iv) Pour off water and wash once or twice with DI water until the rinse water runs clear. Gently transfer the gel from the container to the white gel-imager tray.

Taking a gel image:

1. Wash off the white gel-imager tray with DI water and dry with Kimwipes.
2. Remove the gel from the water and place it in the center of the white tray.
3. Read the image using BioRad ImageLab software (may be called just "ImageLab"). On the Start page, select Protocol 1. *Make sure Gel Imaging is checked on the left column. You do not need to checkmark the "Analyze Image" or "Generate Output" sections.*
4. Insert the tray into the imager machine and click the green "Run Protocol" button.
5. To save, click file, **export -> export for publication** (choose 300 dpi resolution) and save your file as a **.tif** in the 251 spring folder (make sure name your file with names and date) *You can also save the image file, by choosing 'Save as', but be aware you must also **export** the image, or you won't be able to open it on another computer.*

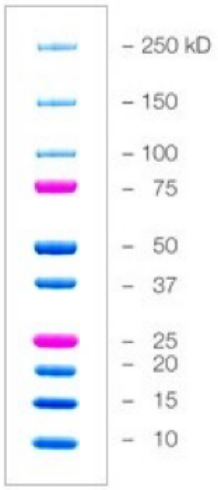


Example screenshot of imaging software. Application should be "Coomassie Blue", Image Exposure should be set to "Intense Bands", Display Options Image Color should be set to Coomassie Blue.

Annotating you gel

Use an image manipulation software, such as Powerpoint, Biorender or photoshop to add labels to your gel.

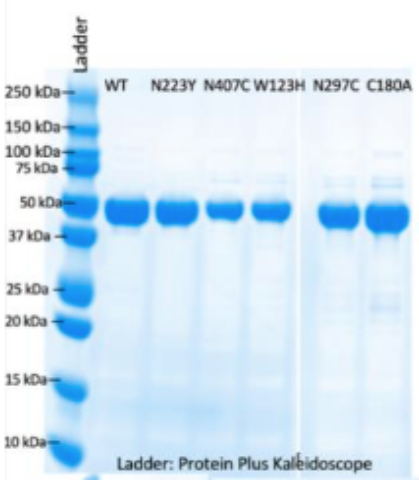
1. Download your gel from the Files section of Canvas.
2. First, import the respective gel image file, and crop to display just your lanes.
3. Annotate on the ladder according to the manufacturer's image below.



BioRad marker guide for precision protein plus dual color marker.

4. Annotate the names of the samples in each lane
5. You may also wish to add a small arrow alongside your gel indicating where the BglB protein is.

   Below is an example annotated figure and legend, and *here is a link to Google Slidedeck with a number of example annotations.*



***Example figure labeling**: 4-12% SDS-PAGE for BglB mutants and wild type (WT) using Protein Plus Kaleidoscope as ladder.*[1]

[1] This is an example from the recent publication to show how to present gel image https://pubs.acs.org/doi/abs/10.1021/acsomega.9b04105#

Protein quantification by Bradford assay (cuvette reading)

Preparation

Material	Storage notes	Amount per student
Purified protein	4℃, or -80˚C if for >2 weeks	300 µL
BioRad Protein Assay Reagent	4˚C	25 mL
Diluent *(DI water is fine)*		25 mL
Bovine Serum Albumin standard (1 mg/mL)	4˚C	1200 µL

1. Working with your lab partner(s), prepare one set of standard protein solutions between you, as shown in Table 1 in 1.5 mL

Vial	Vol of Diluent (µL)	Vol of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 µL of Stock	1000
B	125	375 µL of Stock	750
C	325	325 µL of Stock	500
D	175	175 µL of vial B	375
E	325	325 µL of vial C	250
F	325	325 µL of vial E	125
G	325	325 µL of vial F	62.5
H	400	-	0

Table 1: volumes for creating standard curve dilutions

2. You will be testing your protein concentration both in an undiluted and diluted sample. To make the diluted sample, ***each person*** must make a 60 µL of a 1 in 3 dilution (into water) of their protein sample in a 1.5 mL Eppendorf tube. (Use the elution fraction with the highest concentration from your A280 readings). Mix well by inversion.
3. Add 20 µL of each standard and each person's sample (diluted and undiluted) into fresh 1.5 mL Eppendorf tubes. Repeat this process so that you have **duplicates** for each BSA standard solution and each BglB protein sample. Between you and your partner(s), y*ou should collectively end up with 16 standard tubes (8x2) plus 2 tubes of <u>each</u> undiluted sample, and 2 tubes of <u>each</u> 1 in 3 diluted sample.*
4. Add 1 mL of the Bradford reagent to each tube, then mix well by inversion. *If splitting the work within your group, be sure to add the Bradford reagent at roughly the same time, so you can start the incubations around the same time.*
5. Incubate samples for at least 10 minutes at room temperature (RT).
6. Pipette each sample (1 mL) into a clean cuvette. *Take care not to knock over your cuvettes, you can use an empty cuvette box as a rack!*
7. With the spectrophotometer set to 595 nm, zero the instrument on a cuvette filled only with 1 mL water.
8. Measure the absorbance of all the standards then all samples. Note down each value below and copy or stick into your lab book- *you may wish to take a picture of this for the at-home assignment.*

9. Calculate the mean from each duplicate set then subtract the mean 595 nm measurement for the BSA 0 standard (H) from the mean 595 nm measurements of all other individual standard and unknown sample replicates. This is your Corrected mean A595 for each.

Record your readings here:

Vial	Final BSA Concentration (µg/mL)	A595 Rep 1	A595 Rep 2	MeanA 595	Corrected Mean A595
A	1000				
B	750				
C	500				
D	375				
E	250				
F	125				
G	62.5				
H	0				
Undiluted sample (20 µL)					
1 in 3 diluted sample (20 µL)					

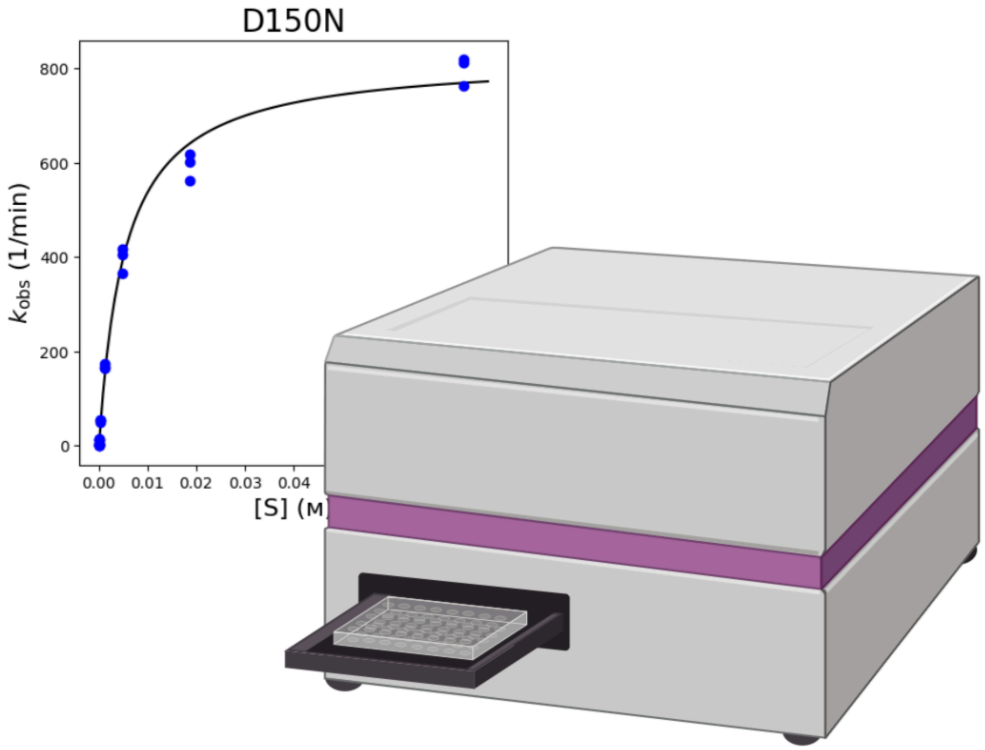
Analysis

You will need to use a graphing software to create a standard curve from which you can then generate the linear equation, which you can use to calculate your unknown protein sample concentrations. There are many options, but this guide shows you how to do it using Excel. *Remember that once you have calculated the concentration values of the protein samples, you will need to multiply the concentration of the 1 in 3 diluted sample by three to correct for the dilution.*

Ideally, the undiluted and diluted-then-corrected values should be pretty similar, however the accuracy of these measurements will be reduced if they are either end of the limit of detection for this assay (in this case <200 µg/mL or >1000 µg/mL). Therefore, if the concentration value you calculate for the undiluted sample is >1000 µg/mL, or your diluted sample is <200 µg/mL, it may be out of range, and thus less reliable. Choose the value which best falls within this range and note down your choice, or, if both values are similar, take the average.

Module 3: Test

Enzyme Characterization



Kinetic Assay

This assay provides data on the catalytic efficiency of your mutant enzyme by observing color change in the enzyme-substrate-product solution over time. The substrate that we use is 4-nitrophenyl-β-D-glucopyranoside; this molecule consists of a sugar attached to a nitrophenyl group. When the sugar and nitrophenyl are connected, the solution is colorless; however, once the nitrophenyl is cleaved from the sugar (this is what our enzyme does) and is free floating, the solution will appear yellow. We can measure yellow intensity as an indicator of product formed by the enzymatic reaction. We do so by using a spectrophotometer plate reader and shooting light at wavelength 420 nm through our samples. A yellow solution will absorb light in the violet range (420 nm), which is on the opposite side of the color wheel.

The faster the solution turns yellow, the more catalytically efficient the enzyme. We interpret catalytic efficiency through the Michaelis-Menten model. To get data that the model supports, we need to vary the concentration of substrate. We do this by setting up a 96-well plate serial dilution. We use triplicate sample replication for data validation purposes, and we always run the wild type in parallel with our three mutants. **More info on how we interpret the kinetic assay data can be found here.**

Preparation

Material	Storage notes	Amount per 96-well plate
Purified Protein (mutant protein plus an aliquot of wild-type for pair/group)	4℃	25 µL per well of 3 µg/mL stock
Substrate Stock (4-nitrophenyl-β-D-glucopyranoside 0.1 M)*	-20℃	2 mL
Protein Elution Buffer	4℃	~ 8 mL
“Protein Plate” (Corning 96-Well Clear-Bottom Half-Area Microplates, #3884)		1 plate
“Substrate plate” can be any half-area 96-well plate		1 plate

**Note: Thaw your substrate stock and vortex if necessary until completely clear (i.e., no precipitate). It may look a little bit yellow due to background pNPG hydrolysis, which is normal.*

Equipment

- 50-300 µL multichannel pipette, plate reader with monochronometer or A420 filter, computer with plate reader software installed.

Instructions

Determining Dilution Factors for Purifications

1. Calculate the volume required (V1) to dilute both the wild-type and your mutant protein samples to a final concentration (C2) of **3 µg/mL in a final volume of 1 mL** (V2). Calculate the appropriate volume of diluent (elution buffer) to make up the final volume. Dilute and keep on ice.

Use the equation: V1.C1=V2.C2 *take care with your units and check with an instructor if you are unsure.*

Preparing the Substrate Plate

First, work with your lab partner(s) to make one substrate plate between you. In this section, you will be making a 96 sample serial dilution of the substrate we use in our kinetic assay.

1. Get a 96-well plate and prepare your workstation to use the multichannel pipette and a trough with about 8 mL of elution buffer. Using a single-channel pipette, dispense 150 µL of substrate stock into row A (12 wells).
2. Using a multi-channel pipette, dispense 100 µL of elution buffer into the rest of the rows (B, C, D, E, F, G, H).
3. Using a multi-channel pipette, transfer 50 µL of the concentrated Substrate Stock from row A to row B. Mix gently by pipetting up and down 10 times.
4. Repeat this transfer step for all following rows, except row H.

I.e. next transfer 50 µL from row B to row C. Continue working your way down the plate. After you've completed mixing row G, withdraw 50 µL. But instead of adding that to row H, just discard in the waste along with the tips. This is a quick and simple method for making serial dilutions in a plate format.

5. Verify by eye that every well in the plate has the same volume (100 µL).

Table 18.1. Substrate concentrations in mM, note the final dilution with protein solution has been factored in here. New 3X dilutions are expected to generate curves with better, more accurate resolution at lower concentrations.

	3X Dilutions substrate concentration mM
A	75
B	25
C	8.33
D	2.78
E	0.93
F	0.31
G	0.10
H	0

Preparing the Protein Plate

Now that you have created the gradient of substrate concentrations in the substrate plate, aliquot your proteins into the protein plate (the other plate, **DO NOT add your protein to the same plate as your substrate plate!**). Add wild type protein to the first three columns. You will be sharing your protein plate with your lab partner(s).

