ChE 5535:
BioEngineering Lab Techniques
Lab Manual for Spring 2017

Instructor:

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Made you look!

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Course Syllabus: CHE 5535 BioEngineering Lab Techniques

Instructor: Dr. Jacob Elmer (Jacob.Elmer@Villanova.edu)

Office Hours: M 2:30-4:30 pm, W 11:30-1:30 in 119 White Hall, otherwise available by appointment

Book: N/A – Notes and a lab manual will be provided

Meeting Times and Locations: Monday/Wednesday Lectures in White 120, Wednesday Labs in White or CEER
Monday lectures will cover virtual experiments and concepts that we won't get to do in lab.
Wednesday lectures will cover the experiments that you will conduct during Wednesday labs.

Grading: Letter grades will be assigned based on the following scale. This scale may or may not be curved.

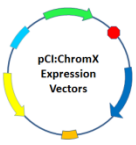
A	A-	B+	B	B-	C+	C	C-	D+	D	D-	F
100%	93	90	87	83	80	77	73	70	67	63	60	0%

Participation	10%
Course Project	15%
Lab Reports (3)	30%
Exams (3)	45%

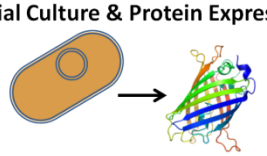
Note: Participation grades will be decided by the instructor and lab partner feedback forms.

Relevance to Curriculum: This is the only course in the ChE curriculum which gives students the opportunity to practice real-world techniques commonly used in the Pharmaceutical Industry and Academia. Since my goal is to prepare you for the workplace, please let me know if there are any additional topics you would like to cover.

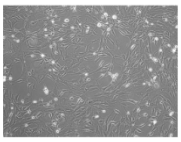
Module 1:
Plasmid Construction



Module 2:
Bacterial Culture & Protein Expression



Module 3:
Animal Cell Culture



Course Overview: Students will learn state-of-the-art techniques in each of three modules: Molecular Genetics, Protein Expression/Purification, and Animal Cell Culture. Students will prepare lab reports for each of these modules to hone their communication skills, while three exams will be given to cover the topics in each module. Students will also complete a course project that requires them to apply concepts learned in class to producing a therapeutic protein of their choice.

Student Learning Objectives: Students will be able to:

- Construct DNA plasmids for the expression of recombinant proteins
- Optimize bacterial cell culture and efficiently purify recombinant proteins
- Culture animal cell lines and use them for assays (e.g. viability, staining, etc.)
- Troubleshoot experiments by suggesting possible solutions to common problems
- Analyze experimental data, organize them into figures, and form conclusions
- Effectively communicate their results in the form of a concise report

Policies:

- Attendance is highly encouraged on Mondays and mandatory on Wednesdays for lab sessions
  - http://www1.villanova.edu/villanova/provost/resources/student/policies/attendance.html
  - https://www1.villanova.edu/villanova/engineering/resources/policies/forms/studentAbsence.html
  - **2% will be deducted from the student's overall grade if they miss a lab without permission.**
- Lab Reports are due one week after the last experiment of each module
  - Lab Reports may be accepted ≤ 24 hours late with a 10% penalty, > 24 hours late = 50% off
  - Plaigirism will not be tolerated and will result in a 50% penalty for the entire lab group
    - Check your partner's work!! You are responsible for your partner's plaigirism.
- Exams will be given on 2/20, 3/27, and 5/10 – missing a test without prior notification will result in a 0%.
  - Make-up exams will be arranged at the instructor's discretion

Emergency Situations:

If you must miss a class, exam, or homework assignment due to serious illness, death in the family, Villanova Athletic obligations, etc., then you MUST NOTIFY the instructor in writing (e.g. email). You must notify me before the class or assignment due date. Athletic absences must be justified with a signed dismissal note.

Accommodations:

It is the policy of Villanova to make reasonable academic accommodations for qualified individuals with disabilities. If you are a person with a disability (non-physical) please register with the Learning Support Office by contacting learning.support.services@villanova.edu or 610-519-5176 as soon as possible. Registration is needed to receive accommodations.

The Office of Disability Services collaborates with students, faculty, staff, and community members to create diverse learning environments that are usable, equitable, inclusive and sustainable. The ODS provides Villanova University students with physical disabilities the necessary support to successfully complete their education and participate in activities available to all students. If you have a diagnosed disability and plan to utilize academic accommodations, please contact Gregory Hannah, advisor to students with disabilities at ods@villanova.edu.

Academic Honesty & Integrity

As Villanova students you recognize that integrity is central to the University's mission. As engineers, our code of conduct requires us to place honor and integrity at the forefront of everything we do. As engineering students, it is expected that you will adopt these values and instill them into your work habits. You are encouraged to consult the Academic Integrity Policy and Code at the beginning of each semester (link below). Finally, any student found to be cheating during an exam or copying another student's work will be given a 0% for the assignment. *(https://www1.villanova.edu/villanova/provost/resources/student/policies/integrity.html).*

The College of Engineering has adopted a series of practices that will be employed during exams:

- *Students must arrive before the start of the exam and will not be allowed to enter if >5 min late.*
- *All cell phones must be turned off and stored away until the student exits the exam room.*
- *The official Villanova class attendance policy must be followed when requesting absences for exams.*
- *Each student must write and sign the following statement:*

  "I have neither given nor received any unauthorized assistance in the completion of this exam."

Lab Safety and Conduct:

This course requires students to perform experiments with hazardous chemicals and biologicals. Therefore, all students will be required to act professionally and safely when in the lab. Students who disobey this rule will be expelled from the lab session with a corresponding penalty in their participation grade.
Students must satisfy all of the following requirements before entering the lab:

- Pass Chemical and Biohazard Safety/Training Quizzes with a score of 100%. Three attempts are allowed.
- Students must wear proper attire at all times, including:
  - Closed-toes shoes, long pants, goggles (available in the lab)
- No food or drink will be allowed in the lab. Personal property may be stored in a closet outside the lab.

Class/Lab Schedule

Dates	Monday Lectures: Virtual Experiments	Wednesday Lectures and Laboratory Experiments	
		Lectures	Experiments
1/16-1/18	NO CLASS (MLK Day)	Class Overview + Lab Safety Introduction to PCR	Primary PCR Reactions + Agarose Gels & Extraction
1/23-1/25	**Traditional Cloning Strategies**	**Circular Polymerase Extension Cloning (CPEC)**	**Agarose Gels & Extraction (ctd.) + Secondary PCR Reactions**
1/30-2/1	**Mutagenesis**	**Bacterial Transformation**	Transformation of Live Bacterial Cells
2/6-2/8	Optimizing Gene Expression/Epigenetics	DNA Purification and Sequencing	Plasmid Isolation and DNA Sequencing Setup
2/13-2/15	RNA: The other nucleic acid - RNAi, etc.	Bioinformatics	DNA Sequencing Analysis and Bioinformatics
2/20-2/22	**EXAM 1: Molecular Genetics**	Bacterial Fermentation Protein Expression	Bacterial Fermentation Protein Overexpression
2/27-3/1	Alternative Expression Strategies	Cell Lysis Introduction to Chromatography	Cell Lysis and Clarification Protein Purification I
3/6-3/8	NO CLASS (Spring Break!)		
3/13-3/15	Protein Analysis Techniques I	Protein Analysis Techniques II	Protein Purification II PAGE gels
3/20-3/22	Video Lesson: Protein Modeling I (FoldIt)	Video Lesson: Protein Modeling II	Intro to Swiss PDB
3/27-3/29	**Exam 2: Bacterial Fermentation**	Introduction to Mammalian Cell Culture	Freezing, Thawing, and Passaging Animal Cells
4/3-4/5	CRISPR/Cas Part I	CRISPR/Cas Part II	Oligo Annealing Cloning: Inserting gRNA into KO plasmid
4/10-4/12	Gene and Cell Therapy I	Gene and Cell Therapy II	CRISPR/Cas: Stable Transfection of Animal Cells
4/17-4/19	NO CLASS Easter Break!	Microscopy Part I	Fluorescent Microscopy and Cell Staining/Viability Assays
4/24-4/26	Microscopy Part II	Animal Cell Culture Methods	Animal Cell Culture Scale Up: WAVE Bioreactor
5/1-5/3	Live Animal Models and Clinical Trials	Additional Topics, Snow Day Makeup, or Review for Final Exam	NO LAB – Monday Class Day
Finals Week	**FINAL EXAM – 5/10/17 at 2:30-5:00 pm**		

No class: 1/16, 3/6, 3/8, 4/17 **Online lessons:** 2/15, 3/20, 3/22 **Individual lab sessions:** 3/29, 4/12, 4/19

Exam Dates: Exam 1 = 2/20/17 Exam 2 = 3/27/17 Exam 3/Final = 5/10/17

Lab Reports are due one week after the last experiment in each module

Note: This schedule is subject to changes at the instructor's discretion.

Course Project Description

In this course, we will cover all of the techniques you need to express a recombinant protein and test it on animal cells *in vitro*. Unfortunately, the chromoproteins that we will be expressing are not clinically useful. The purpose of this project is to give you an opportunity to apply the skills you learn in this class to a real pharmaceutical protein of your choice, just as you may do in your job one day.

Specifically, your assignment is to pick any pharmaceutical protein and propose a strategy for producing it. You will communicate your strategy in a **3 page report** that includes the following information:

1. Select a target protein and explain its clinical application(s).
2. Prepare an optimized sequence for the gene and describe/justify the expression plasmid you will use.
3. Describe the cloning strategy that you will use.
4. Select a cell line for protein expression. Justify your choice.
5. Describe how you will purify the recombinant protein. Justify your choice.
6. Provide at least one way in which you will test the protein to make sure it is functional.

The report should include the following sections:

- **Introduction** – Describe your protein and its applications. Why should we make it?
- **Materials** – Describe the gene you design for the protein and the expression plasmid.
- **Methods** – Describe and justify your choices for cloning strategy, cell line, purification method, and characterization strategy.
- **Conclusion** – Provide a brief summary of your plan – emphasize key decisions and their benefits.
- **Appendix** – See below for required sections, which do not count towards the 3 page limit.
  - *Include a figure that shows the structure of your protein in Swiss PDB. Highlight the active site.*
  - *Include a plasmid map and sequence in the appendix (does not count towards 3 page limit)*
  - *Include a flowsheet that summarizes your cloning and expression/purification strategies.*
  - *Include any references or citations here as well.*

To assist you in developing this report, you will meet with the instructor twice during the semester on the dates shown below. The purpose of these meetings will just be to ensure that you have a feasible plan – no materials need to be turned in at these times, but you should bring some form of notes with you to the meeting.

- **Progress Meeting 1 – 2/22/17**
  - Be prepared to answer questions 1-3 above – have a protein, gene, plasmid, and partial cloning strategy in mind and ready to discuss.
- **Progress Meeting 2 – 3/29/17**
  - Be prepared to discuss questions 4-6 above – have a host cell line, purification method, and characterization strategy in mind and ready to discuss.

First Draft Due: 4/19/17…..50% of overall report grade

I will return your drafts (with revisions/comments) by 4/24/17.