1. For each mutant, aliquot 25 µL of diluted purified protein with a single channel pipette into each well in three adjacent columns of the plate (24 wells).

Table 18.2. 96-well Protein Plate setup. Descriptors in the table represent the arrangement of WT and Mutant samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	WT	WT	WT	Mut1	Mut1	Mut1	Mut2	Mut2	Mut2	Mut3	Mut3	Mut3
B	WT	WT	WT	Mut1	Mut1	Mut1	Mut2	Mut2	Mut2	Mut3	Mut3	Mut3
C	WT	WT	WT	Mut1	Mut1	Mut1	Mut2	Mut2	Mut2	Mut3	Mut3	Mut3
D	WT	WT	WT	Mut1	Mut1	Mut1	Mut2	Mut2	Mut2	Mut3	Mut3	Mut3
E	WT	WT	WT	Mut1	Mut1	Mut1	Mut2	Mut2	Mut2	Mut3	Mut3	Mut3
F	WT	WT	WT	Mut1	Mut1	Mut1	Mut2	Mut2	Mut2	Mut3	Mut3	Mut3
G	WT	WT	WT	Mut1	Mut1	Mut1	Mut2	Mut2	Mut2	Mut3	Mut3	Mut3
H	WT	WT	WT	Mut1	Mut1	Mut1	Mut2	Mut2	Mut2	Mut3	Mut3	Mut3

Kinetic program setup

The following instructions are relevant to the and MPM6 plate reader software available in SCL 147

1. Turn on the plate-reader and computer, and load the MPM6 software
2. Go to Instrument Setup and set up a kinetic cycle to read Absorbance at 420nm every 1 minute for 15 minutes, with a 3s, high speed shake.

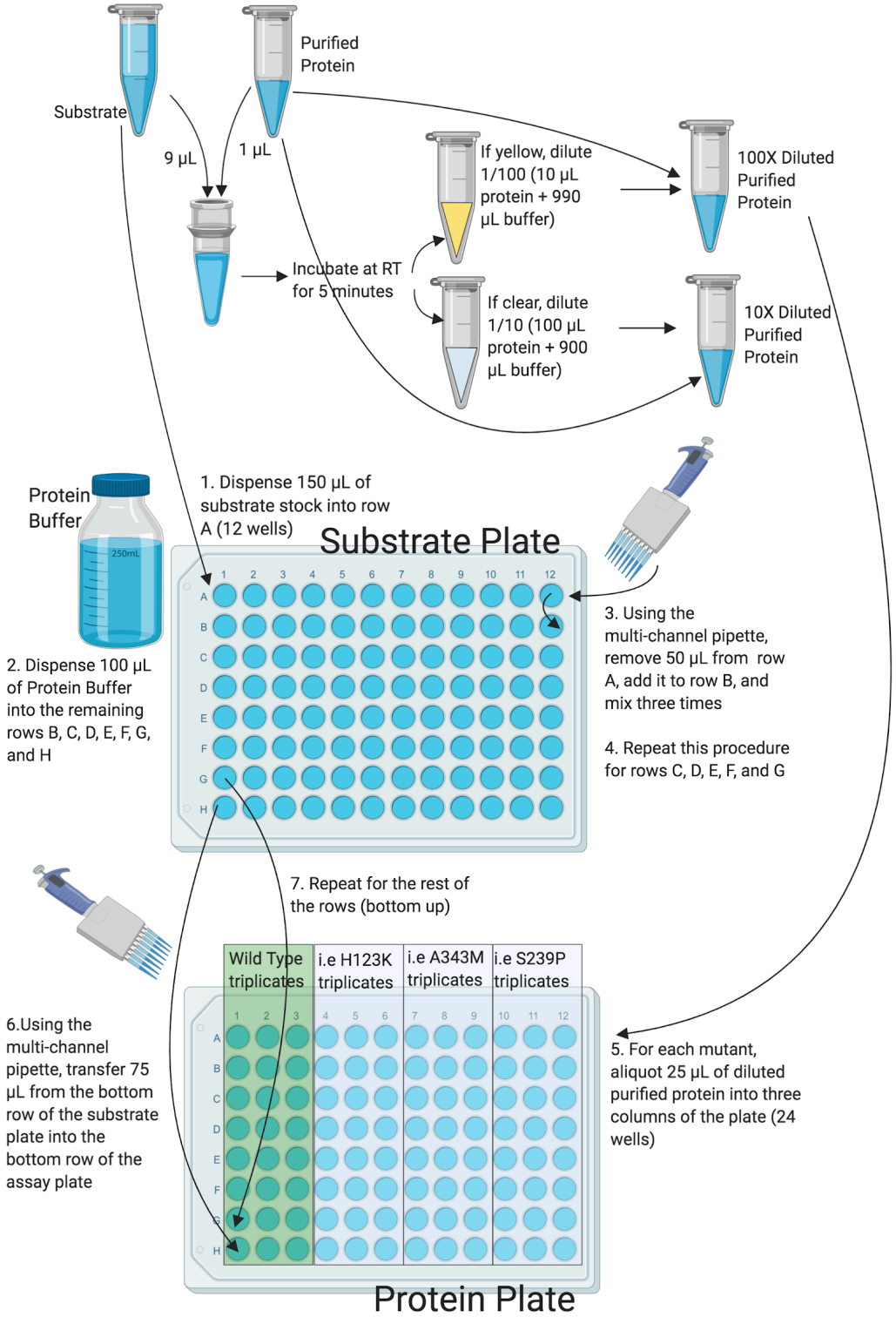
Initiating the Assay and Collecting Data

Ensure that the plate reader is ready and software is open, to the point where you can load your plate into the reader and hit run. As soon as you start to add your protein, the assay is initiated, the "clock starts ticking" and everything is *very time-sensitive.*

To initiate the assay, you will be transferring the solutions from the substrate plate into the protein plate, which will be read by the machine such that the plates are "overlaid".
In other words, well A1 of the substrate plate will be pipetted into well A1 of the protein plate.

1. Move the plate to the substrate addition zone near the plate reader
2. Using the multichannel pipette, transfer 75 µL from the bottom row of the substrate plate into the bottom row of the assay plate.
3. Repeat for the rest of the rows (working your way up the plate, from Row H to A).
4. Place the plate on the prepared plate reader and press "Enter" (run time: 15 minutes).
5. Once the assay is finished, go to "Report" and export the data to Excel.

Kinetic Assay Plate Setup



Simplified Thermal Stability Heat Challenge Assay

This assay provides data on the thermal stability of your mutant enzyme. More specifically, the data generated here answers the question: “what temperature renders our mutant enzyme unable to function properly?” To address this question, we heat the enzyme at a range of temperatures, transfer the heated enzyme to a substrate solution, and observe color change in the solution over time (much like the kinetic assay). We represent this measure of thermal stability as T_{50}, the temperature at which the enzyme loses 50% of its initial activity. The wild type BglB has a T_{50} of 40°C. Therefore, we use a gradient of 30 to 50°C.

We will be using a very simplified method using various heat blocks, but this can be done using a thermocycler to fine-tune the temperature range. More information and examples of enzyme thermal stability are discussed in these papers:

- Ahmad, S., Kumar, V., Ramanand, K. B., & Rao, N. M. (2012). Probing protein stability and proteolytic resistance by loop scanning: a comprehensive mutational analysis. *Protein Science*, *21*(3), 433-446.
- Huang, P., Chu, S. K., Frizzo, H. N., Connolly, M. P., Caster, R. W., & Siegel, J. B. (2020). Evaluating Protein Engineering Thermostability Prediction Tools Using an Independently Generated Dataset. *ACS omega*, *5*(12), 6487-6493.

Material	**Storage notes**	**Amount per 96-well plate**
Purified Protein (3 protein samples / mutants can fit on a plate, along with 1 WT control)	4℃	25 µL per well of 3 µg/mL stock10 or 100 µL
Substrate Stock (4-nitrophenyl-β-D-glucopyranoside, 0.1 M)	-20℃	250 µL

Elution buffer	4˚C	~8 mL
“Protein Plate” (Corning 96-Well Clear-Bottom Half-Area Microplates, #3884)		1 plate

Equipment

50-300 µL multichannel pipette, plate reader with A420 filter, computer with plate reader software installed.

Instructions

Thermal incubations

1. Aliquot 100 µL of your 3 µg/mL protein stock and label them with your initials, mutant name and 30˚C, 40˚C or 50˚C. Store the remainder of your sample on ice.
2. Also set up one **(**3 µg/mL) **wild-type control per substrate plate** for each temperature as above.
3. Incubate samples in their respective condition for 30 minutes, plus one control for each on ice or room temperature (RT)- note down your choice. During the incubation, prepare your substrate. Work in groups of up to 7 people.
4. Once incubation is done, place samples on ice if you aren’t ready to start the kinetic assay right away.



Simplified thermal stability incubations set up

Preparing the Substrate stock Plate

6. Calculate the volume (V1) required to dilute your 0.1M substrate (C1) into a final concentration (C2) of **3 mM, in a final volume of 8 mL**. Calculate the appropriate volume of elution buffer to make up the final volume. Dilute and keep on ice.

Use the equation: V1.C1=V2.C2. take care with your units and check with an instructor if you are unsure.

Preparing the plates

1. Aliquot 25 µL of diluted (3 µg/mL) protein stocks into your designated row in a 96-well plate (you will be sharing with 6 other colleagues this time, and adding wild type as a control, see Table 18.2).

Table 18.2. 96-well Protein Plate setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A - WT	ice	ice	ice	30˚C	30˚C	30˚C	40˚C	40˚C	40˚C	50˚C	50˚C	50˚C
B - Mut1	ice	ice	ice	30˚C	30˚C	30˚C	40˚C	40˚C	40˚C	50˚C	50˚C	50˚C
C - Mut2	ice	ice	ice	30˚C	30˚C	30˚C	40˚C	40˚C	40˚C	50˚C	50˚C	50˚C
D - Mut3	ice	ice	ice	30˚C	30˚C	30˚C	40˚C	40˚C	40˚C	50˚C	50˚C	50˚C
E - Mut4	ice	ice	ice	30˚C	30˚C	30˚C	40˚C	40˚C	40˚C	50˚C	50˚C	50˚C
F - Mut5	ice	ice	ice	30˚C	30˚C	30˚C	40˚C	40˚C	40˚C	50˚C	50˚C	50˚C
G - Mut6	ice	ice	ice	30˚C	30˚C	30˚C	40˚C	40˚C	40˚C	50˚C	50˚C	50˚C
H - Mut7	ice	ice	ice	30˚C	30˚C	30˚C	40˚C	40˚C	40˚C	50˚C	50˚C	50˚C

2. Move the plate to the substrate addition zone near the plate reader and ensure the reader it is set up, as for the kinetic assay.
3. Pour the 3 mM diluted substrate stock into a multichannel trough and use a multi-channel pipette to transfer 75 µL of diluted substrate into all 96 wells of the assay plate.
4. Immediately transfer to the plate reader and begin the kinetic cycle, as for the kinetic assay.
5. Once the assay is finished, go to “Report” and export the data to Excel.

Thermal Stability Fluorescence-Based Protein Unfolding Assay

This assay provides data on the thermal stability of your mutant enzyme. More specifically, the data generated here answers the question: “at what temperature will this enzyme become half way unfolded?” We represent this measurement as T_m. We are able to elucidate T_m by tagging the internal, hydrophobic residues of the enzyme with a fluorescent molecule; in other words, when the molecule is tightly folded in its normal, functional state, the internal hydrophobic residues are tucked away and not able to bind to the fluorescent dye. However, with increased temperature, the protein will start to unfold, exposing the hydrophobic residues and the dye will bind, which results in observable fluorences and can be quantified by a qPCR machine. The wild type BglB has a T_m of 42 to 46°C; therefore, we use a gradient of 30 to 80°C. In general, you can adjust the gradient of temperatures on the qPCR machine to center on the T_m of your protein.

Material	Part number	Amount per sample
Purified protein: Mutants + WT (positive control)		100 µL
(MicroAmp) Optical strip (tubes)		1

(MicroAmp) Optical caps		1
Elution buffer		< 2 mL
1000X Protein Thermal Shift Dye kit	4461146	< 1 µL

Equipment

- Thermal cycler, quantitative PCR machine, computer with (a) QuantaStudio Analysis Software and (b) thermal shift assay software.

Instructions

Diluting Your Protein

☐ Dilute each of your mutant proteins and WT in PCR tubes using the table below. According to their respective A280 concentration, choose **only one** dilution in the table:

Table Page 20.1. Protein dilution table

Protein concentration (A280)	Dilution	Dilution notes
< 0.1 mg/ml	NA	Do not dilute
0.1 to 1 mg/ml	2X	Mix 50 µL protein with 50 µL elution buffer
1 to 5 mg/ml	10X	Mix 10 µL of protein with 90 µL of elution buffer

For example, if the WT has an A280 of 2.345 mg/mL, then make a 10X dilution for the WT. Follow this process for all mutants.

☐ Prepare and label new MicroAmp tubes so that each mutant has three tubes (a triplicate). MicroAmp tubes are **not** the same as PCR tubes, have numbers on their necks, and use flat caps. **Double check you are using MicroAmp tubes before loading your diluted proteins**.

☐ Load 17.5 µL of each diluted protein into their respective tubes. Remember to do a triplicate for your wild type.

For example, if you have 3 mutants and the wild type, you will have a total of 12 samples and need 12 PCR tubes.

Preparing Thermal Dye

Prepare a solution of 16X Protein Thermal Shift (PTS) dye (1000X stock).
You can prepare this dilution in a PCR tube or 1.5-mL tube. You will need three replications per sample (three wells per sample).

Calculate the Final Volume You Will Need of PTS Dye.

☐ Count the number of mutants and wild type samples you will work with.
☐ Multiply this number by three (because you will be working in triplicate).
☐ Add two extra samples in for pipetting error, etc.
☐ Each sample requires 2.5 µL of diluted dye.

For example, if I have 3 mutants and the wild type, I should make enough diluted dye for 14 samples. So, in my example, I will need 35 µL of diluted dye (e.g., $14 \times 2.5\ \mu L = 35\ \mu L$).

☐ Use $M_1V_1 = M_2V_2$ to find how much 1000x PTS dye (M_1) you need.

For example: if $35\ \mu L = V_2$, $16X = M_2$, $1000X = M_1$, find V_1 $(0.56\ \mu L)$.

☐ Make the needed amount of 16X PTS dye by diluting 1000X dye with elution buffer.
For example: $35\ \mu L - 0.56\ \mu L$ *of* 1000X PTS dye $= 34.44\ \mu L$ of elution buffer. Combine this with the 0.56 uL of PTS dye with the elution buffer to make the diluted dye.

Reaction Setup

Add the components in the order listed to the PCR tubes.

1. Add 17.5 µL of the protein sample.
2. Add 2.5 µL of diluted 16X PTS dye.
3. Pipette each reaction up and down 10 times to mix.
4. Cap the plate with the MicroAmp optical cap and spin it at 1000 rpm for one minute.
5. Turn the qPCR on, load the “Bagel_Thermal_protocal” file, and name your experiments (*For example: Peishan Mutant-Batch1 MM/DD/YY)*.

Program Setup

1. Open the QuantaStudio Analysis Software.
2. Open the existing experiment.
3. Click "*Computer*" > "*AB SW&Data*" > "load *Standard_BAGEL_Thermo.edt*" file
4. Give the experiment a name (e.g., *PH_mutant1_date)* and descriptions of the experiment. Click "Next."
5. No change to the *Experiment Method*, click *next.*
6. Click Next again at the *Assign Targets and Samples.* You can fill in your sample locations if you would like but this has no bearing on the graph that will be generated.
7. Load your samples and close the qPCR.
8. Click the blue START RUN button on the top right and click the number that appears below it. You will be prompted to save this data. Save the data under *Computer* > *AB SW&DATA* > *Bagel_thermo_data* folder.
9. It should take about 20 minutes for the run to complete. Make sure to take the plate out after completion.

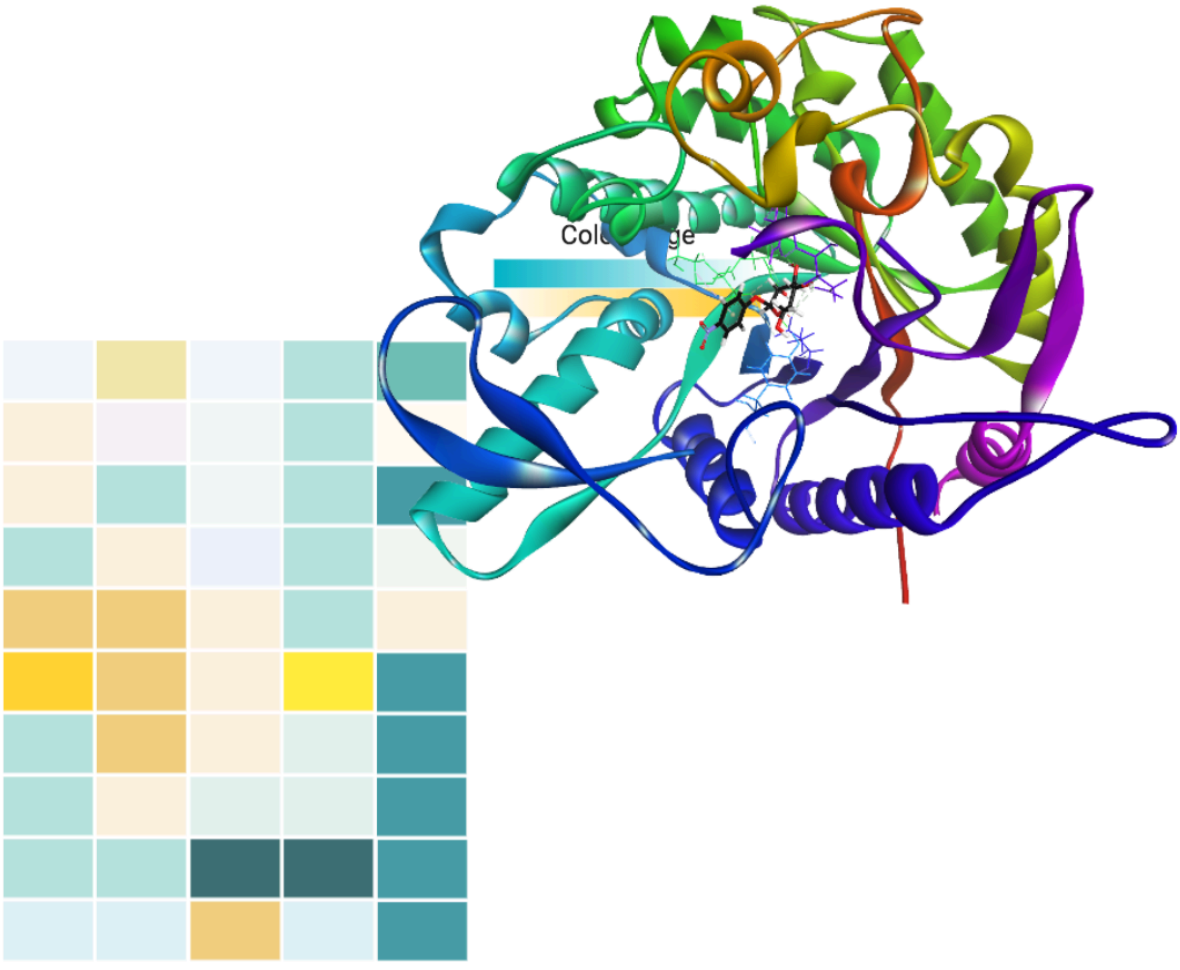
Analyzing and Retrieving Data

1. On Desktop, click on *Protein Thermal Shift Software*
2. Click *Create Study* on the very top left.
3. In Study Properties, name your file and add a description of what samples are present. Hit Save just above the Study Properties name.
4. Click *Experiment files*, then click the green plus sign Import button on the upper left corner and load your .eds file for data analysis.
5. Define your samples by clicking *Assign* under Plate Setup or by right clicking the well and hitting *Assign Well Content*.
6. At the very top, near the save buttons, hit *Analyze* with the bright green play button.
7. Two graphs and a table should appear. Your WT should be around 42C to 46C. Check in with your instructor to verify the results. The top graph should look exponential and the second graph should have an obvious peak. The table that is generated shows you the individual Tm values for your mutants. To see only one at a time, click on their sample name to highlight only that curve.
    a. Note: If you would like to reanalyze a certain region of a graph, click the *Define a region of analysis* button (two lines and a green plus sign) under Melt Curve Plots. Click and drag to select a particular region then hit the main Analyze button at the top to regenerate readings. The new value will show up under the arm D column of the table.
8. Export the file by clicking *Export* on the left. On the same left column, click *Analysis data*

9. In the Export directory (Top column), change the location to a USB drive.
10. Change the file type to .csv (not .txt) and also file name.
11. Then click *export* on the top right corner.

Module 4: Learn

Data analysis and interpretation



Analyzing kinetic assay data

Data Analysis Resource List

- Guide to interpreting colorimetric data assay data with enzyme kinetics
- Details on BglB protein and PNP standard curve
- Sample Kinetic and Thermal Assay Data

Processing plate reader data from the kinetic and thermal assays is an exciting and terminal component of the D2D workflow. In this section, you will be generating the values that we use to characterize catalytic efficiency and thermal stability of your mutants and wild types. In other words, we are using the data collected to figure out (1) the rate at which the enzyme converts substrate into product, and (2) the temperature that the enzyme is rendered inactive. Calculate the rates of absorbance over time for each well, wherein the data has a linear trend; this is often called the *initial rate*. The details for *why* we collect this data are here: Guide to interpreting colorimetric data assay data with enzyme kinetics

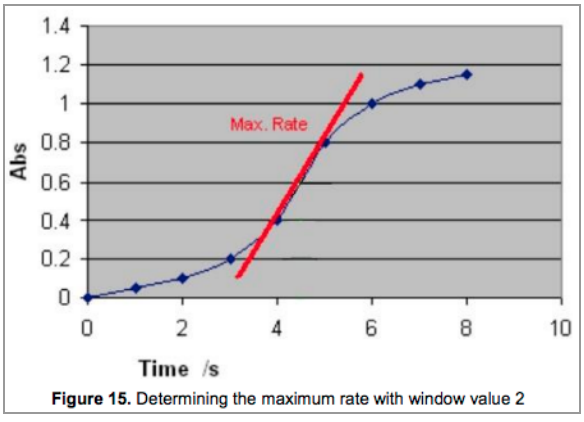


Figure 21.1. Maximum rate determined from linear portion of reaction over time, our rate is determined over. 3-point window (Thermo, 2015).

Cleaning and averaging our replicate data

We need to start by getting all our data into one sheet and calculating the averages from our triplicate data.

1. Save your raw data sheet to your OneDrive (to ensure its constantly backed up)
2. Make a new sheet and name it [WT+your mutant]_raw. If your data is spread across multiple sheets, use this sheet to collate it all (i.e the wild type reading from your plate, plus only your mutant data).

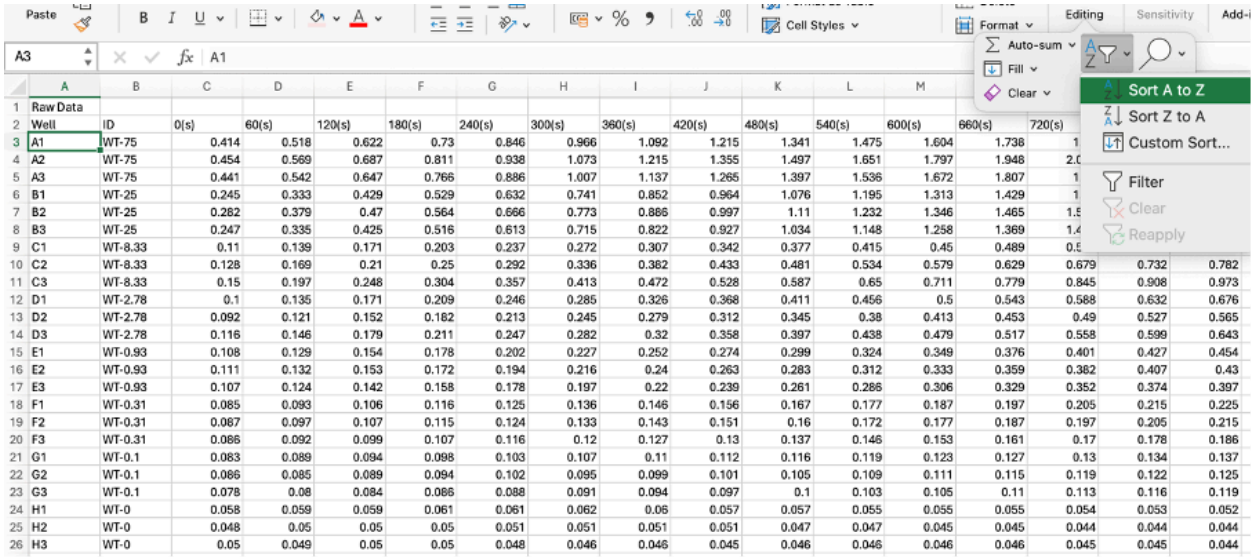
If your raw data already looks like the screenshot in step 3, simply rename your current sheet as above, ensure you have added in the ID column and labels, and go to step 4.