Final Draft Due: 5/3/17…..50% of overall report grade

Lab Safety Training Checklist

CHE 5535, White 111

01/11/17

CHE 5535 students should be trained in the following methods and concepts to ensure lab safety:

General

- ☐ Name of lab safety officer (instructor)
- ☐ Location of MSDS sheets and Chemical Inventory
- ☐ Dress code
- ☐ Contaminated clothing disposal policy

Laboratory Safety

- ☐ Location of spill kits
- ☐ Location of first aid kits
- ☐ Location/Operation of eyewash and safety showers
- ☐ Location of fire extinguisher
- ☐ Emergency exit and evacuation routes
- ☐ Non-exit doors
- ☐ Designated carcinogen and toxic chemical areas.
- ☐ Proper handling of carcinogenic materials, i.e. ethidium bromide
- ☐ Proper protection procedure for systems employing UV light, including UV lamps
- ☐ Proper handling and storage of flammable and explosive chemicals.
- ☐ Proper storage and handling of Level 1 and 2 biohazards, including cultured cells, cultured animal tissue, and biomolecule solutions related to their culture.
- ☐ Safe procedures for utilizing the -80C freezer.
- ☐ Proper procedure for operating centrifuges.

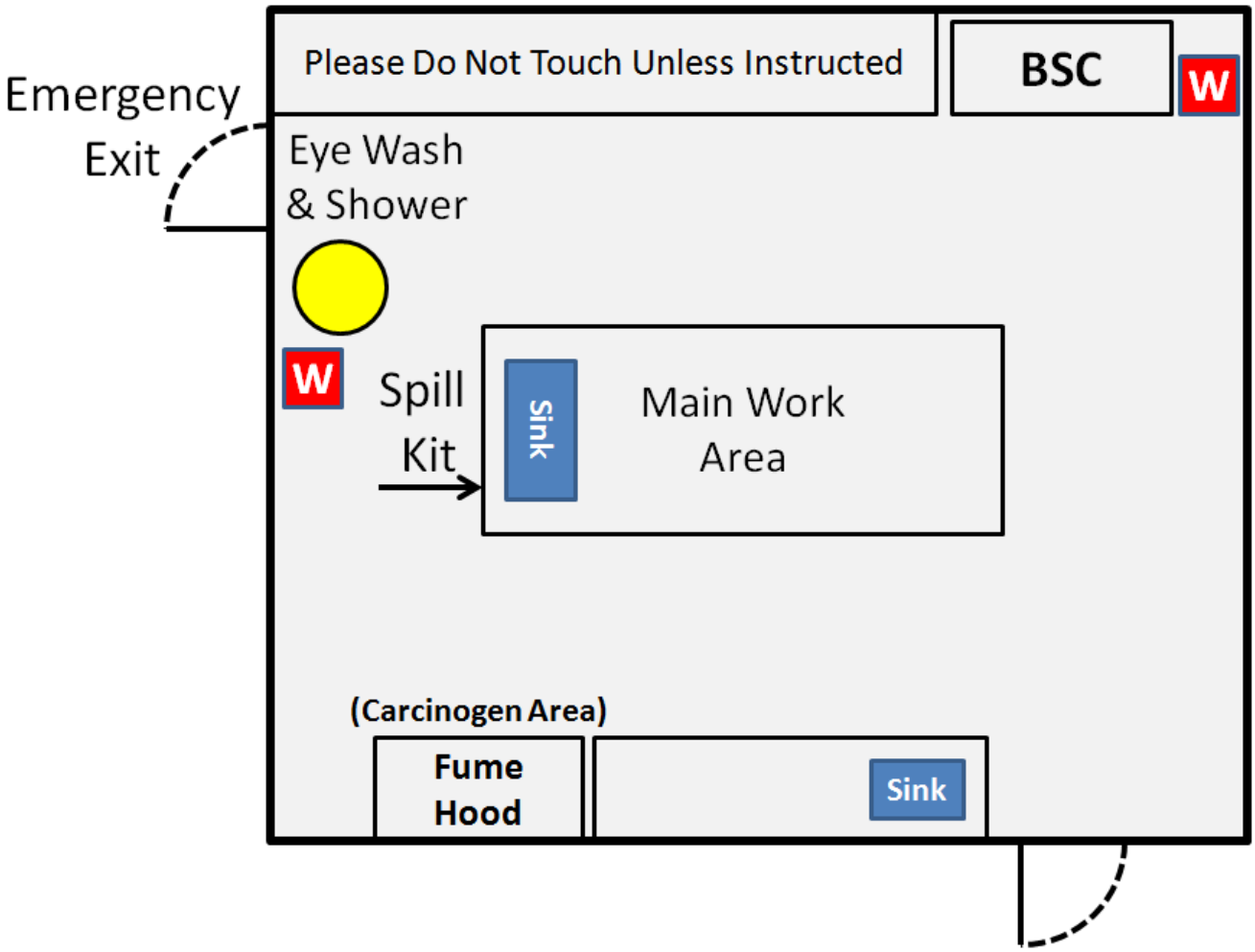
I certify that _____(print) was trained by me _____(print)

and completed the above checklist on _____/_____/_____ (date).

_____(sign) trainer

_____(sign) trainee

Lab Layout: White Hall Room 111



Important Notes:

- This lab is designated as Biosafety Level 2, which means that bloodborne pathogens (e.g. HIV, Salmonella, etc.) may exist there. We will not work with these pathogens, but they could potentially contaminate our cell cultures.
- No food or drink is allowed in the lab, especially in the lab fridge/freezers.
- In an emergency situation, evacuate the lab through the main door into the hallway. If that door is blocked, exit through the alternate door in the SW corner of the lab that leads to Dr. Coe's lab.
- Emergency eye wash and shower are located near the entrance to the lab. Rinse eyes for at least 15 minutes and disrobe if possible before using the safety shower.
- Carcinogens (ethidium bromide) will be used in the fume hood, so use extra caution in that area.
- Shutoff valves are located directly beneath each sink.
- A spill kit is under the sink in the center of the lab.
- The lab does not have a fire extinguisher.

Two biohazard waste boxes will always be maintained in the lab. Dispose of any materials that have come into contact with cells in these waste boxes, except for pipette tips, which should be discarded into sharps boxes.

Guidelines for Proper Waste Disposal

You will generate many different types of waste during this course. The definitions of each waste type and their proper disposal techniques are listed below:

- **Normal Waste**
  - Definition: Any materials which are dry and have not come into contact with chemicals or biologicals.
  - Disposal: Normal trash can near main entrance or the biohazard box, if in doubt.

- **Chemical Waste**
  - Definition: Any liquid, solvent, or dry chemical used in the lab.
  - Disposal: Liquids should be collected in a properly labeled glass container. EH&S will retrieve these containers once per semester and provide new containers upon request.
  - Hazards: Do not mix waste chemicals which will violently react with each other (acids/bases).

- **Pipette Tips**
  - Definition: 0.1-1000 μL pipette tips (not 2-50 mL pipettes)
  - Disposal: All tips should go in sharps boxes. Do not put them in biohazard boxes (bags will rip!)

- **Broken/Used Glass**
  - Definition: Glass = silicon dioxide. Enough said.
  - Disposal: Any broken glass or used Pasteur pipettes should be discarded in a labeled glass disposal box.

- **Biohazardous Waste**
  - Definition: Any solid or liquid that comes into contact with live cells (bacteria or animal cells)
  - Solids Disposal: Solid dry waste should be collected in biohazard boxes. These boxes must be transported to Mendel for autoclaving and dropped off in the basement of Mendel for EH&S.
  - Liquids Disposal: Liquid biohazard waste may be sterilized with household bleach and poured down the sink drain, as long as the waste does not contain any bleach-reactive chemicals.
  - **DO NOT AUTOCLAVE any solution containing BLEACH**!!!

- **Ethidium Bromide**
  - Definition: Any tips used to transport EtBr, gels containing EtBr, gel extraction waste
  - Disposal: EtBr-contaminated materials will be collected in a marked container under the fume hood.

- **Recombinant DNA**
  - Definition: Any plasmid containing antibiotic resistance genes.
  - Disposal: Recombinant DNA plasmids must be autoclaved and incinerated to prevent the spread of antibiotic resistance to pathogenic microbes.

Guidelines for Effective Aseptic Technique

- **Sterilization Methods**
  - Autoclave any solids or solutions that are not temperature sensitive (e.g. water, saline, tips, etc)
  - Filter (0.2 μm pore size) liquids that are temperature sensitive, requires a syringe or vacuum
  - 70% Ethanol (***never use 100%***) can sterilize dry surfaces, but don't use on cell culture flasks
  - 5-10% Bleach also sterilizes dry surfaces, but may react with some materials (ex: metal)
  - UV light sterilizes whatever surface it touches, but never achieves 100% sterility

- **Sterilization of the Biological Safety Cabinet (BSC)**
  - The UV light in the BSC should be set to turn on for 30 minutes after every use
  - Spray every surface with 70% Ethanol ***before*** and ***after*** each use
  - Only store necessary equipment (pipettes, pump, etc.) in the BSC to maintain sterility
    - Storing supplies in the BSC limits the effectiveness of the UV light/ethanol sterilization
  - Spray all hood surfaces with bleach once every few months to completely decontaminate it
  - Routinely empty the aspiration waste bottle and used tip jar to remove potential contaminants
  - TRY NOT TO BLOCK THE VENTS IN THE FRONT/BACK OF THE BSC!!!

- **Proper work habits in the Biological Safety Cabinet (BSC)**
  - Spray the hood and all materials (except cells) with 70% Ethanol before putting them in the BSC
  - Spray your gloves with 70% Ethanol and vigorously rub them until dry before working in the BSC
    - Re-spray your gloves every time you re-enter the BSC as well
  - Try to work at least 6" deep in the hood at all times to avoid contamination
  - Keep the sash as low as possible to prevent contaminants (e.g. sneezes) from entering the BSC
  - Remove contaminated materials as soon as possible and don't accumulate waste in the BSC.
  - Some people simultaneously sterilize their supplies and the BSC with UV before working
    - Don't use UV light to sterilize radiation-sensitive materials (e.g. DNA!!)

- **Once something is sterilized, keep it sterile by minimizing the time it is open to the air in the BSC**
  - Replace the caps on bottles as soon as possible
  - Eject pipette tips if they have been out for longer than 10 seconds
  - Eject pipette tips if they touch any surface, even if you are unsure that they touched something

- **Visually check to see if solutions are contaminated – shake them to see if they are turbid**
  - Unless solutions must be kept in the fridge or freezer, keep them at RT to quickly detect contamination
  - It is also good practice to re-sterilize some solutions (e.g. PBS) every 6 months

- **Working with Bacteria on the Bench**
  - NEVER work with bacteria in the BSC – only work with them in a designated hood or the bench
  - Prepare the bench area by spraying it with ethanol first, then lighting the Bunsen burner flame
    - DO NOT use ethanol after the flame is lit – the ethanol is flammable!
  - Spray all your materials and gloves with 70% Ethanol (away from the flame)
  - Keep the bacteria as close to the flame as possible and work quickly
  - Try not to talk over the samples or breath onto them to avoid contamination

Lab 0 – Using a Micropipetter

Pipetman Basics:

- Pipetmans usually come in four ranges:
  - 0.2-2.0 µL, 2.0-20 µL, 20-200 µL, 200-1000 µL
  - Others include: 10-100 µL, 1000-5000 µL, etc.

- A pipetman is designated by its maximum volume (ex: 0.2-2 µL is a P2) and it is assumed that the minimum volume is 10% of the maximum volume

- Always use the appropriate pipetman for the desired volume
  - DON'T use a P20 to pipette 1.7 µL – you will get the wrong volume

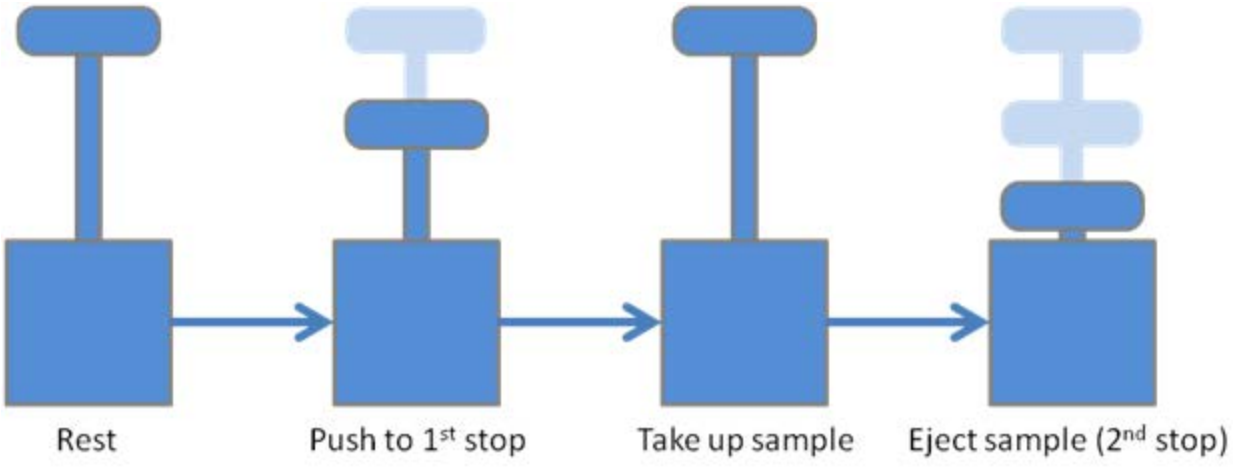
- Never let liquid touch the white piston and never set the pipette down horizontally.

- Pipetmans should be calibrated every 1-2 years
  - Always look at the volume you're pipetting to make sure it is roughly correct
  - Most pipette tips have graduations you can use as a visual check

- Make sure the tip is secure so it doesn't fall off as you're pipetting 1 mL of 1M HCl…

- On the volume indicator, µL digits are always in black
  - nL and mL are usually in red
  - Ex: on a 200-1000 µL pipetman, 1000 µL would read 1 (red) 00 (black)

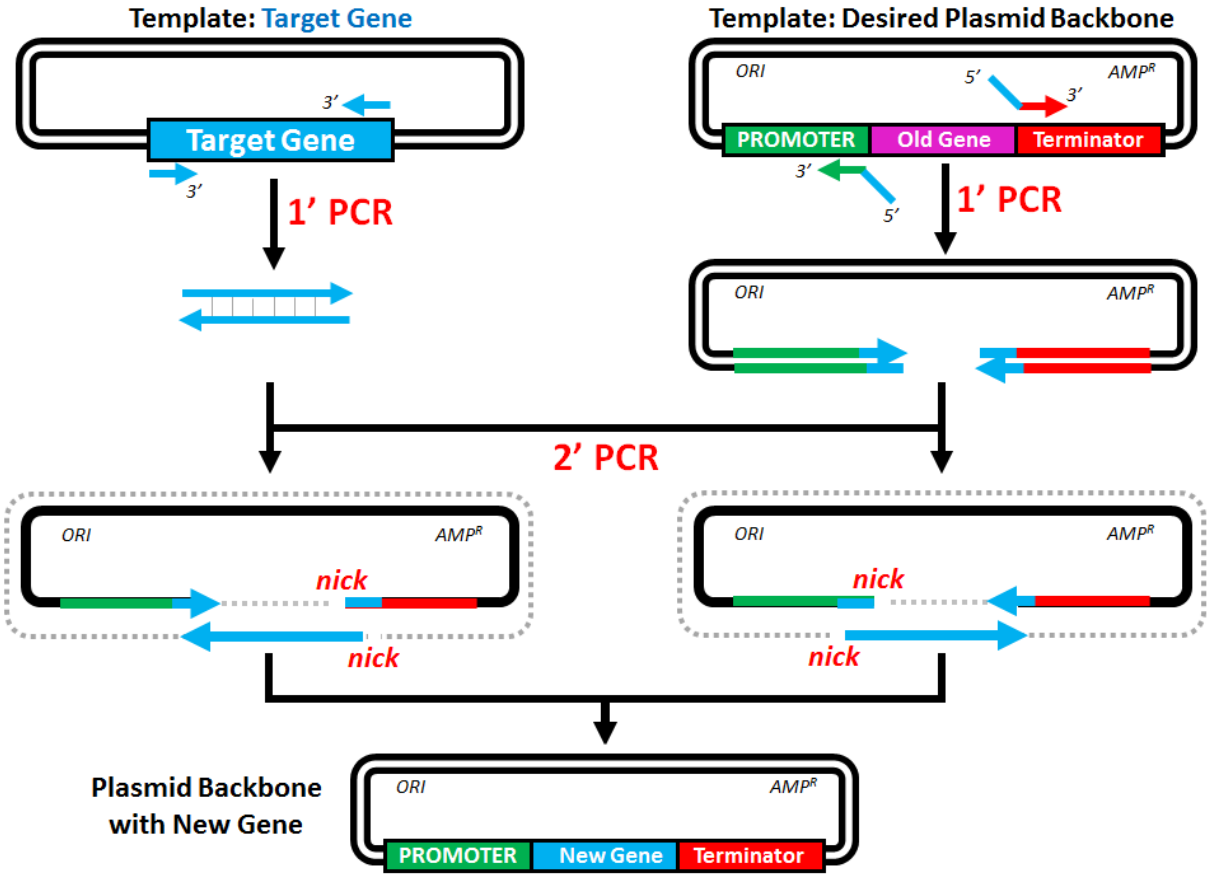
The two stop system:



1. Push to the first stop before submerging the pipette tip in the liquid to avoid creating bubbles
2. Slowly release the plunger to take up the liquid volume – do not do this too fast or bubbles will form
3. Put the tip into the destination tube/liquid and push the plunger to the 2nd stop to release the liquid
4. Remove the tip from the tube and then release the plunger. Eject the tip if necessary.

Module 1: Plasmid Construction

In this module, you will learn how to combine two (or more) pieces of DNA to create a new plasmid. While there are several different methods available to make (i.e. clone) new plasmids, the technique that we will be using is called Circular Polymerase Extension Cloning (CPEC). CPEC stands out from other cloning techniques because (1) it does not require specific sequences (e.g. restriction sites), (2) it only requires 1 enzyme (Phusion Polymerase), and (3) it is a relatively quick and mostly simple process, and (4) the new plasmid can be visualized on an agarose gel. An overview of CPEC is shown in the figure below:



Note: Each new plasmid will have two nicks in the phosphodiester backbone, but those will be repaired when they are transformed into the *E. coli*

Overview of Circular Polymerase Extension Cloning (broken down into our labs):

Lab 1: Amplify the gene of interest and the desired plasmid backbone (containing a promoter, terminator, antibiotic resistance gene, and origin of replication) in two separate primary PCR reactions (1′ PCR).

a. You will use conventional PCR primers to amplify the gene. (100% complimentarity, T_m = 65°C)
b. The instructor will use bipartite primers to amplify the target plasmid:
   i. Forward Primer = 5′ – (Complimentary to Gene) – (Complimentary to Terminator) – 3′
   ii. Reverse Primer = 5′ – (Complimentary to Gene) – (Complimentary to Promoter) – 3′

Lab 2: Combine the 1′ PCR products for a 2′ PCR reaction that is primed by their overlapping sequences.

c. In this step, the 1′ PCR products use each other as templates to complete the desired plasmid.
d. However, this process does leave nicks in the phosphodiester backbone.

Lab 3: Transform the nicked plasmids into DH5α *E. coli* to repair the nicks and replicate plasmid for sequencing.

Lab 4: Isolate the repaired/replicated plasmid from *E. coli*, then ship it off for DNA sequencing.

Lab 1a – PCR Primer Design

Purpose: *Design single stranded DNA oligomers that will prime a PCR reaction.*

Instructions:
Use the worksheet below to design primers that amplify your chromoprotein gene. The sequences of each chromoprotein gene are provided in the Appendix.

Forward Primer		**Reverse Primer**	
Copy 30 bases from the beginning (5' end) of the target sequence.		**Copy 30 bases from the end (3' end) of the target sequence.**	
		Take the reverse complement at: www.reverse-complement.com	
Truncate each primer until they both have T_m ~ 65C using NEB's calculator: http://tmcalculator.neb.com/#!/ *Note: Leave a G or C on the 3' ends to make the duplex more stable. However, do not leave more than 2-3 consecutive G's or C's at the 3' end of a primer, since they can form problematic secondary structures. Note: Primer Tm's should never differ more than 5°C (to prevent non-specific binding).*			
	T_m =		**T_m =**
Check your primers for secondary structure formation at https://www.idtdna.com/calc/analyzer *Note: You will always get some results for each analysis, but don't worry about results with ΔG ≤ -10 kcal/mol Note: Be especially wary of secondary structures that form with ≥5 bases at the 3' end of a primer.*			
Hairpins?		**Hairpins?**	
Self-Dimers?		**Self-Dimers?**	
Hetero-Dimers?		**Hetero-Dimers?**	
Check your primers for off-target binding: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch *Click "Compare two or more sequences", then paste your primer sequence in the first field and paste the template plasmid sequence into the second field. Click BLAST to search for primer binding sites.*			
Confirm target sequence binding:		**Confirm target sequence binding:**	
Binding to off-target sites?		**Binding to off-target sites?**	

Troubleshooting:

If you detect any secondary structures or off-target binding, you may consider making your amplicon bigger. Moving the forward primer upstream (or reverse primer downstream) will allow you to utilize new sequences that may not have these issues. However, in our specific case, the amplicon must start with the beginning of the gene. Therefore, your only option would be to move the reverse primer downstream.

Primers should ideally have ~40-60% G/C content. If the G/C content is too high, the primer sequence will likely be too short and prone to off-target binding.

Lab 1b – The Polymerase Chain Reaction (PCR)

Purpose: *Make billions of copies of a target sequence. The ends of the sequence can also be modified.*
Instructions:

1. **Thaw frozen primers or resuspend new primers. Also thaw template DNA, reaction buffer, and dNTPs.**
    a. Resuspend new primers by adding 1 mL of ultrapure water to the primer, then vortex for 2 min.

2. **Prepare the PCR reaction mixture according to the table below. Note that we will be running 5 identical reactions at different annealing temperatures, so we will prepare a "master mix" and split it into 5 tubes.**
    a. The reagents must be mixed in the order shown below – always add enzyme last.
    b. Keep the enzyme on ice and return it to the freezer as soon as you are finished with it.

Reagents	Stock Concentration	Required Amount or Concentration	Individual (1x) Reaction Volume	Master Mix Volume (5.5x)
Forward Primer	**25 μM**	**0.5 μM**	*μL*	*μL*
Reverse Primer	**25 μM**	**0.5 μM**	*μL*	*μL*
Template DNA	**50 ng/μL**	**25 ng**	*μL*	*μL*
HF Buffer	**5x**	**1x**	*μL*	*μL*
dNTP's	**10 mM**	**200 μM**	*μL*	*μL*
Water	*Add enough to reach 25 μL total volume*		*μL*	*μL*
Polymerase	**2,000 U/mL**	**0.5 U**	*μL*	*μL*
		Total:	*25 μL*	*137.5 μL*

Note: *The stock concentrations listed above correspond to the kit that we will be using (NEB Phusion Polymerase PCR Kit). These values may differ in other kits.*
Note: *In some cases, $MgCl_2$ may also be added to help denature large templates or templates with high GC content. It is also used to reduce secondary structure formation in sub-optimal primers.*

3. **Mix the reaction by pipetting (50 μL) up and down 5-10 times, then put 25 μL aliquots into 5 PCR tubes.**
    a. Be careful to avoid making bubbles in the tubes.