3. In [WT+your mutant]_raw, extract and arrange your raw data according to your plate layout, as shown below, with all timepoints and substrate concentrations in one sheet

Ensure that you have included plate co-ordinates for your mutants!

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	Raw Data																
2	Well	ID	0(s)	60(s)	120(s)	180(s)	240(s)	300(s)	360(s)	420(s)	480(s)	540(s)	600(s)	660(s)	720(s)	780(s)	840(s)
3	A1	WT-75	0.414	0.518	0.622	0.73	0.846	0.966	1.092	1.215	1.341	1.475	1.604	1.738	1.87	2.007	2.143
4	B1	WT-25	0.245	0.333	0.429	0.529	0.632	0.741	0.852	0.964	1.076	1.195	1.313	1.429	1.55	1.671	1.795
5	C1	WT-8.33	0.11	0.139	0.171	0.203	0.237	0.272	0.307	0.342	0.377	0.415	0.45	0.489	0.525	0.56	0.597
6	D1	WT-2.78	0.1	0.135	0.171	0.209	0.246	0.285	0.326	0.368	0.411	0.456	0.5	0.543	0.588	0.632	0.676
7	E1	WT-0.93	0.108	0.129	0.154	0.178	0.202	0.227	0.252	0.274	0.299	0.324	0.349	0.376	0.401	0.427	0.454
8	F1	WT-0.31	0.085	0.093	0.106	0.116	0.125	0.136	0.146	0.156	0.167	0.177	0.187	0.197	0.205	0.215	0.225
9	G1	WT-0.1	0.083	0.089	0.094	0.098	0.103	0.107	0.11	0.112	0.116	0.119	0.123	0.127	0.13	0.134	0.137
10	H1	WT-0	0.058	0.059	0.059	0.061	0.061	0.062	0.06	0.057	0.057	0.055	0.055	0.055	0.054	0.053	0.052
11	A2	WT-75	0.454	0.569	0.687	0.811	0.938	1.073	1.215	1.355	1.497	1.651	1.797	1.948	2.097	2.257	2.409
12	B2	WT-25	0.282	0.379	0.47	0.564	0.666	0.773	0.886	0.997	1.11	1.232	1.346	1.465	1.587	1.712	1.835
13	C2	WT-8.33	0.128	0.169	0.21	0.25	0.292	0.336	0.382	0.433	0.481	0.534	0.579	0.629	0.679	0.732	0.782
14	D2	WT-2.78	0.092	0.121	0.152	0.182	0.213	0.245	0.279	0.312	0.345	0.38	0.413	0.453	0.49	0.527	0.565
15	E2	WT-0.93	0.111	0.132	0.153	0.172	0.194	0.216	0.24	0.263	0.283	0.312	0.333	0.359	0.382	0.407	0.43
16	F2	WT-0.31	0.087	0.097	0.107	0.115	0.124	0.133	0.143	0.151	0.16	0.172	0.177	0.187	0.197	0.205	0.215
17	G2	WT-0.1	0.086	0.085	0.089	0.094	0.102	0.095	0.099	0.101	0.105	0.109	0.111	0.115	0.119	0.122	0.125
18	H2	WT-0	0.048	0.05	0.05	0.05	0.051	0.051	0.051	0.051	0.047	0.047	0.045	0.045	0.044	0.044	0.044
19	A3	WT-75	0.441	0.542	0.647	0.766	0.886	1.007	1.137	1.265	1.397	1.536	1.672	1.807	1.95	2.09	2.233
20	B3	WT-25	0.247	0.335	0.425	0.516	0.613	0.715	0.822	0.927	1.034	1.148	1.258	1.369	1.486	1.6	1.718
21	C3	WT-8.33	0.15	0.197	0.248	0.304	0.357	0.413	0.472	0.528	0.587	0.65	0.711	0.779	0.845	0.908	0.973
22	D3	WT-2.78	0.116	0.146	0.179	0.211	0.247	0.282	0.32	0.358	0.397	0.438	0.479	0.517	0.558	0.599	0.643
23	E3	WT-0.93	0.107	0.124	0.142	0.158	0.178	0.197	0.22	0.239	0.261	0.286	0.306	0.329	0.352	0.374	0.397
24	F3	WT-0.31	0.086	0.092	0.099	0.107	0.116	0.12	0.127	0.13	0.137	0.146	0.153	0.161	0.17	0.178	0.186
25	G3	WT-0.1	0.078	0.08	0.084	0.086	0.088	0.091	0.094	0.097	0.1	0.103	0.105	0.11	0.113	0.116	0.119
26	H3	WT-0	0.05	0.049	0.05	0.05	0.048	0.046	0.046	0.045	0.046	0.046	0.046	0.046	0.045	0.045	0.044

4. To sort your values by triplicate, use the ‘Sort’ function on the Well column (column A) to sort A-Z. Check in column B that your triplicates are now grouped.



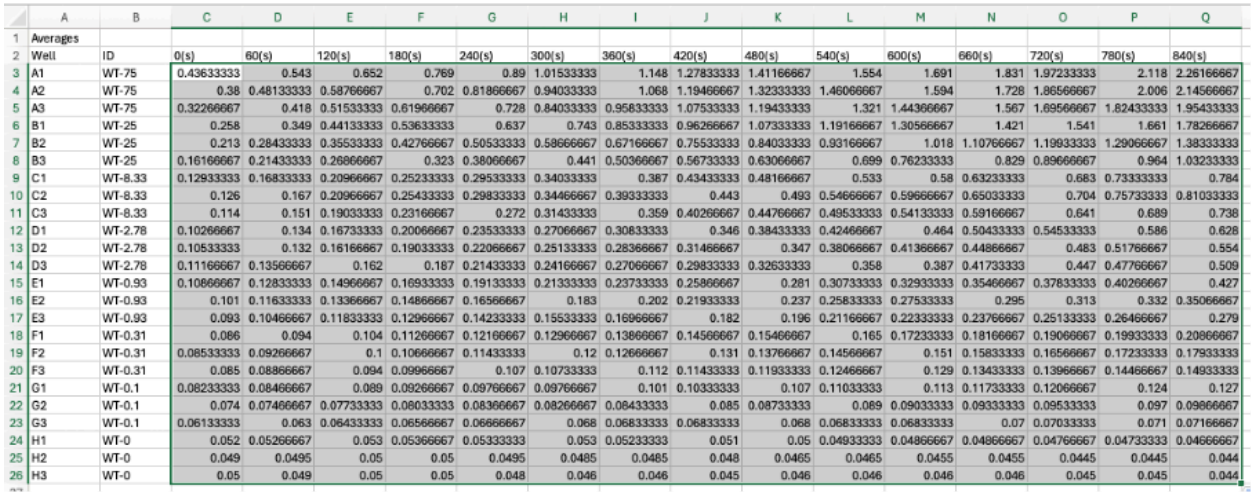
	A	B	C	D	E	F	G	H	I	J	K	L	M		
1	Raw Data														
2	Well	ID	0(s)	60(s)	120(s)	180(s)	240(s)	300(s)	360(s)	420(s)	480(s)	540(s)	600(s)	660(s)	720(s)
3	A1	WT-75	0.414	0.518	0.622	0.73	0.846	0.966	1.092	1.215	1.341	1.475	1.604	1.738	1
4	A2	WT-75	0.454	0.569	0.687	0.811	0.938	1.073	1.215	1.355	1.497	1.651	1.797	1.948	2.0
5	A3	WT-75	0.441	0.542	0.647	0.766	0.886	1.007	1.137	1.265	1.397	1.536	1.672	1.807	1
6	B1	WT-25	0.245	0.333	0.429	0.529	0.632	0.741	0.852	0.964	1.076	1.195	1.313	1.429	1
7	B2	WT-25	0.282	0.379	0.47	0.564	0.666	0.773	0.886	0.997	1.11	1.232	1.346	1.465	1.5
8	B3	WT-25	0.247	0.335	0.425	0.516	0.613	0.715	0.822	0.927	1.034	1.148	1.258	1.369	1.4
9	C1	WT-8.33	0.11	0.139	0.171	0.203	0.237	0.272	0.307	0.342	0.377	0.415	0.45	0.489	0.5
10	C2	WT-8.33	0.128	0.169	0.21	0.25	0.292	0.336	0.382	0.433	0.481	0.534	0.579	0.629	0.679	0.732	0.782
11	C3	WT-8.33	0.15	0.197	0.248	0.304	0.357	0.413	0.472	0.528	0.587	0.65	0.711	0.779	0.845	0.908	0.973
12	D1	WT-2.78	0.1	0.135	0.171	0.209	0.246	0.285	0.326	0.368	0.411	0.456	0.5	0.543	0.588	0.632	0.676
13	D2	WT-2.78	0.092	0.121	0.152	0.182	0.213	0.245	0.279	0.312	0.345	0.38	0.413	0.453	0.49	0.527	0.565
14	D3	WT-2.78	0.116	0.146	0.179	0.211	0.247	0.282	0.32	0.358	0.397	0.438	0.479	0.517	0.558	0.599	0.643
15	E1	WT-0.93	0.108	0.129	0.154	0.178	0.202	0.227	0.252	0.274	0.299	0.324	0.349	0.376	0.401	0.427	0.454
16	E2	WT-0.93	0.111	0.132	0.153	0.172	0.194	0.216	0.24	0.263	0.283	0.312	0.333	0.359	0.382	0.407	0.43
17	E3	WT-0.93	0.107	0.124	0.142	0.158	0.178	0.197	0.22	0.239	0.261	0.286	0.306	0.329	0.352	0.374	0.397
18	F1	WT-0.31	0.085	0.093	0.106	0.116	0.125	0.136	0.146	0.156	0.167	0.177	0.187	0.197	0.205	0.215	0.225
19	F2	WT-0.31	0.087	0.097	0.107	0.115	0.124	0.133	0.143	0.151	0.16	0.172	0.177	0.187	0.197	0.205	0.215
20	F3	WT-0.31	0.086	0.092	0.099	0.107	0.116	0.12	0.127	0.13	0.137	0.146	0.153	0.161	0.17	0.178	0.186
21	G1	WT-0.1	0.083	0.089	0.094	0.098	0.103	0.107	0.11	0.112	0.116	0.119	0.123	0.127	0.13	0.134	0.137
22	G2	WT-0.1	0.086	0.085	0.089	0.094	0.102	0.095	0.099	0.101	0.105	0.109	0.111	0.115	0.119	0.122	0.125
23	G3	WT-0.1	0.078	0.08	0.084	0.086	0.088	0.091	0.094	0.097	0.1	0.103	0.105	0.11	0.113	0.116	0.119
24	H1	WT-0	0.058	0.059	0.059	0.061	0.061	0.062	0.06	0.057	0.057	0.055	0.055	0.055	0.054	0.053	0.052
25	H2	WT-0	0.048	0.05	0.05	0.05	0.051	0.051	0.051	0.051	0.047	0.047	0.045	0.045	0.044	0.044	0.044
26	H3	WT-0	0.05	0.049	0.05	0.05	0.048	0.046	0.046	0.045	0.046	0.046	0.046	0.046	0.045	0.045	0.044

5. Check that your triplicates group together nicely. If you have one reading which looks like a big outlier (e.g. triplicates of 0.12, 0.11 and 0.04, 0.04 is likely an outlier). *You may consider removing outliers; however, you must record which values were removed and justify why.*
6. Make a duplicate sheet, by right-clicking on the sheet tab, selecting ‘Move or Copy’, selecting your current sheet and ticking ‘Create a copy’. Rename your duplicate sheet with [WT+your mutant]_averages
7. Clear the data from your averages sheet and put the cursor into C3.
    a. Type in: =AVERAGE(then **without pressing enter**, use your mouse to navigate back to your raw sheet.
    b. Click on C3 in the raw data table. Navigate with the mouse back to the formula bar **(without clicking anywhere else)** and enter a colon (:) then click on C5 in the raw table. Hit enter.
    c. You should be sent back to your averages sheet. Check the formula is correct:

C3 =AVERAGE('WT-raw'!C3:'WT-raw'!C5)

	A	B	C	D	E	F
1	Averages					
2	Well	ID	0(s)	60(s)	120(s)	180(s)
3	A1	WT-75	0.43633333			
4	A2	WT-75				
5	A3	WT-75				
6	B1	WT-25				

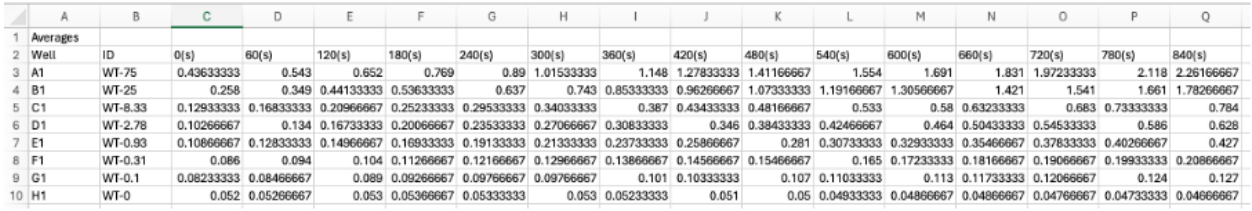
Click on the square in the bottom right of the cell and double click. This should flash-fill the whole column with this formula. Once all rows are filled, left click+hold this square, and drag right to fill the rest of the table.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	Averages																
2	Well	ID	0(s)	60(s)	120(s)	180(s)	240(s)	300(s)	360(s)	420(s)	480(s)	540(s)	600(s)	660(s)	720(s)	780(s)	840(s)
3	A1	WT-75	0.43633333	0.543	0.652	0.769	0.89	1.01533333	1.148	1.27833333	1.41166667	1.554	1.691	1.831	1.97233333	2.118	2.26166667
4	A2	WT-75	0.38	0.48133333	0.58766667	0.702	0.81866667	0.94033333	1.068	1.19466667	1.32333333	1.46066667	1.594	1.728	1.86566667	2.006	2.14566667
5	A3	WT-75	0.32266667	0.418	0.51533333	0.61966667	0.728	0.84033333	0.95833333	1.07533333	1.19433333	1.321	1.44366667	1.567	1.69566667	1.82433333	1.95433333
6	B1	WT-25	0.258	0.349	0.44133333	0.53633333	0.637	0.743	0.85333333	0.96266667	1.07333333	1.19166667	1.30566667	1.421	1.541	1.661	1.78266667
7	B2	WT-25	0.213	0.28433333	0.35533333	0.42766667	0.50533333	0.58666667	0.67166667	0.75533333	0.84033333	0.93166667	1.018	1.10766667	1.19933333	1.29066667	1.38333333
8	B3	WT-25	0.16166667	0.21433333	0.26866667	0.323	0.38066667	0.441	0.50366667	0.56733333	0.63066667	0.699	0.76233333	0.829	0.89666667	0.964	1.03233333
9	C1	WT-8.33	0.12933333	0.16833333	0.20966667	0.25233333	0.29533333	0.34033333	0.387	0.43433333	0.48166667	0.533	0.58	0.63233333	0.683	0.73333333	0.784
10	C2	WT-8.33	0.126	0.167	0.20966667	0.25433333	0.29833333	0.34466667	0.39333333	0.443	0.493	0.54666667	0.59666667	0.65033333	0.704	0.75733333	0.81033333
11	C3	WT-8.33	0.114	0.151	0.19033333	0.23166667	0.272	0.31433333	0.359	0.40266667	0.44766667	0.49533333	0.54133333	0.59166667	0.641	0.689	0.738
12	D1	WT-2.78	0.10266667	0.134	0.16733333	0.20066667	0.23533333	0.27066667	0.30833333	0.346	0.38433333	0.42466667	0.464	0.50433333	0.54533333	0.586	0.628
13	D2	WT-2.78	0.10533333	0.132	0.16166667	0.19033333	0.22066667	0.25133333	0.28366667	0.31466667	0.347	0.38066667	0.41366667	0.44866667	0.483	0.51766667	0.554
14	D3	WT-2.78	0.11166667	0.13566667	0.162	0.187	0.21433333	0.24166667	0.27066667	0.29833333	0.32633333	0.358	0.387	0.41733333	0.447	0.47766667	0.509
15	E1	WT-0.93	0.10866667	0.12833333	0.14966667	0.16933333	0.19133333	0.21333333	0.23733333	0.25866667	0.281	0.30733333	0.32933333	0.35466667	0.37833333	0.40266667	0.427
16	E2	WT-0.93	0.101	0.11633333	0.13366667	0.14866667	0.16566667	0.183	0.202	0.21933333	0.237	0.25833333	0.27533333	0.295	0.313	0.332	0.35066667
17	E3	WT-0.93	0.093	0.10466667	0.11833333	0.12966667	0.14233333	0.15533333	0.16966667	0.182	0.196	0.21166667	0.22333333	0.23766667	0.25133333	0.26466667	0.279
18	F1	WT-0.31	0.086	0.094	0.104	0.11266667	0.12166667	0.12966667	0.13866667	0.14566667	0.15466667	0.165	0.17233333	0.18166667	0.19066667	0.19933333	0.20866667
19	F2	WT-0.31	0.08533333	0.09266667	0.1	0.10666667	0.11433333	0.12	0.12666667	0.131	0.13766667	0.14566667	0.151	0.15833333	0.16566667	0.17233333	0.17933333
20	F3	WT-0.31	0.085	0.08866667	0.094	0.09966667	0.107	0.10733333	0.112	0.11433333	0.11933333	0.12466667	0.129	0.13433333	0.13966667	0.14466667	0.14933333
21	G1	WT-0.1	0.08233333	0.08466667	0.089	0.09266667	0.09766667	0.09766667	0.101	0.10333333	0.107	0.11033333	0.113	0.11733333	0.12066667	0.124	0.127
22	G2	WT-0.1	0.074	0.07466667	0.07733333	0.08033333	0.08366667	0.08266667	0.08433333	0.085	0.08733333	0.089	0.09033333	0.09333333	0.09533333	0.097	0.09866667
23	G3	WT-0.1	0.06133333	0.063	0.06433333	0.06566667	0.06666667	0.068	0.06833333	0.06833333	0.068	0.06833333	0.06833333	0.07	0.07033333	0.071	0.07166667
24	H1	WT-0	0.052	0.05266667	0.053	0.05366667	0.05333333	0.053	0.05233333	0.051	0.05	0.04933333	0.04866667	0.04866667	0.04766667	0.04733333	0.04666667
25	H2	WT-0	0.049	0.0495	0.05	0.05	0.0495	0.0485	0.0485	0.048	0.0465	0.0465	0.0455	0.0455	0.0445	0.0445	0.044
26	H3	WT-0	0.05	0.049	0.05	0.05	0.048	0.046	0.046	0.045	0.046	0.046	0.046	0.046	0.045	0.045	0.044

8. Hold down Ctrl and click on the row numbers on the right to highlight all rows except the top row for each replicate (e.g. leave A1, B1, C1 unhighlighted, but highlight A2, A3, B2, B3, C2, C3). Right click on one of the highlighted row numbers and press Delete. You should be left with just your correct averages.

Double check the formulae of a couple of different cells to be sure its correct.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	Averages																
2	Well	ID	0(s)	60(s)	120(s)	180(s)	240(s)	300(s)	360(s)	420(s)	480(s)	540(s)	600(s)	660(s)	720(s)	780(s)	840(s)
3	A1	WT-75	0.43633333	0.543	0.652	0.769	0.89	1.01533333	1.148	1.27833333	1.41166667	1.554	1.691	1.831	1.97233333	2.118	2.26166667
4	B1	WT-25	0.258	0.349	0.44133333	0.53633333	0.637	0.743	0.85333333	0.96266667	1.07333333	1.19166667	1.30566667	1.421	1.541	1.661	1.78266667
5	C1	WT-8.33	0.12933333	0.16833333	0.20966667	0.25233333	0.29533333	0.34033333	0.387	0.43433333	0.48166667	0.533	0.58	0.63233333	0.683	0.73333333	0.784
6	D1	WT-2.78	0.10266667	0.134	0.16733333	0.20066667	0.23533333	0.27066667	0.30833333	0.346	0.38433333	0.42466667	0.464	0.50433333	0.54533333	0.586	0.628
7	E1	WT-0.93	0.10866667	0.12833333	0.14966667	0.16933333	0.19133333	0.21333333	0.23733333	0.25866667	0.281	0.30733333	0.32933333	0.35466667	0.37833333	0.40266667	0.427
8	F1	WT-0.31	0.086	0.094	0.104	0.11266667	0.12166667	0.12966667	0.13866667	0.14566667	0.15466667	0.165	0.17233333	0.18166667	0.19066667	0.19933333	0.20866667
9	G1	WT-0.1	0.08233333	0.08466667	0.089	0.09266667	0.09766667	0.09766667	0.101	0.10333333	0.107	0.11033333	0.113	0.11733333	0.12066667	0.124	0.127
10	H1	WT-0	0.052	0.05266667	0.053	0.05366667	0.05333333	0.053	0.05233333	0.051	0.05	0.04933333	0.04866667	0.04866667	0.04766667	0.04733333	0.04666667

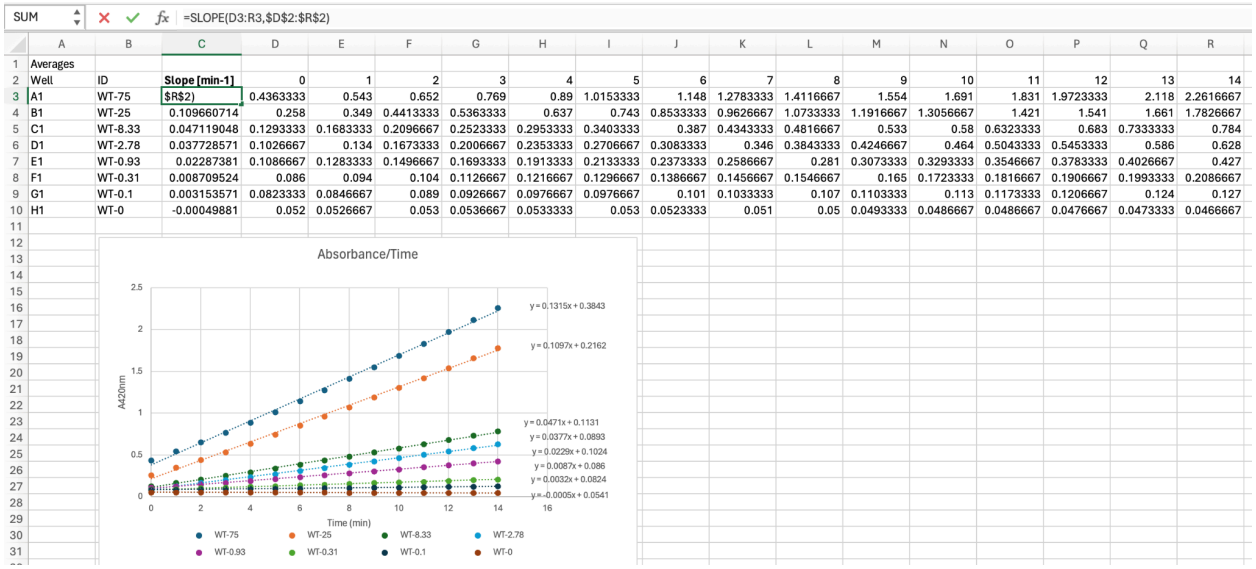
Generating initial velocities (V_0)

As our software did not generate rate values for each concentration, we will be doing this manually (also doing it manually is the best way to understand what you are doing).

1. Highlight all the data from B2:Q10 and got to Insert> Chart >XY Scatter to create a scatter graph.
2. Go to Chart Design> Add Chart Elements to add X and Y axis and a title.
3. Go to Chart Design> Add Chart Elements and add a linear trendline for your WT-75 data. Double click on the trendline, and in the Format Trendline box on the right, scroll down and select ‘Display Equation on Chart’. Repeat for all

your other concentrations. Play around with the size of the chart and drag the elements around so that you can clearly see all the equations.

4. Highlight column C, right-click and hit Insert column. Title this new column Slope [min^{-1}], extract the slopes from each equation and add them here using the SLOPE function (see the equation bar):



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	Averages																	
2	Well	ID	Slope [min-1]	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
3	A1	WT-75	R2)	0.4363333	0.543	0.652	0.769	0.89	1.0153333	1.148	1.2783333	1.4116667	1.554	1.691	1.831	1.9723333	2.118	2.2616667
4	B1	WT-25	0.109660714	0.258	0.349	0.4413333	0.5363333	0.637	0.743	0.8533333	0.9626667	1.0733333	1.1916667	1.3056667	1.421	1.541	1.661	1.7826667
5	C1	WT-8.33	0.047119048	0.1293333	0.1683333	0.2096667	0.2523333	0.2953333	0.3403333	0.387	0.4343333	0.4816667	0.533	0.58	0.6323333	0.683	0.7333333	0.784
6	D1	WT-2.78	0.037728571	0.1026667	0.134	0.1673333	0.2006667	0.2353333	0.2706667	0.3083333	0.346	0.3843333	0.4246667	0.464	0.5043333	0.5453333	0.586	0.628
7	E1	WT-0.93	0.02287381	0.1086667	0.1283333	0.1496667	0.1693333	0.1913333	0.2133333	0.2373333	0.2586667	0.281	0.3073333	0.3293333	0.3546667	0.3783333	0.4026667	0.427
8	F1	WT-0.31	0.008709524	0.086	0.094	0.104	0.1126667	0.1216667	0.1296667	0.1386667	0.1456667	0.1546667	0.165	0.1723333	0.1816667	0.1906667	0.1993333	0.2086667
9	G1	WT-0.1	0.003153571	0.0823333	0.0846667	0.089	0.0926667	0.0976667	0.0976667	0.101	0.1033333	0.107	0.1103333	0.113	0.1173333	0.1206667	0.124	0.127
10	H1	WT-0	-0.00049881	0.052	0.0526667	0.053	0.0536667	0.0533333	0.053	0.0523333	0.051	0.05	0.0493333	0.0486667	0.0486667	0.0476667	0.0473333	0.0466667

We now need to normalize the absorbance/time values (which are arbitrary and depend on the plate reader) into concentration/time.