4. **Run the reactions through the thermal cycler with the routine shown below. Adjust volume to 25 μL.**

Steps		Temperature	Time	
Initial Denaturation		98°C	30 sec	
30 Cycles	**Cycle Denature**	98°C	10 sec	
	Cycle Anneal	50-70°C	20 sec	
	Cycle Extend	72°C		30 sec/kb
Final Extension		72°C	5 min	
Final Hold		4°C	*Indefinite*	

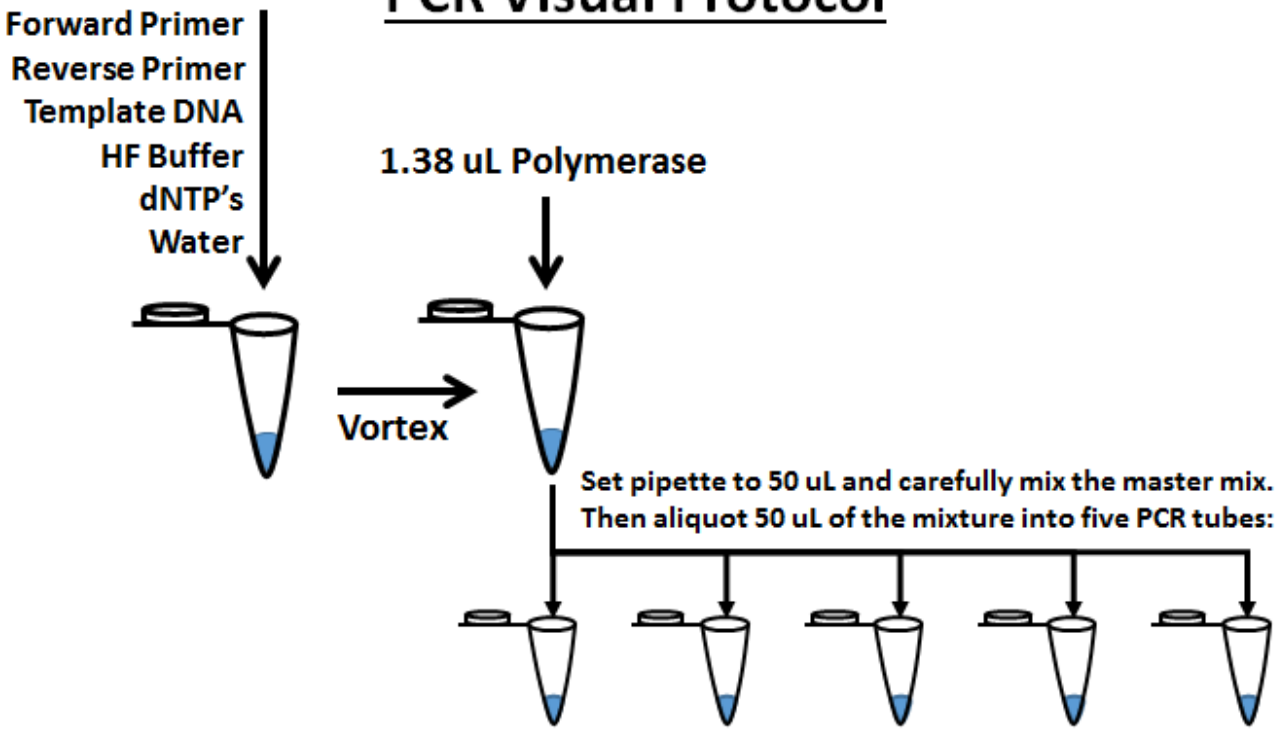
Note: *The temperatures and times shown above are optimized for our specific primers and the kit that we are using. If you use different primers or kits, these values may need to be adjusted.*

Record the annealing temperatures you used for each tube (A, B, C, D, and E) in the table below:

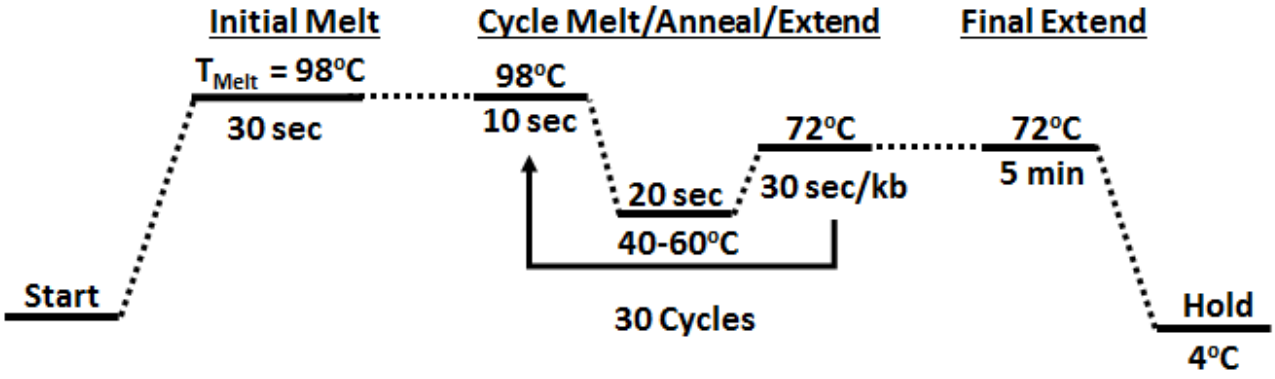
A	B	C	D	E

5. **After the routine has finished, immediately proceed to the gel electrophoresis step or freeze the samples.**

PCR Visual Protocol



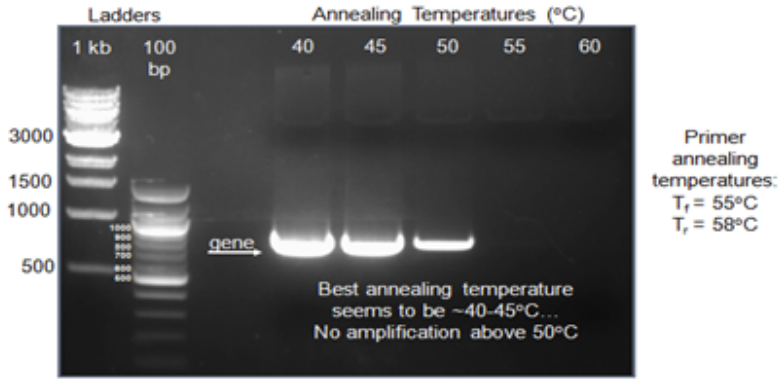
PCR Thermal Cycler Routine:



Record the annealing temperatures you used for each tube (A, B, C, D, and E) in the table below:

A	B	C	D	E

Expected Results:



Lab 1c – Agarose Gel Electrophoresis

Purpose: *Prepare a 1% agarose gel to separate and visualize DNA fragments based on their size/length. This gel will be used to purify your amplified PCR products (Lab 1b).*

Instructions:

Pre-made gels have already been prepared for you, but Dr. Elmer will demonstrate Steps 1-7 below.

CAUTION: Agarose gels contain ethidium bromide, a DNA intercalating agent that is a known carcinogen. Do not allow the gel or the dye to touch your skin and immediately dispose of your gloves after handling gels.

----------***Making an Agarose Gel***----------

1. **Gels must be prepared and run in Tris-Acetic Acid-EDTA (TAE) buffer. Prepare a 10x TAE stock as follows:**
    a. Tris Base (400 mM) 48.4 g
    b. EDTA (10 mM) 2.92 g
    c. Glacial Acetic Acid (1.14% v/v) 11.4 mL
    d. IMPORTANT: You must use Tris base, not Tris-HCl. Also, do not titrate the buffer.

2. **Prepare one liter of 1X TAE buffer by mixing:**
    a. 10X TAE Buffer 100 mL
    b. Ultrapure Water 900 mL

3. **Prepare a 1% agarose gel (Note: 1% = 1 gram in 100 mL) by mixing:**
    a. Agarose Powder 750 mg
    b. 1X TAE Buffer 75 mL

4. **Microwave the agarose solution until it is completely clear (~90 seconds).**
    a. CAUTION: The agarose mixture may get superheated in the microwave and spontaneously boil when you remove it. Use protective gloves when handling the gel solution to prevent burns.

5. **Thoroughly clean a gel cast and comb by spraying it with 75% ethanol and wiping it dry.**

6. **Pour the molten gel into the cast and immediately add 5 μL of ethidium bromide stock. Mix thoroughly.**

7. **Let the gel solidify for approximately 15-30 minutes. It will become opaque when it is completely solid.**

---------- ***Running an Agarose Gel*** ----------

1. **Add the appropriate amount of loading dye concentrate to your sample and mix thoroughly.**
    a. Ex: Add 5 μL of 10X dye to a 45 μL sample

2. **Carefully pipette 10 mL of ladder into Lane 1, then pipette all of your samples into Lanes 3-7.**

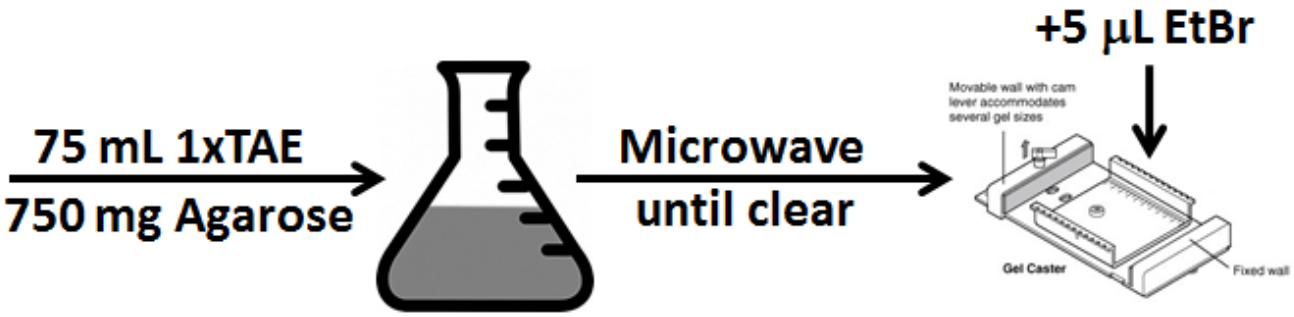
3. **Run the gel at 115V for 15-30 minutes. Stop the gel when the dye front has migrated ~1-2" into the gel.**

4. **Visualize the bands with the UV transilluminator and take a picture of the gel for your lab report.**
    a. **Note:** UV light can mutate DNA (e.g. create thymine dimers), so try to minimize UV exposure.