1. Create a new sheet and name it calculations
2. Copy columns B and C from the [WT+your mutant]_averages sheet and input the constants required for the next step. These are the pathlength of the plate reader, *l* = 0.572 cm, the extinction coefficient of the substrate, εPNP = 10660 $M^{-1}cm^{-1}$:
3. Calculate the initial velocity of the reaction at each substrate concentration, using each of your slopes and the following equation:

$$v = \frac{slope}{\epsilon_{PNP} \cdot l} = \frac{0.140\ \text{min}^{-1}}{(10{,}660\ \text{M}^{-1}\cancel{\text{cm}^{-1}})(0.572\ \cancel{\text{cm}})} = 2.30 \times 10^{-5}\ \text{M/min}$$

Example equation

1	Calculate Initial Velocity				
2	Substrate (mM)	Slope (min-1)	Pathlength (cm)	Ext Coff. (M-1cm-1)	Vo (M/min)
3	75	0.131503571	0.572	10660	2.157E-05
4	25	0.109660714	0.572	10660	1.798E-05
5	8.33	0.047119048	0.572	10660	7.728E-06
6	2.78	0.037728571	0.572	10660	6.188E-06
7	0.93	0.02287381	0.572	10660	3.751E-06
8	0.31	0.008709524	0.572	10660	1.428E-06
9	0.1	0.003153571	0.572	10660	5.172E-07

Calculating k_{obs} values and plotting the Michaelis-Menten curve

We can now calculate the k_{obs}, the observed rate constant. For this, we need to know the enzyme concentration in the reaction (E_0).

1. Calculate the E_0 using the following equation, and input as a new column in your sheet. *The concentration in µg/mL is the final concentration in the reaction well!*

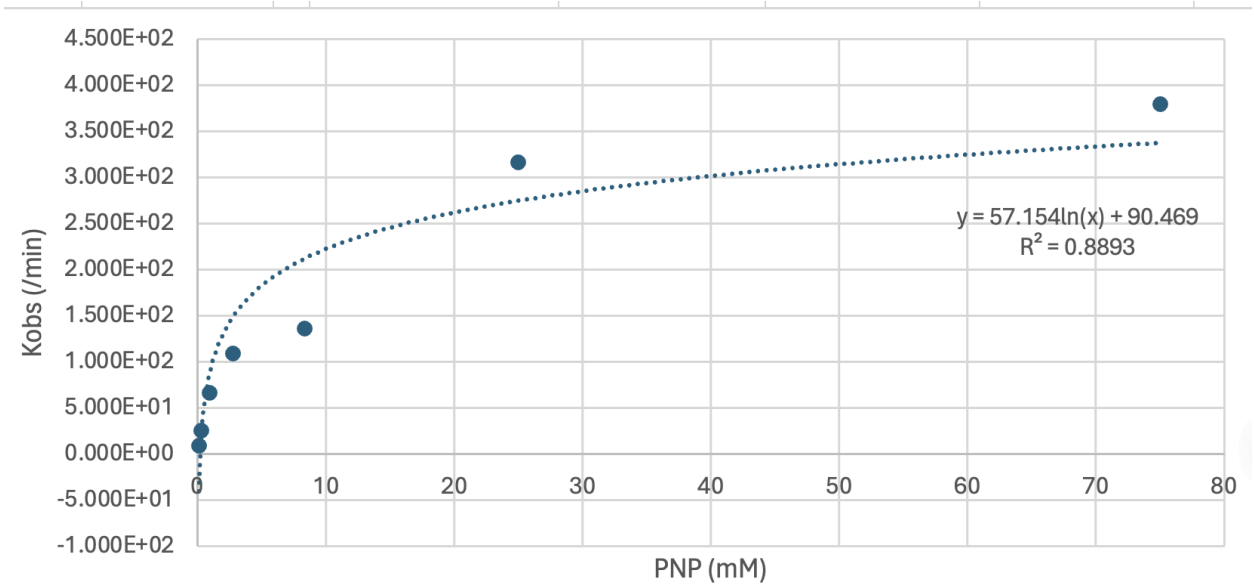
$$\text{Conc. BglB (µM)} = \frac{\text{Conc. BglB (µg/mL)}}{\text{MW BglB (kDa)}}$$

2. Use the following equation to calculate your k_{obs}, and input this as a new column. **Be sure to convert the BglB concentration into M!**

$$k_{\text{obs}} = \frac{v}{[\text{E}]_0} = \frac{\text{M/min}^{-1}}{\text{M}} = \text{min}^{-1}$$

3. Add a new column after column A, title it: Substrate (mM), and input the substrate concentrations for each ID. Plot an XY scatter graph of X= concentration, Y= K_{obs}. Add in axis labels, a logarithmic trendline and the R^2 and equation. This is your Michaelis-Menten plot.

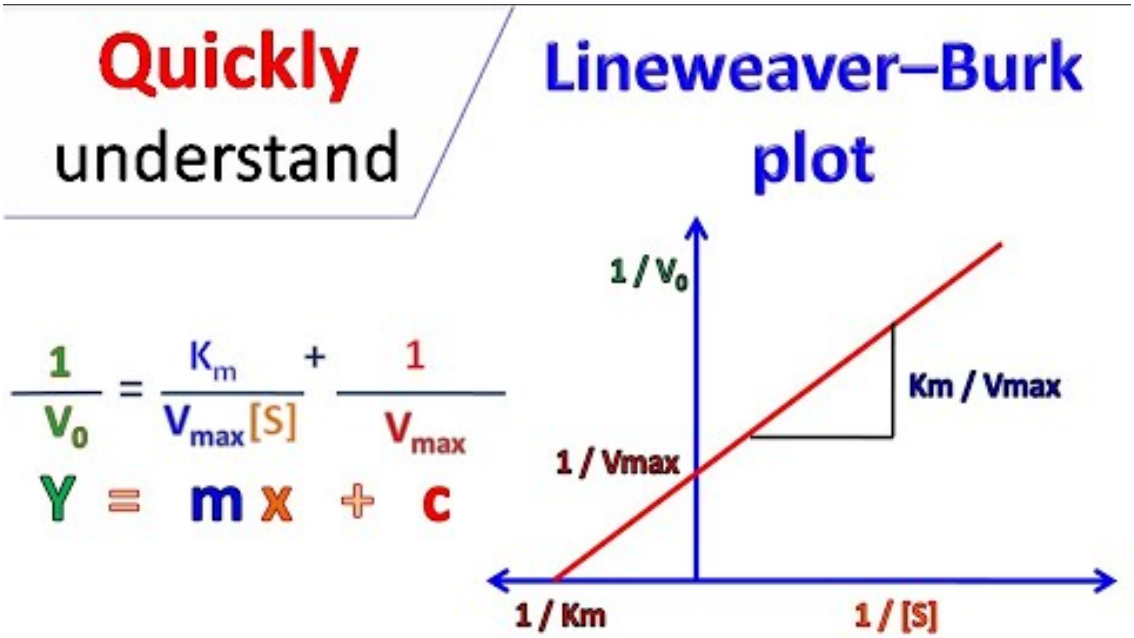
Calculate Initial Velocity						Michealis-Menten plot		
ID	Substrate (mM)	Slope (min-1)	Pathlength (cm)	Ext Coff. (M-1cm-1)	Vo (M/min)	BglB concentration (M)	Kobs (min-1)	Kobs (sec-1)
WT-75	75	0.131503571	0.572	10660	2.157E-05	1.42093E-08	1.518E+03	2.530E+01
WT-25	25	0.109660714	0.572	10660	1.798E-05		1.266E+03	2.109E+01
WT-8.33	8.33	0.047119048	0.572	10660	7.728E-06		5.438E+02	9.064E+00
WT-2.78	2.78	0.037728571	0.572	10660	6.188E-06		4.355E+02	7.258E+00
WT-0.93	0.93	0.02287381	0.572	10660	3.751E-06		2.640E+02	4.400E+00
WT-0.31	0.31	0.008709524	0.572	10660	1.428E-06		1.005E+02	1.675E+00
WT-0.1	0.1	0.003153571	0.572	10660	5.172E-07		3.640E+01	6.066E-01



Using a non-linear regression model, you can use this plot to calculate your Km/Vmax, however we will use a method to give an approximation in absence of these models, which relies only on the V_0

Lineweaver-Burk plot to estimate Km and Vmax

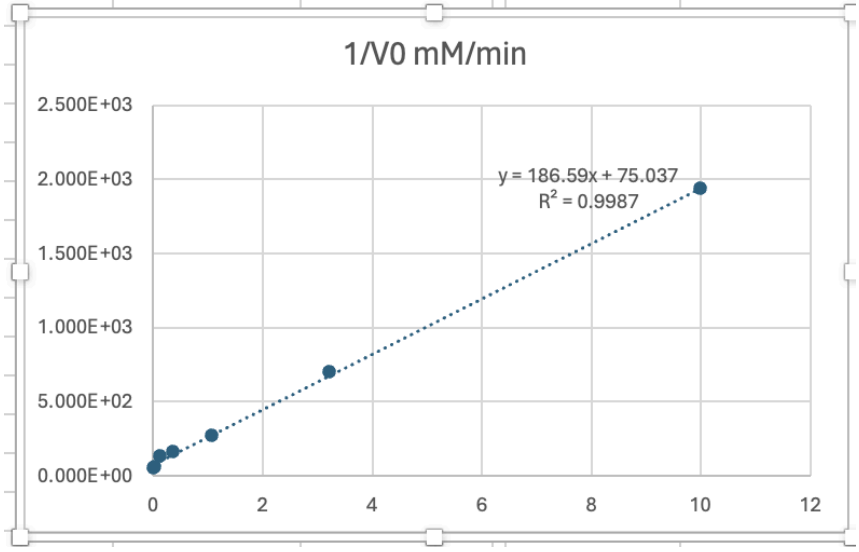
Without access to non-linear regression models, it is difficult to generate the values we need from this plot. Therefore, we will use a simplified method, using the Lineweaver—Burk model to estimate these as follows:



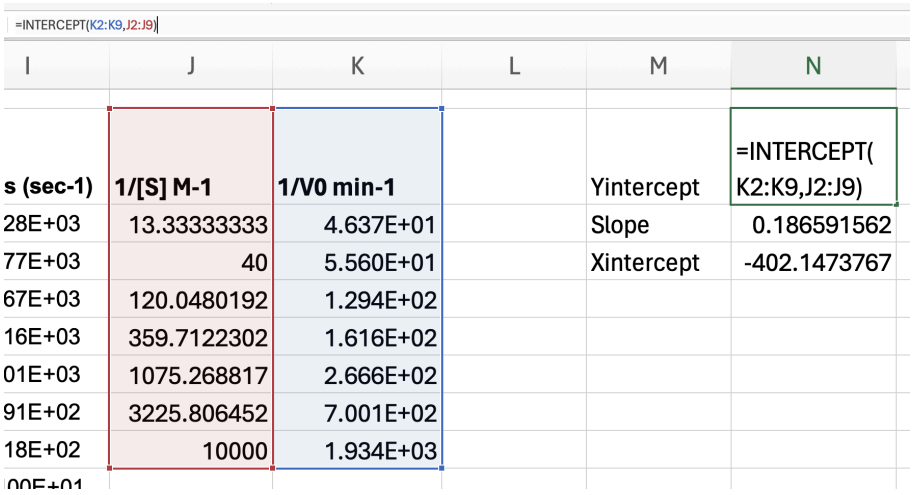
Check out this video, for more information

1. Add in new columns in J and K, called 1/S and 1/V0. Calculate the inverse of each substrate concentration (1/S) and initial velocity.
2. Plot an XY scatter graph of X= 1/S, Y= $1/V_0$. Add in axis labels, a linear trendline and the R^2 and equation. *NB. If the R^2 is <0.998, try removing data points to improve the fit, you don't plot the zero concentration so you can delete it.*