5. **Clean a spatula with 75% ethanol and cut out your desired band(s) and transfer to a clean tube.**

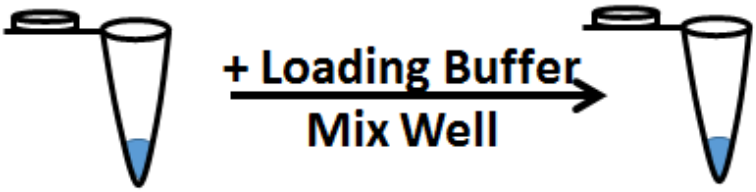
6. **Proceed immediately to gel extraction or save gel slices in the freezer for up to 1-2 weeks.**

Agarose Gel Electrophoresis Visual Protocol

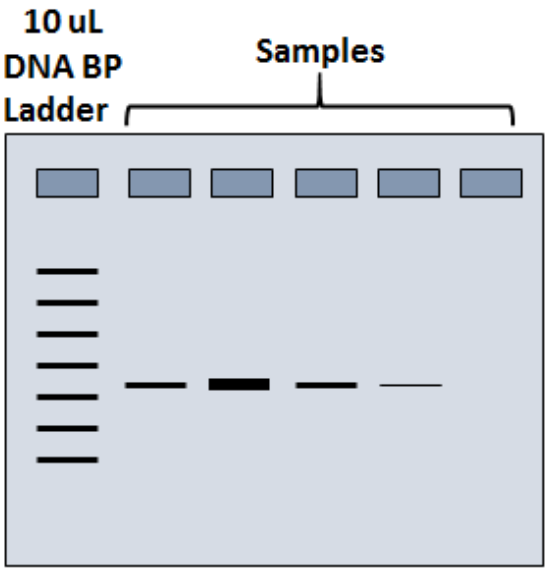


Make sure the comb is in place and EtBr has been added,
Then let the gel solidify COMPLETELY (~30 min)

Sample Preparation



Submerge the gel completely in 1xTAE (not water!)
Carefully load samples into each well.



Electric Field

(-)

115V

15-30 min

(+)

Lab 1d – Gel Extraction

Purpose: *Extract the desired DNA bands from an agarose gel for downstream applications.*

Instructions:

Note: Ethanol must be added to the Wash Buffer. Add 48 mL of 100% ethanol to the 12 mL of Wash Buffer.

1. **Preheat a water bath to 55°C. Do this before running your gel, since it may take a while.**

2. **Weigh your gel slice(s). For every 1 mg of gel, add 3 uL of Gel Solubilization Solution.**
    a. Split the gel slice into multiple tubes if the solubilization solution volume exceeds the tube volume.

3. **Incubate the sample at 55°C until it melts completely. Vortexing every 2 min will accelerate this process.**
    a. The solution should remain yellow. If it changes to red, add 10 uL aliquots of 3M Sodium Acetate until it is yellow again.

4. **Add 1 uL of 100% isopropanol for every 1 mg of gel originally in the tube.**
    a. Split into multiple tubes if necessary.

5. **Insert a spin column into a collection tube for each of your samples.**

6. **Add 500 uL of Column Preparation Solution to each column.**
7. **Centrifuge at 12,000g for 1 minute.**
8. **Discard the flow through liquid.**

9. **Add 700 uL of your sample to the column.**
10. **Centrifuge at 12,000g for 1 minute.**
11. **Collect the flow through liquid as ethidium bromide waste. Repeat steps 9-11 for all remaining sample.**

12. **Add 700 uL of Wash Buffer to the column.**
13. **Centrifuge at 12,000g for 1 minute.**
14. **Discard the flow through liquid.**

15. **Centrifuge the column one more time at 12,000g for 1 minute.**

16. **Transfer the spin column to a new clean Eppendorf (1.7 mL) tube.**

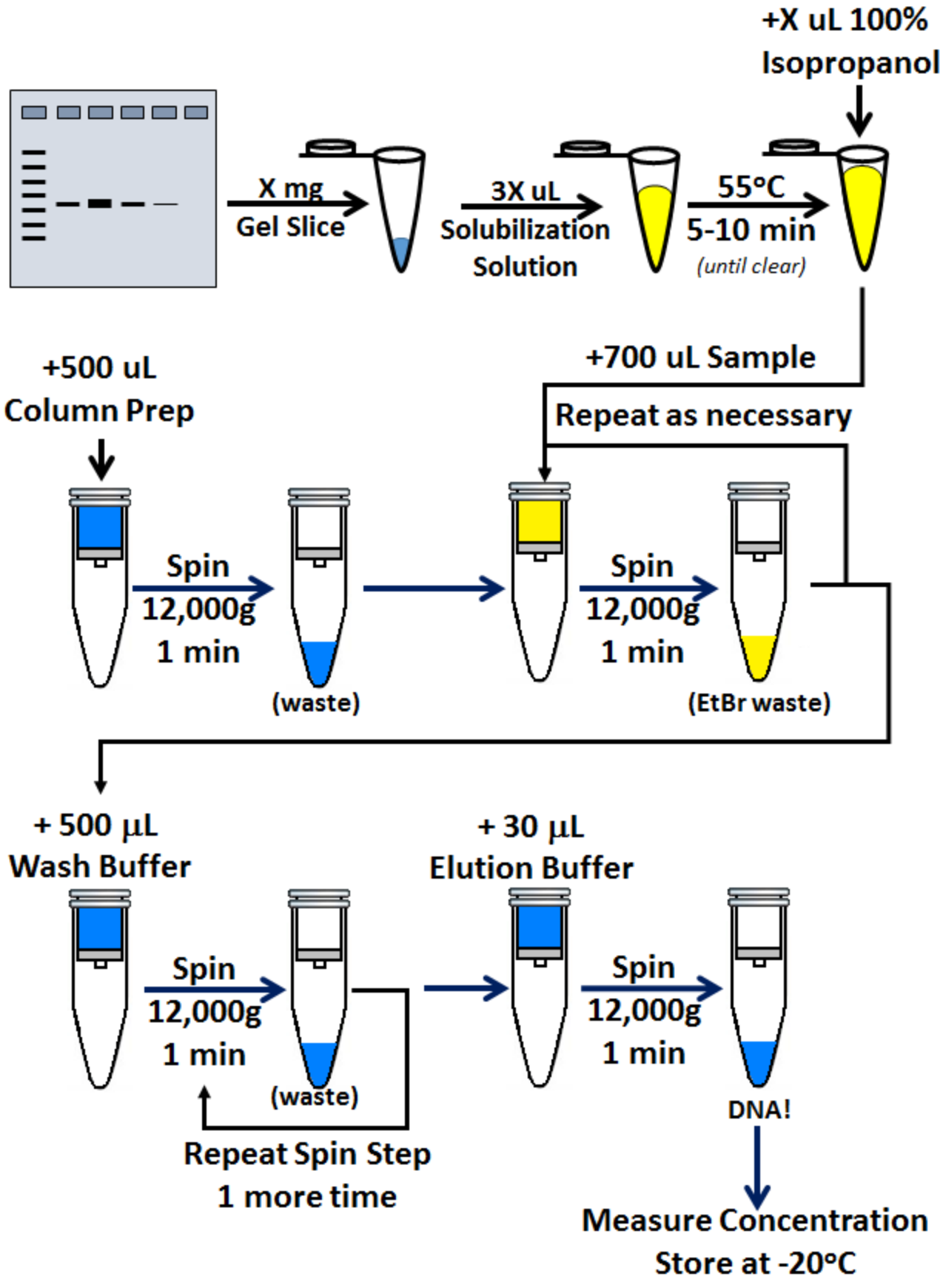
17. **Add 30 uL of Elution Buffer or Ultrapure Water directly to the center of the spin column.**
18. **Centrifuge at 12,000g for 1 minute.**
19. **Store the flow through liquid (DNA) for Lab 2 at -20°C after measuring its concentration (see below).**

<u>Using the TAKE3 plate to measure DNA Concentration:</u>

1. **Add 2 uL of Elution Buffer or Ultra Pure water to position B1. Add 2 uL of sample to the other wells.**

2. **Measure the absorbance of the blank (i.e. water) sample and then measure sample concentrations.**
    a. Concentrations should be in the range of 1-100 ng/ul for PCR and digests.
    b. The ratio of A_{260}/A_{280} should be equal to or greater than 1.80. (A_{260} = DNA, A_{280} = Protein)
        i. If this value is less than 1.8, you may have significant protein contamination.

Gel Extraction Visual Protocol



Lab 2a – CPEC Primer Design

You will need two sets of primers for CPEC: (1) one set that amplifies the target gene and (2) one set that amplifies the desired plasmid backbone (promoter, terminator, etc.). The primers used for the gene in Lab 1 were traditional PCR primers – they are 100% complimentary to the target sequence. In contrast, the primers used for the plasmid backbone are bipartite – the 5' ends are also complimentary to the gene, while the 3' ends are complimentary to the expression plasmid. A detailed protocol for designing all of these primers is given below:

1) Primers for the Gene: (Use the primers designed in Lab 1a or follow the directions below):
   - **a.** Forward Primer:
     - **i.** Copy 30 bp downstream from the 5' end of the target sequence (e.g. 1st codon of gene).
     - **ii.** Truncate the 3' end of the sequence until T_m ~ 65°C. Try to leave a G or C at the 3' end.
     - **iii.** Check for secondary structures and off-target binding.
   - **b.** Reverse Primer:
     - **i.** Copy 30 bp upstream from the 3' end of the target sequence (e.g. stop codon TAA).
     - **ii.** Take the reverse complement of this sequence.
     - **iii.** Truncate the 3' end of the sequence until T_m ~ 65°C. Try to leave a G or C at the 3' end.
     - **iv.** Check for secondary structures and off-target binding.

2) Primers for the Plasmid Backbone
   - **a.** Forward Primer:
     - **i.** Take the reverse complement of the reverse primer for the gene.
       - **1.** This is the 5' sequence of your forward primer.
     - **ii.** Next, copy the first 30 bp downstream from the insertion site (e.g. after the stop codon).
     - **iii.** Use the T_m calculator to truncate the 3' end of this sequence until T_m ~ 65°C
       - **1.** This is the 3' sequence of your forward primer. Try to leave a G or C at the 3' end.
     - **iv.** Combine the sequences from steps i and iii to obtain your final forward primer.
   - **b.** Reverse Primer:
     - **i.** Take the reverse complement of the forward primer for the gene.
       - **1.** This is the 5' sequence of your reverse primer.
     - **ii.** Next, copy the 30 bp upstream of the insertion site (e.g. beginning of old gene).
     - **iii.** Obtain the reverse complement at http://reverse-complement.com/
     - **iv.** Use the T_m calculator to truncate the 3' end of this sequence until T_m ~ 65°C
       - **1.** This is the 3' sequence of your reverse primer. Try to leave a G or C at the 3' end.
     - **v.** Combine the sequences from steps i and iv to obtain your final reverse primer.

Use the table below to design your primers. Start by copying the primer sequences you used to amplify the gene in Lab 1b. Next, refer to the expression plasmid sequence in the Appendix to design the primers for the plasmid. Recall that our goal is to fuse the chromoprotein to the 3' end of the chitin binding domain/intein fusion tag. To do so, we will need to replace the existing old gene (highlighted in yellow in the appendix) using CPEC.

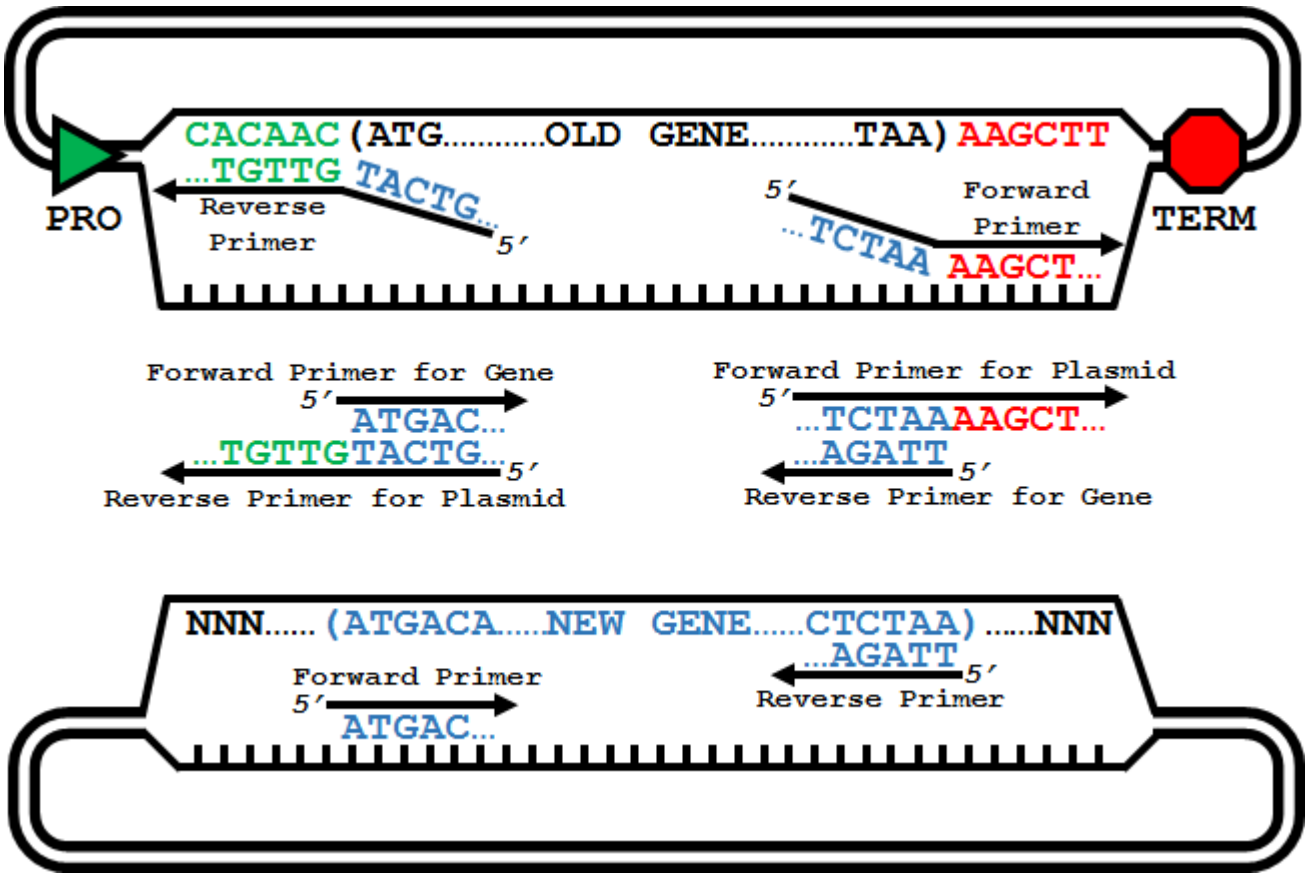
Example: CPEC Primer Design Table

1' PCR for Gene	Tm	Complimentary to Gene		
Forward Primer				
Reverse Primer				
1' PCR for Plasmid	**Tm**	**Complimentary to Gene**	**Complimentary to Plasmid**	**Tm**
Forward Primer				
Reverse Primer				

Note: Sequences highlighted in similar colors are the reverse compliments of each other.

Reminder: If possible, try to have a C or G at the 3' ends of each primer.

Diagram of CPEC Primer Design



Notes:

Lab 2b – CPEC: 2' PCR

Purpose: *Combine the 1' PCR products (gene and backbone) to yield a new (nicked) expression plasmid.*

Instructions:

1. **Measure the concentration of your megaprimers.**
   a. See the instructions for using the Take3 plate in Lab 1d (Agarose Gel Extraction).
   b. Dr. Elmer has already prepared the plasmid amplicon for you (with a reaction similar to Lab 1).

2. **Prepare the 2' PCR reaction mixture according to the table below.**
   a. The reagents must be mixed in the order shown below – always add enzyme last.
   b. Keep the enzyme on ice and return it to the freezer as soon as you are finished with it.
   c. Use the equation below to calculate the volume of gene required:

$$V_{gene} = \left(\frac{BP_{gene}}{BP_{plasmid}}\right)\left(\frac{C_{plasmid}V_{plasmid}}{C_{gene}}\right) = \left(\frac{BP_{gene}}{BP_{plasmid}}\right)\left(\frac{\mathbf{200}\boldsymbol{ng}}{C_{gene}}\right)$$

Reagents	Stock Concentration	Required Amount or Concentration	Reaction Volume (μL)	3.5x Master Mix (μL)
Gene Amplicon		**$Moles_{GENE}$ = $Moles_{PLASMID}$**	μL	
Plasmid Amplicon		**200 ng**	μL	
HF Buffer	**5x**	**1x**	μL	
dNTP's	**10 mM**	**200 μM**	μL	
Water	*Add up to a final total volume of 50 μL*		μL	
Polymerase	**2,000 U/mL**	**1 U**	μL	
		Total:	50 μL	

Note: *The stock concentrations listed above correspond to the NEB Phusion Polymerase PCR Kit.*

Note: *If the volumes of gene and plasmid amplicon samples may exceed 50 uL, then you may (1) repeat the PCR reactions and gel extractions to get higher yields or (2) decrease the amount of plasmid amplicon to 100 ng.*

3. **Aliquot the master mix into 3 separate tubes (50 uL each).**

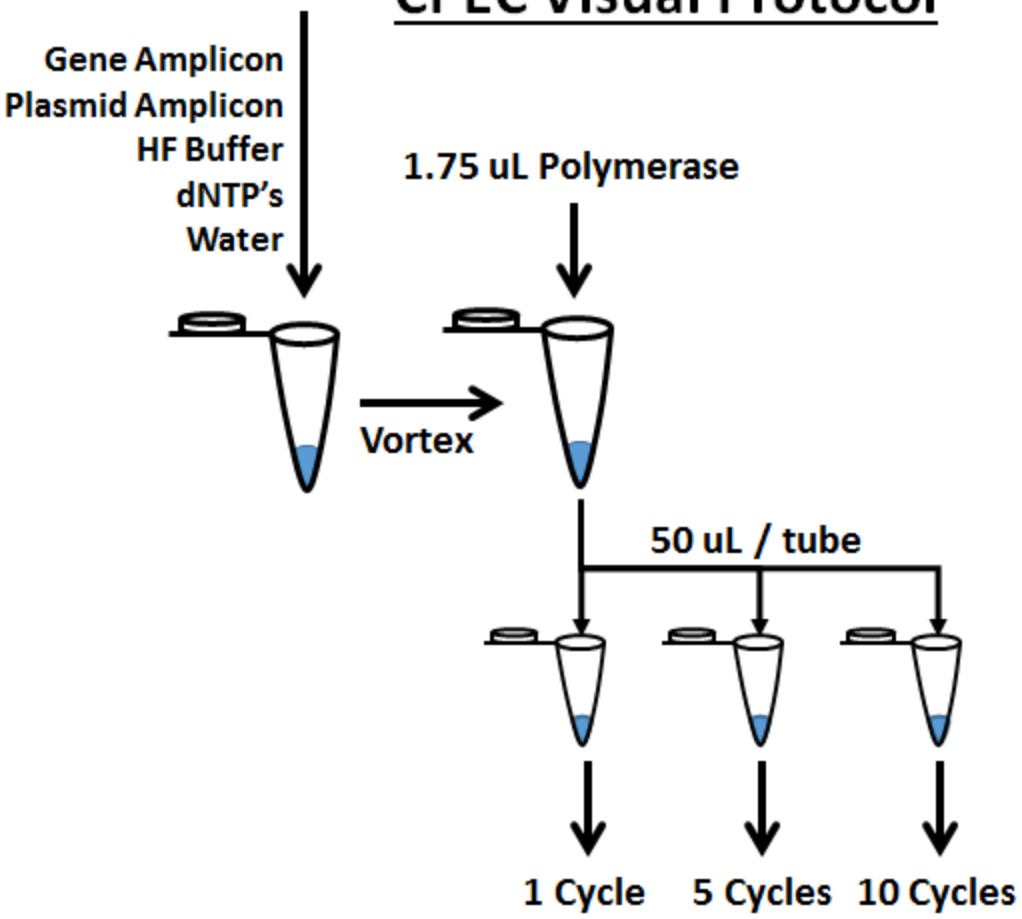
4. **Run the reactions through the thermal cycler with the routine shown below.**
   a. Run your first tube through 1 cycle, the second aliquot for 5 cycles, and the third aliquot for 10 cycles.

Steps		Temperature	Time	
Initial Denaturation		98°C	3 min	
1-10 Cycles	**Cycle Denature**	98°C	30 sec	
	Cycle Anneal	60°C	30 sec	
	Cycle Extend	72°C		1 min/kb
Final Extension		72°C	15 min	
Final Hold		4°C	*Indefinite*	

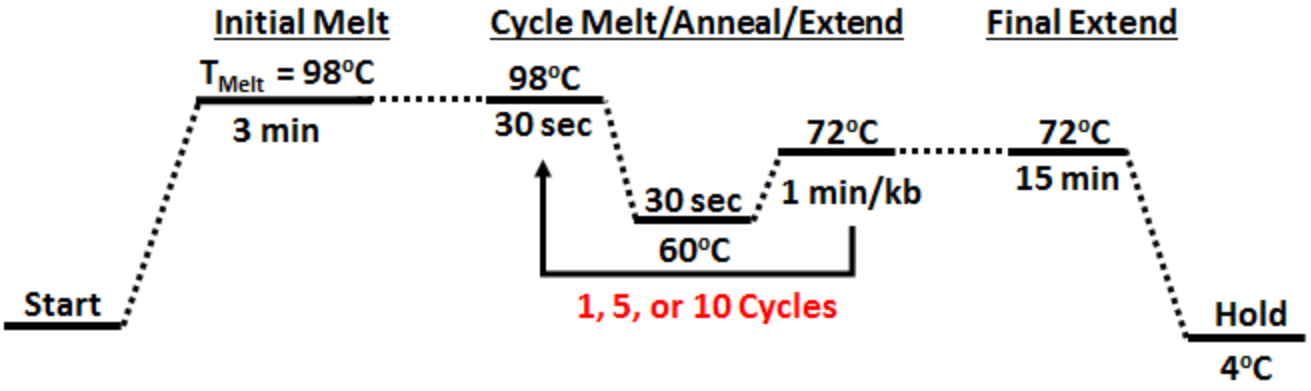
Note: *The temperatures and times shown above are optimized for our specific primers and the kit that we are using. If you use different primers or kits, these values may need to be adjusted.*

5. **After all of the routines have finished, run 45 uL of each reaction on a gel and save the other 5 uL.**

CPEC Visual Protocol



CPEC Thermal Cycler Routine:



Lab 3 – Transformation of DH5α *E. coli*

Purpose: Insert your plasmids from Lab 2 into DH5α *E. coli* to produce large quantities of new plasmid for sequencing (Labs 4 and 5) and protein expression in BL21 *E. coli* (Lab 6).