1/[S] mM-1	1/V0 mM/min
0.013333333	4.637E+01
0.04	5.560E+01
0.120048019	1.294E+02
0.35971223	1.616E+02
1.075268817	2.666E+02
3.225806452	7.001E+02
10	1.934E+03



3. You now need to gather the Y and X intercept values and Slope. Use the In INTERCEPT function to get the Y-intercept:



4. Use the SLOPE function to extract the slope

=SLOPE(K2:K9,J2:J9)

I	J	K	L	M	N
s (sec-1)	1/[S] M-1	1/V0 min-1		Yintercept	75.03730712
28E+03	13.33333333	4.637E+01		Slope	K9,J2:J9)
77E+03	40	5.560E+01		Xintercept	-402.1473767
67E+03	120.0480192	1.294E+02			
16E+03	359.7122302	1.616E+02			
01E+03	1075.268817	2.666E+02			
91E+02	3225.806452	7.001E+02			
18E+02	10000	1.934E+03			
00E+01					

5. Calculate the X-intercept using: Y-int/Slope.

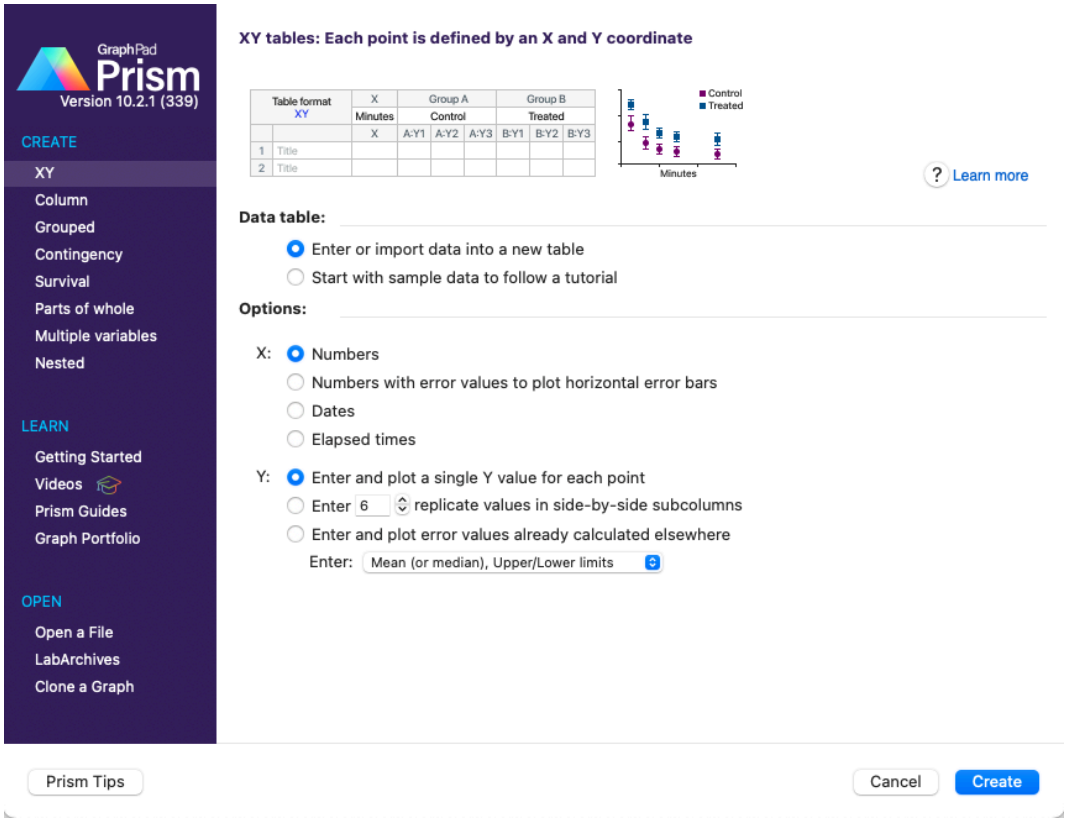
			Linewaver Burk plot
1/[S] mM-1	1/V0 mM/min	Yintercept	75.03730712
0.013333333	4.637E+01	Slope	=SLOPE(M2:M9,L2:L9)
0.04	5.560E+01	Xintercept	-0.402147377
0.120048019	1.294E+02		
0.35971223	1.616E+02	**Km (mM)**	2.486650561
1.075268817	2.666E+02	**Vmax (mM)**	0.013326704
3.225806452	7.001E+02	**Kcat (mM min-1)**	1.518E+03
10	1.934E+03	**Kcat/Km (mM-1 min-1)**	6.104E+02

The Vmax = 1/Y intercept
The Km = -1/X intercept
These values will need to be converted to mM

6. Finally, though imperfect, the K_{cat} can be approximated as the K_{obs} from the highest substrate concentration.
7. You can now calculate the efficiency of your enzyme as Kcat / Km.
8. Repeat all these steps with your mutant, to calculate your mutant Km and Vmax.

Alternative: Michaelis-Menten analysis using Graphpad Prism

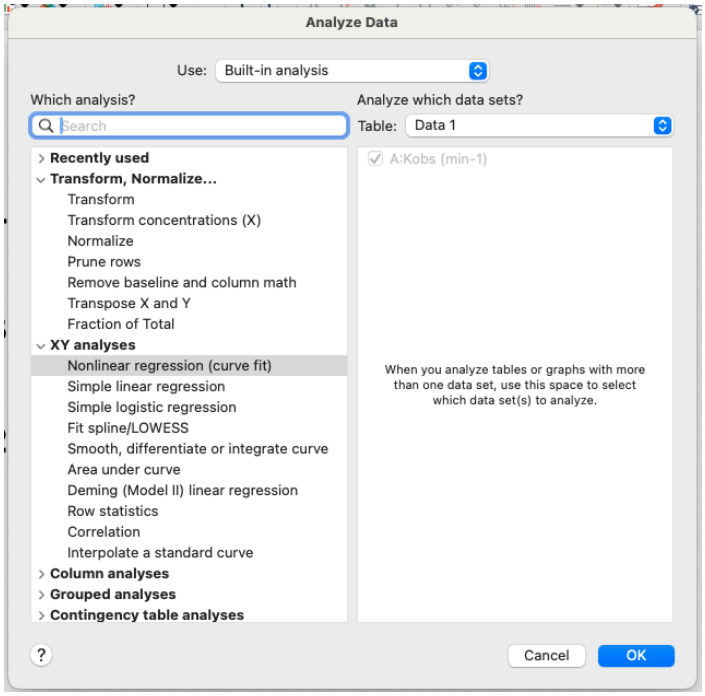
1. Load up GraphPad Prism and under Create, select XY, specifying X as “Numbers” and Y as “Enter and plot a single Y value for each point”

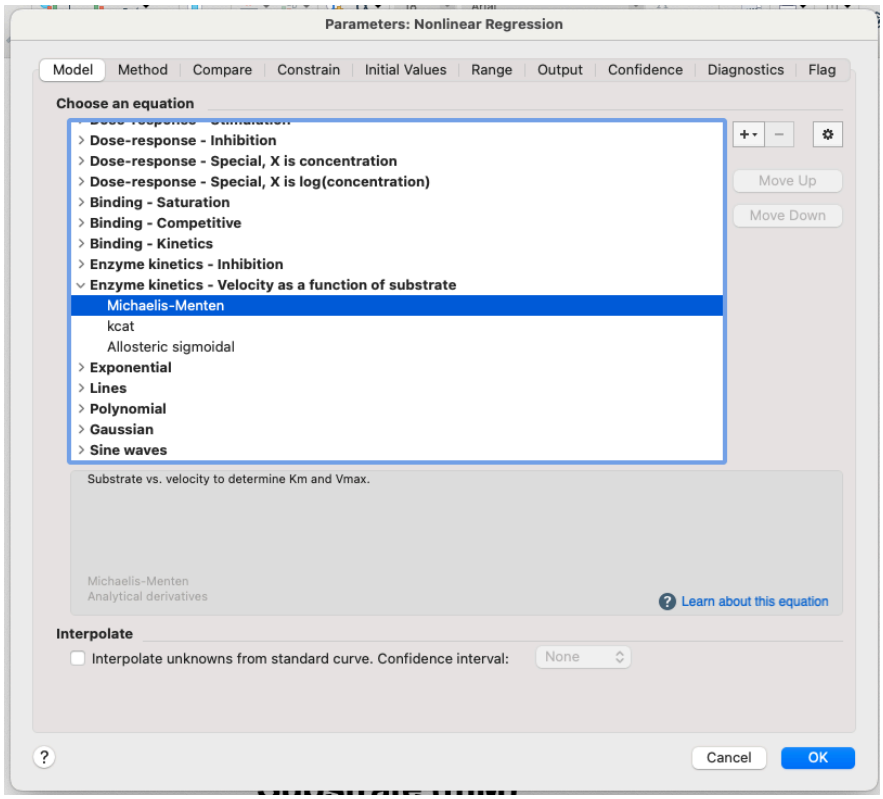


2. Populate the X column with substrate concentration (mM) and the Y column A with the Kobs (min^{-1}).

		X	Group A
		Substrate (mM)	Kobs (min-1)
		X	Y
1	Title	75.00	379.400
2	Title	25.00	316.400
3	Title	8.33	136.000
4	Title	2.78	108.900
5	Title	0.93	66.000
6	Title	0.31	25.130
7	Title	0.10	9.099
8	Title		

3. Navigate to the Graphs tab on the left hand panel and select the tab for Data 1. Select an XY scatter plot. Select the Analyze button in the top left of the tool bar.
4. Select non-linear regression, then under Enzyme Kinetics- Velocity as a function of substrate, select Michaelis-Menten.

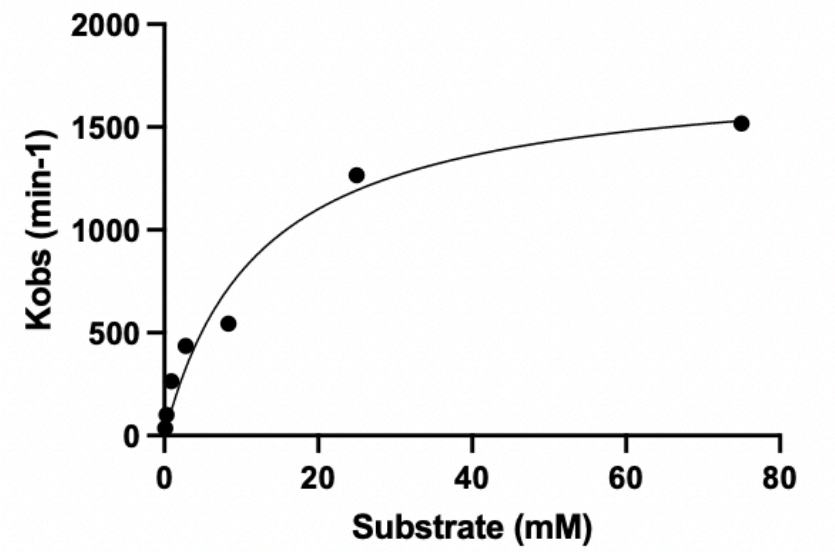




5. Navigate to the Results tab and extract your Km and Vmax values.
    a. In these analyses, because you are plotting the Kobs rather than the V0, you have already corrected for enzyme concentration, *therefore the Vmax here is*

actually your Kcat. See how it compares with the Lineweaver-Burk analysis if you completed both.

6. Navigate back to the Graph tab and using a text box, add your Km and Vmax values to the graph (e.g as a table to one side). Once you've ensured all axes are properly labeled and the graph is titled and formatted correctly, export your graph using File > Export and export as a JPEG or TIF file.



7. Repeat all these steps with your mutant, to calculate your mutant Km and Vmax and similarly export the graph.

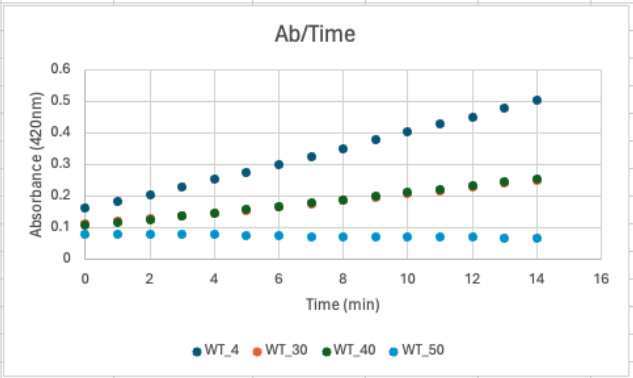
You can bring up the dialog box to edit the axis of the graph by double clicking on either axis, or edit data points by double clicking on the data or graph area. You can have a play around with point size, color etc. You can edit the text of axis titles simply by clicking on them.