Instructions:

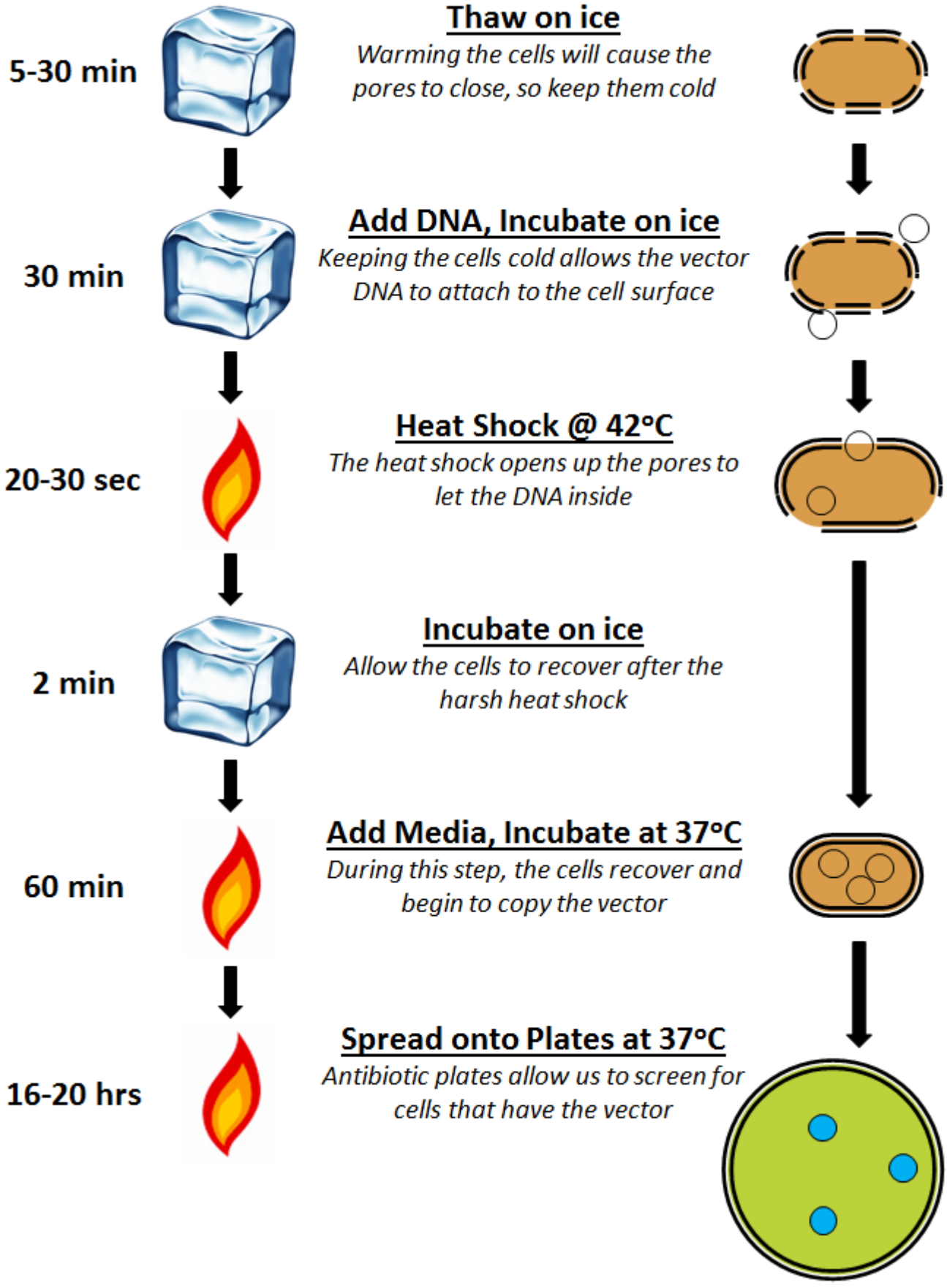
---------- ***Transformation of DH5α E.coli*** ----------

1. **Thaw out an aliquot of DH5α cells on ice**
2. **Add 5 μL of the <u>CPEC sample with the best complete plasmid yield</u> (1, 5, or 10 cycles) to the tube**
3. **Incubate the tube on ice for 30 minutes**
4. **Heat shock the cells in the 42°C water bath for 20 seconds**
5. **Incubate the cells on ice for 2 minutes**
6. **Add 350 μL of LB media to the cells**
7. **Tape the tubes down in the shaker and incubate @ 37°C and 225 rpm for 1 hour**
8. **Meanwhile, take the ampicillin plates out of the fridge and set them in the hood to dry.**
9. **Add 50 μL of your cells to each plate**
10. **Using a sterile cell spreader, spread the cells on each plate until it appears dry**
11. **Sterilize the spreader by spraying it with ethanol and igniting it, then letting it cool ~20 sec**
12. **Incubate the plates at 37°C overnight (~16 hrs, no more than 20 hrs)**
13. **The plates will be stored in the fridge until we use them to replicate the plasmid next week.**

----------***Preparation of LB Agar Plates*** ----------

1. **In a 1L bottle, mix the following ingredients:**
   - **a.** Water 400 mL
   - **b.** LB agar powder 16 g
2. **Autoclave the mixture at 121°C for 20 minutes.**
3. **Immediately after the autoclave cycle is finished, remove the molten agar and cool it down in the sink.**
4. **Cool the agar with tap water until it is lukewarm to the touch, but don't let it solidify in the bottle.**
5. **Dry off the bottle and sterilize it with 75% ethanol, then bring it into the biosafety cabinet.**
6. **Quickly add 400 uL of ampicillin stock solution to the agar and mix well.**
   a. 100 mg/mL Ampicillin stock = 200 mg ampicillin powder + 2 mL ultrapure water
   b. Sterilize the ampicillin stock by passing it through a 0.22 um syringe filter
   c. Ampicillin solutions can be stored in the refrigerator for up to 2 months
7. **Fill petri dishes 1/3-1/2 full (30-50 mL) with agar and let them solidify for 20-30 minutes.**
   a. Ampicillin-agar plates can be stored in a bag at 4°C for up to 2 months.

Transformation Visual Protocol



Lab 4 – Plasmid Purification/Miniprep

Purpose: Use your transformed *E. coli* colonies to produce (relatively) large quantities of plasmid DNA that will be used for DNA sequencing (Lab 5) and Protein Expression (Lab 6).

Instructions:

Note: *The inoculation of bacterial cell culture for miniprep is described on the following page, along with protocols for preparing glycerol stocks of E. coli strains for long term storage and preparing samples for DNA sequencing. The instructor inoculated your cultures yesterday to allow you to complete the miniprep and glycerol stocks today.*

----------***Isolation of Plasmid DNA***----------

Ethanol must be added to the Wash Buffers and RNase must be added to the Resuspension Buffer in new kits.

1. **Centrifuge the cells at 3000g for 5 minutes**
2. **Decant the supernatant media into bleach**
3. **Resuspend the cell pellet by adding 200 µL of resuspension buffer and vortexing**
4. **Transfer the cell suspension to a fresh 1.5 mL tube**

5. **Add 200 µL of lysis buffer to the tube and mix by inversion 5-7 times**
    a. The sample should change color from tan to white-ish

6. **Incubate the lysis reaction at RT for exactly 5 minutes**
    a. Longer incubation will damage the sample and reduce final purity

7. **Add 350 µL of neutralization buffer and mix by inversion 5-7 times**
    a. Proteins and genomic DNA will precipitate to form a white "snot"

8. **Spin the tubes at 12,000g for 10 minutes**

9. **Prepare a new spin column by inserting it into a fresh 1.5 mL tube**
10. **Add 500 µL of Column Preparation Buffer to the columns**
11. **Spin the tubes at 12,000g for 1 minute**

12. **Transfer the supernatant to the spin column by pipetting/decanting**
13. **Spin the tubes at 12,000g for 1 minute**
14. **Discard the flow through**

15. **Add 500 µL of Wash buffer 1 to the columns**
16. **Spin the tubes at 12,000g for 1 minute**
17. **Discard the flow through**

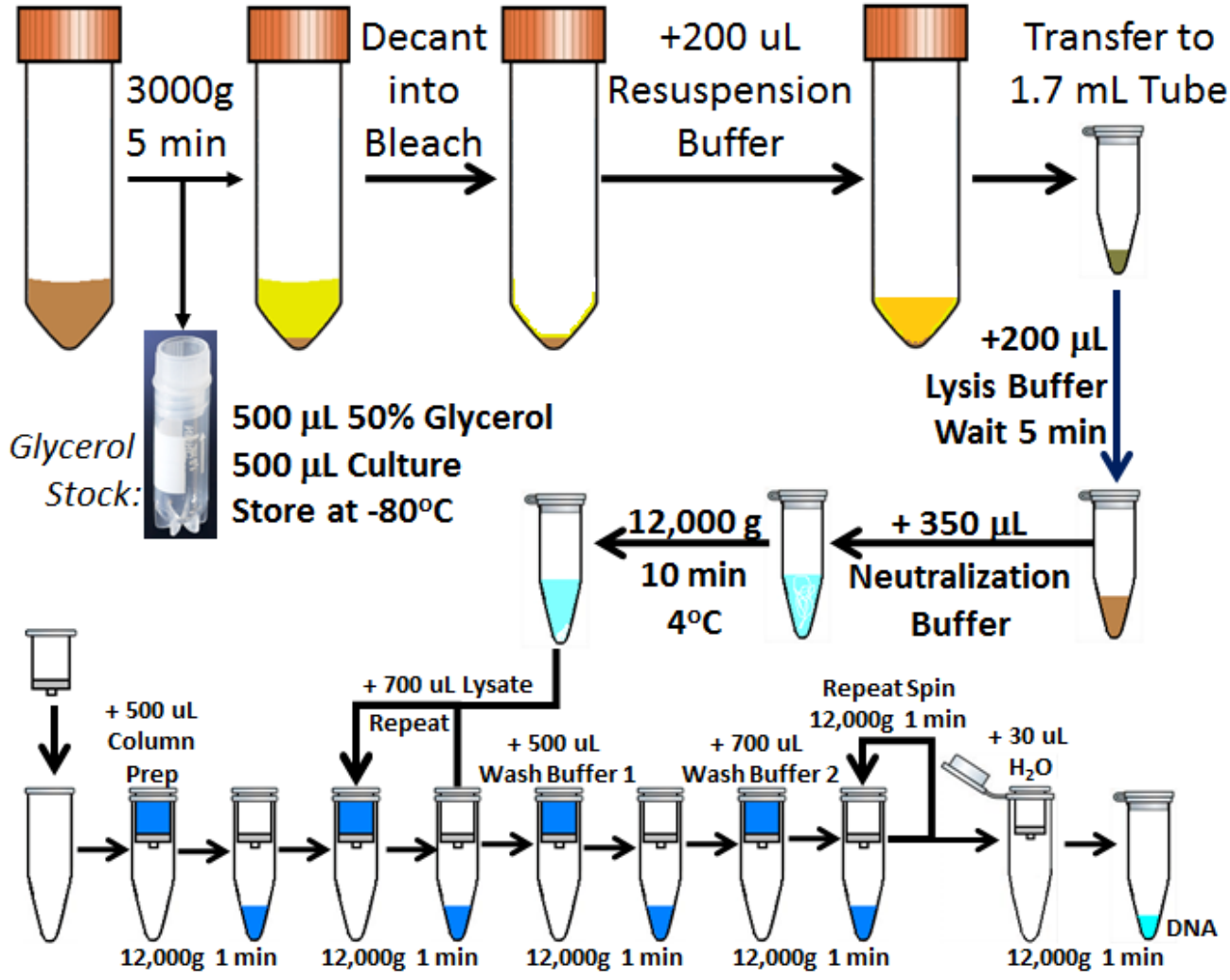
18. **Add 700 µL of Wash buffer 2 to the columns**
19. **Spin the tubes at 12,000g for 1 minute**
20. **Discard the flow through**
21. **Spin the tubes again at 12,000g for 1 minute to remove any remaining EtOH**

22. **Transfer the dry spin column to a new 1.5 mL tube**
23. **Add 30 µL of elution buffer to the center of the spin column**
24. **Incubate at RT for 1 minute**
25. **Spin the tubes at 12,000g for 1 minute**
26. **The flow-through is pure plasmid DNA – measure the DNA concentration and store at -20°C**

MiniPrep and Sequencing Visual Protocol

Inoculation of Small-Scale (5 mL) Bacterial Culture for MiniPrep

Overview of MiniPrep Procedure:



Sequencing Prep = 800 ng Plasmid DNA + 25 pmol primer in 15 uL

---------- ***Inoculation of Small-Scale (10 mL) E. coli Culture*** ----------

1. **Prepare fresh LB media:**
   - **a.** Water 500 mL
   - **b.** LB media powder 12.5 g
   - **c.** Mix well, then autoclave at 121°C for 20 minutes. LB Media can be stored at 4°C for ≤ 6 months.