Analyzing thermal stability data (simplified method)

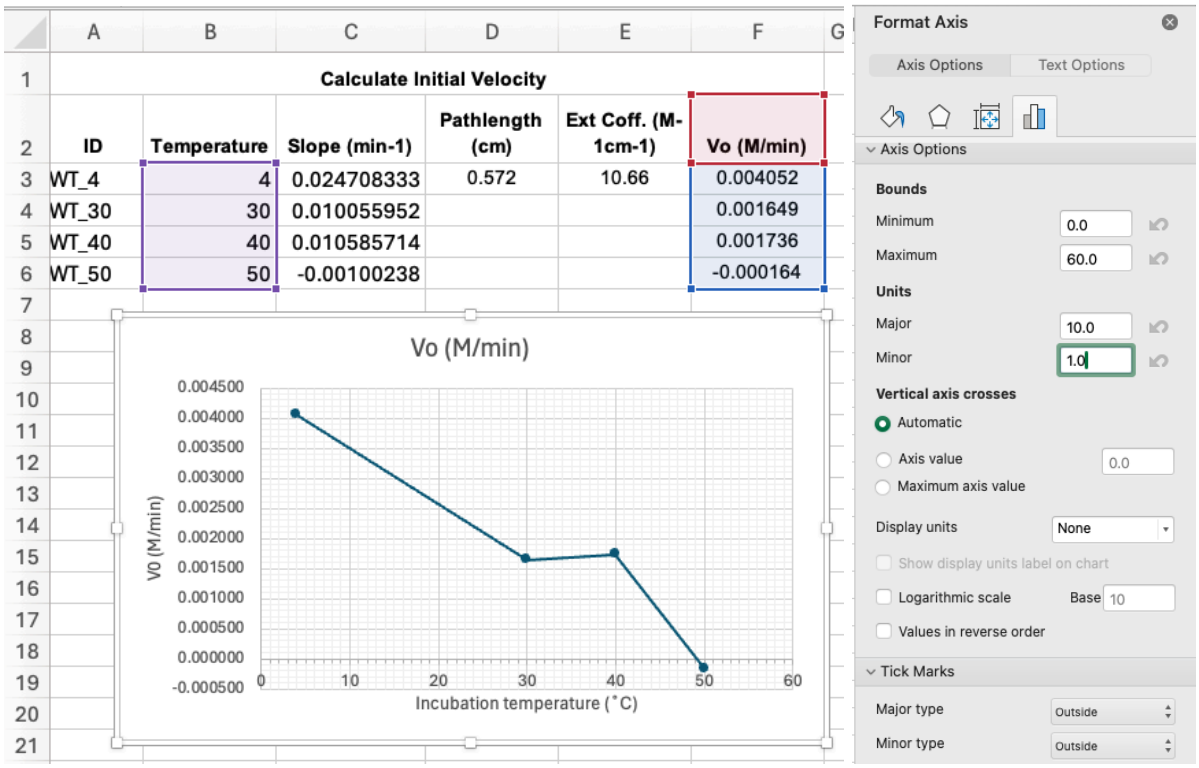
1. Repeat steps for Cleaning and Averaging data and calculating initial velocities as for the kinetic data, however rather than grouping your triplicates by substrate concentration, you will group them by incubation temperature.

You should end up with an averages table as below:

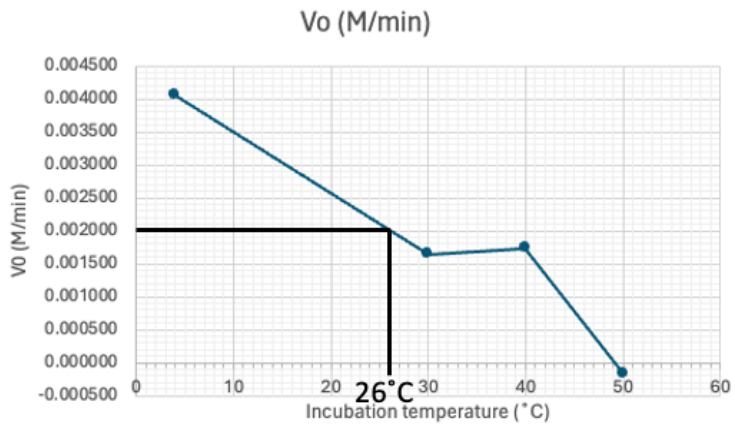
	A	B	C	D	E	F	G	H
1	Averaged data							
2	Well	ID	**Slope [min-1]**	0	1	2	3	
3	A1	WT_4	0.024708333	0.15933333	0.18133333	0.20333333	0.22666667	0
4	B1	WT_30	0.010055952	0.11033333	0.11933333	0.128	0.137	0
5	C1	WT_40	0.010585714	0.10633333	0.116	0.125	0.135	0.1453
6	D1	WT_50	-0.001002381	0.07833333	0.07766667	0.07733333	0.07666667	0.0766



2. Create an XY scatter plot (with interconnecting lines) with the temperature (˚C) on the X axis and V_0 on the Y axis. Alter the minor axis tick type to be 1.0 and and choose for both major and minor ticks to be displayed. Also go into add Chart Elements to select display of minor horizontal and vertical gridlines.



3. Draw a line from Y= (WT_4 V_0) / 2 to the connecting line, then read off the corresponding X-value to give you an estimated T_{50}, the temperature at which you enzyme loses 50% of activity
4. Repeat all these steps with your mutant, to calculate your estimated mutant T_{50}.



This is a crude measure, but should allow you to roughly compare your WT and mutant thermal stability. Bear in mind that typically to read off a T_{50} or similar values, you would fit a regression model. This plot cannot be fit with a simple regression model, because of its shape and lack of data points, meaning that the connecting line does not represent a trend line, but simply a connection of your data points. This is a limitation of this method and it's why we can only say this is an estimation, rather than a mathematically derived value.

Evaluating results: supporting or failing to support your hypothesis

Refreshing your memory

Now that you have the quantitative data that describes your mutant's functionality, in terms of catalytic efficiency and thermal stability, it's time to compare your results with your hypothesis.

At this point, we recommend reopening up Foldit and refreshing your memory of what your mutation looks like and what intermolecular interactions changed (or didn't change) in making that mutation

Recall the structure of your hypothesis, it should include a because statement and should explicitly state at least one testable parameter.

> I hypothesize that β-glucosidase mutant _____ will demonstrate increased/decreased/the same catalytic efficiency (K_{cat}/K_M), enzyme turnover rate (k_{cat}), estimated binding affinity (K_M), and or Thermal Stability (T_m) in comparison to the wild type because its Foldit score suggests a high likelihood of expression, and Foldit analysis also points to increased hydrogen bonding/molecular interaction of

interest; furthermore, previously published data on similar mutations _____, _____ supports this hypothesis

Organizing your ideas and your data

In your notes, you should create a table that looks like this:

Variant	ΔFoldit Energy Score	Description of intermolecular interaction changes	Hypothesis	Expression/ purification yield (mg/mL)	Km (mM)	Kcat (min^{-1})	Kcat/Km (min^{-1} mM^{-1})	T_{50}	Results support your hypothesis? (Yes /No)

With your hypothesis, and the model of your mutation in front of you, assess: do my results support my hypothesis or do they indicate that my hypothesis should be rejected?

Writing up your findings: the lab report

For MB&B 251L/MCDB 301L, you will need to write these into a final lab report, guidance for which can be found here.

Notes for instructors

Grading rubrics

1. 5 minute presentation
2. Lab report

Foldit installation guide

Installation for Windows users

▫ Navigate to the Foldit Standalone Download Page
▫ Login: `Wiki`, Password: `welcome1` (case sensitive!)
▫ Select the appropriate page for your computer (e.g. Win x64)
▫ Select the most recent version
Unless prompted otherwise by your instructor, ***use the most recent version.*** *The versions are dated: YYYYMMDD (i.e. 20200723, meaning year 2020, month = July, day = 23) The 20230408 version has the very important "shift+Q" feature and works!*
▫ Follow the prompts to download and install the program on your computer using the windows install wizard.

- You may have to be "pushy" and insist that windows install the software. It will probably complain that it's not from a trusted source.

Installation for Mac Users

▫ Navigate to the Foldit Standalone Download Page
▫ Login: `Wiki`, Password: `welcome1` (case sensitive!)
▫ Select the appropriate page for your computer (e.g. macos_x64)

Follow the instructions with care, the order of operations is essential!

1. Download the newest version of Foldit Standalone and move it into your applications folder but DO NOT try to open it yet.
   Unless prompted otherwise by your instructor, use the most recent version. The versions are dated: YYYYMMDD (i.e. 20200723, meaning year 2020, month = July, day = 23) The 20230408 version has the very important "shift+Q" feature and works!
2. Download and "run"/open the setup zip drive, you will be prompted to open it after a message from your mac asking "if you are sure because this is a file from the internet". Go ahead and open. It will say "complete" when done.
3. Finally, open Foldit Standalone from your applications!

Some Other things you might have mac issues with: Sometimes Mac computers reject the Foldit file and generate a security error message. If you see this, click on the question mark located on the lower left-hand corner of this message and a page will pop up that explains how to change your privacy settings (Figure 15). From here, to open Foldit Standalone, navigate to your "Applications", right click (or control click) on Foldit Standalone to get it to "open" that way. Another security message may pop up, when prompted: click "Open Anyway" and it should work.

Deep Prep Recipes

This is deep prep: making the stock solutions that you will use throughout the procedures in this repository. For ideas of where to source parts, check out the parts list.

LB-Kan Plates

Consumable Spreadsheet

Materials	Amount per Reaction
LB Broth Powder (Millers*)	25 g
Agar Powder	15 g
Ultrapure Water	1000 mL
Kanamycin	1 mL of 1000X kan stock solution**
Autoclave Bottle	1 L

*Build Transformation LB (miller's formula) ThermoFisher (#12795027), alternatively we use LB with Agar Pellets in Siegel Lab. Some LB mixes will contain agar too, use manufacturer recipe.
** See deep prep Kan Stock solution

Instructions and Methods

1. Obtain a 1 Liter autoclavable bottle
2. Add a little (~100 mL) DI or milliQ water to the bottom of the bottle
3. Add 25 g of LB broth powder (ensure it is the "Miller's" formula)
4. Add 15 g (1.5%) of bacteriological agar

5. Swirl to mix roughly.
6. Fill with DI or milliQ water to 1 Liter line on the bottle
7. Cap bottle and swirl to mix
   Note: Powder will not dissolve completely.
8. Loosen the cap so the bottle doesn't explode in the autoclave
9. Add a fresh piece of aluminum foil to cover the top. Then, add a fresh piece of autoclave tape.
10. Add water to the autoclave basin and autoclave at 121° C for at least 20 minutes on LIQUID cycle.
11. After the cycle is complete, remove to the benchtop to cool to approximately 50°C.
12. Add 50 µg/mL of Kanamycin to the flask and swirl the flask vigorously for several seconds to mix.
13. Remove the petri dishes from the plastic sleeves carefully! The plastic sleeves will be used in a future step.
14. Pour out the media into the petri dishes.
    a. Remove the lid to a petri dish and remove the foil cover to the flask.
    b. Pour just enough LB agar into the petri dish to completely cover the bottom of the dish. (5-10mL ish, I think).
    c. Replace the lid.
15. Store the plates back in the plastic sleeves the petri dishes come in.
16. The dishes should be stored UPSIDE DOWN in the cold room.

Notes and Helpful Tips

- Work quickly when pouring out the agar into the petri dishes because agar will harden.
    - Finish pouring dishes within five minutes of adding antibiotics.
- To avoid condensation, pour the plates in small stacks of 4 to 5, then stack up to 10 plates together.
    - The only plates that will accumulate condensation on the lid are the ones on the top of the stack!

Lysis Mix (100 reactions) aka "Dry Lysis Mix"

Combine 80 mg of lysozyme, 10 mg of DNAse, and 10 mg of PMSF in a Falcon tube and shake well to combine.

1 M Isopropyl-beta-D-thiogalactopyranoside (1000X IPTG) (1 mL)

Dissolve 240 mg of IPTG in 1 mL of Wash Buffer or water. Store 25 µL aliquots at –20°C.

Wash Buffer (1 L)

Dissolve 11.9 g of HEPES, 8.7 g of sodium chloride, and 0.68 g of imidazole in water and adjust the pH to 7.50.

Elution Buffer (1 L)

Dissolve 11.9 g of HEPES, 8.7 g of sodium chloride, and 7.31 g of EDTA in water and adjust the pH to 7.50.

0.1 M 4-nitrophenyl-β-D-glucopyranoside (substrate) (33 mL)

Dissolve 1 g of 4-nitrophenyl-β-D-glucopyranoside in 33 mL of elution buffer and store 1 mL aliquots at -20°C.

50 mg/mL Kanamycin Stock (1000X kanamycin) (10 mL)

Dissolve 500 mg of kanamycin in 10 mL of water and store 250 µL aliquots at -20°C.