2. **Sterilize the bench with 75% EtOH and light a Bunsen Burner**

3. **Transfer 5 mL of LB media into a sterile 50 mL tube**

4. **Add 10 uL of sterile ampicillin stock solution (100 mg/mL) to the LB media**

5. **Spray a pipette with 75% EtOH to sterilize it and then pick up a sterile pipette tip**

6. **Scrape a colony off of the agar plate with the pipette tip, then eject the tip into the LB-amp solution**

7. **Incubate the culture at 37°C and 225 rpm for 16-20 hrs (or until the culture appears turbid)**

---------- ***Glycerol Stocks*** ----------

1. **Sterilize the bench with EtOH and light a Bunsen burner**

2. **Label a cryo-tube and loosen the cap**

3. **Add 500 µL of sterile 50% glycerol to each tube**

4. **Add 500 µL of *E. coli* culture to the tube**

5. **Freeze at -80°C until needed**

---------- ***DNA Sequencing Prep*** ----------

1. **Measure the concentration of your plasmid DNA sample.**

2. **Prepare a mixture of plasmid DNA and the pCI:X sequencing primer with the guidelines below:**

Reagent	Stock Concentration	Required Amount/ Concentration	Volume to be Added
Plasmid DNA		800 ng	uL
Sequencing Primer	25 nmol/mL	25 pmol	uL
DNase-Free Water	*Up to a final total volume of 15 uL*		uL
		Total:	15 uL

Note: Dilute your plasmid DNA with ultrapure DNase free water or elution buffer, if necessary.

3. **Mail the samples to the sequencing company (GenScript) and wait about 2 days.**

We will be using GenScript, a global DNA sequencing company based in New Jersey:

http://www.genscript.com/sequencing_guidelines.html

Lab 5 – Sequencing Analysis and BLAST

Purpose: Align your experimental sequence with the predicted sequence to check for mutations.

Instructions:

---***DNA Sequencing Alignment*** ---

1. **Download your experimental sequence and the predicted sequence**
2. **Navigate to the Basic Local Alignment Search Tool (BLAST) on the NCBI website:**

   http://blast.ncbi.nlm.nih.gov/Blast.cgi
3. **Select "nucleotide blast" in the list of BLAST programs**
4. **Click on the button that says "Align two or more sequences"**
5. **Copy and paste the expected sequence into the top dialog box (Query)**
6. **Copy and paste the actual sequence into the bottom dialog box (Subject)**
7. **Click the blue "BLAST" button at the bottom of the page….and cross your fingers….**
8. **Scroll down to the Alignments window to see your sequence alignment**
   - **a.** Copy and Paste the alignment into a word file
   - b. Include the alignment for your sequence in your lab report

--- ***Homology Search – Part I***---

1. **Go back to the BLAST homepage: http://blast.ncbi.nlm.nih.gov/Blast.cgi**
2. **Click on "tblastx" instead of nucleotide BLAST. This tool compares translated amino acid sequences.**
3. **Click the "align two or more sequences" button.**
4. **Copy your gene's sequence into the Query Field, then copy another group's gene into the Subject field**
5. **Click BLAST to compare the sequences of the proteins**. How similar are they?

---***Homology Search – Part II*** ---

1. **Go back to the BLAST homepage: http://blast.ncbi.nlm.nih.gov/Blast.cgi**
2. **Click on "tblastx". This tool translates your gene into amino acids and searches the protein database for similar sequences.**
3. **Click the BLAST button to search for proteins with similar sequences.** What kind of results do you see?

Guidelines for Lab Report 1

Synopsis

It is of paramount importance that you are able to effectively communicate your ideas in a succinct and correct manner. In the interest of honing that skill, your lab reports will be limited to 4 pages (single spaced, 12pt Times New Roman, Margins = 1" on all sides).

The first two pages will only contain text and must include the following sections:

- **Title/Authors** – Include a descriptive and concise title, along with a list your group members
- **Abstract** – A summary that consists of 1-2 sentences for each of the following sections (≤100 words).
- **Introduction** – Describe how CPEC works and discuss its advantages/disadvantages.
  - See figure 1 below - refer to this figure in your Introduction
- **Methods** – Explain the variables you tested to optimize CPEC (T_{anneal} and cycle #) and their importance.
  - DO NOT rewrite the protocols.
- **Results** – Describe the results obtained in Labs 1-5.
  - **Lab 1**: What was the effect of annealing temperature? Which was best? Why? Was your PCR product the expected size?
  - **Lab 2**: What products did you obtain after 1, 5, and 10 cycles of CPEC? Which was best? Why? Was your CPEC product the expected size?
  - **Lab 3**: Was the transformation successful? Compare your results to other groups' results.
  - **Labs 4/5**: Describe your sequencing results – is your gene present? Is it mutated?
- **Conclusion** – Summarize your findings – give optimum conditions and indicate if reactions succeeded.

The last 1-2 pages will contain figures and citations. For this report, you must include the following figures:

- **Figure 1: Schematic of CPEC Reaction**
  - Make your own figure. DO NOT copy my illustrations from the notes.
  - A picture is worth 1,000 words – make a good figure so you can keep the Introduction short.
- **Figure 2: Effects of Annealing Temperature on PCR yield (Lab 1)**
  - Completely annotate the gel – label the ladder, each band, and the individual lane #'s
  - Indicate the observed length (in base pairs) of the PCR products
- **Figure 3: Effects of Cycle Number on CPEC yield (Lab 2)**
  - Completely annotate the gel – label the ladder, each band, and the individual lane #'s
  - Indicate the observed length (in base pairs) of the CPEC products
- **Figure 4: Picture of transformed *E. coli* on agar plate, with table containing colony counts (Lab 3)**
- **Figure 5: Sequence Alignment of Gene in Plasmid vs. Expected Gene Sequence (Labs 4/5)**
  - Indicate the beginning and end of the gene sequence. Also highlight any mutations, if present.
- **Include your references in the remaining space.**
- **Note:** Include captions for each figure. Each caption should include enough information for a well educated/trained reader to understand its significance, but it should also be succinct.

Note: You do not need to take up the entire 4 pages. Just be sure to address all of the prompts listed above.

Your report will also be graded for spelling, grammar, and quality (e.g. logical and smooth flow, good organization, etc.). I take it as a personal offense when a paper is not spell checked prior to submission!

For your reference, some additional guidelines on preparing each section of your lab report are given below.

Abstract

The abstract is like an abbreviation of the entire paper. A good way to approach writing an abstract is to use 1-2 sentences to describe each of the sections in the paper. For example, begin your abstract by summarizing your motivation/introduction in a single sentence (remember that you're trying to catch the reader's attention, so make it sensational!). Then mention how your most important methods were used to measure quantity X or make substance Y. The remaining sentences will then report your results and discuss how they verify your conclusion in the first sentence. Most importantly, an abstract must be concise – most journals require abstracts be **250 words or less**!

Introduction

The abstract grabbed the reader's attention, but the purpose of the introduction is to keep their attention. A good introduction should educate the reader in topics relevant to your work, but it shouldn't be saturated with an excess of information. It should provide examples of other pertinent research that has been done and show the motivation for your experiments.

Methods

This section is the shortcoming of some published papers. Its goal is to allow others to easily repeat your work, however, space limits prevent us from publishing long, elaborate protocols. You should try to be as succinct as possible, but place an emphasis on important variables that were studied in your work.

Results

The purpose of the Results section is to effectively communicate the raw data which you have collected. It should be an impartial communication of the results of your experiment. DO NOT draw any conclusions or make any assumptions in the Results section – that will be done in the Conclusion section. Avoid words like "Therefore,…" Instead, simply try describing the figures and data in words. It is still OK to compare values though (e.g. the reading for sample X was 10 times higher than the reading for sample Y).

For example, you might say: ***"Figure 1 shows that a raisin diet decreased tumor cell growth by 10%."***
But don't make assumptions/conclusions like: ***"Raisins cure cancer."***

Conclusion

This section is your chance to rationalize your results into meaningful conclusions. It is a good opportunity to compare results from your separate experiments to make a single conclusion. For example, you could say "Both magnesium and calcium increased enzyme activity, so the enzyme may have a binding site for divalent cations." Do not present any new information in the Discussion, but it is a good idea to include references (if possible) that reinforce any conclusions that you make from your results.

The conclusion is also meant to summarize what you have learned from your research. It should briefly highlight the new information that has been discovered, how others can use this discovery, and any future work that you believe might be necessary to perfect the method or investigate it further.

References

References should be listed at the end of each lab report in the Nature Journal format. You may manually keep track of references, but I recommend a citation manager like Mendeley (free!) or EndNote ($$ and dumb…).

Lab Report 1 Rubric

Abstract			
Overall Quality: Briefly summarize the entire report.	1 *Incorrect Info*	2 *Sections Missing*	3 *Complete Summary*
Introduction			
Description of CPEC reaction.	1	2	3
Advantages of CPEC	1	2	3
Disadvantages of CPEC	1	2	3
Methods			
Which variables were tested?	1	2	3
Why are they important?	1	2	3
Results			
What was the effect of T_{anneal}?	1	2	3
Which T_{anneal} was best? Why?	1	2	3
Was the product the right size?	1	2	3
Effect of CPEC cycle # on products?	1	2	3
Which # of cycles is best?	1	2	3
Was the CPEC product the right size?	1	2	3
Were the transformations successful?	1	2	3
Describe the sequencing results.	1	2	3
For all prompts above, 3 = correctly addressed, 2 = partially correct, 1 = incorrect, 0 = missing			
Conclusion			
Overall Quality: Draw conclusions based on the results	1 *Missing parts*	2 *Partially incorrect*	3 *Correct conclusions*
Figures			
1: Reaction Scheme for CPEC	1	2	3
2: Effects of Annealing Temperature	1	2	3
3: Effects of CPEC Cycle Number	1	2	3
4: Transformation Plate and Colony #'s	1	2	3
5 - Sequence Alignment	1	2	3
3 = Perfect resolution/annotation/caption, 2 = labels or caption missing, 1 = no labels/caption			
References			
Overall Quality: Appropriate references to support text	1 *1 reference*	2 *2 references*	3 *≥3 references*

Spelling and Grammar ***10 = no spelling or grammar errors, -1 pt for every error***											
Negative pts: -	0	1	2	3	4	5	6	7	8	9	10
Organization and Flow *10 = Logical order and proper segues between sections, -1 pt for every instructor comment*											
Negative pts: -	0	1	2	3	4	5	6	7	8	9	10

Total = /83

The instructor reserves the right to modify this rubric at any time.

Notes

Module 2: Protein Expression, Purification, and Characterization

Introduction

Now that we have verified the sequences of our plasmids, we are ready to begin some protein expression. All recombinant protein production processes consist of 4 vital steps – fermentation/expression, cell lysis, protein purification, and protein characterization. These steps will be covered in detail in the following labs in Module 2:

Lab 6: Grow up large amounts of *E. coli* containing your expression plasmid, then change the culture conditions (i.e. add IPTG and/or decrease temperature) to activate/maximize protein expression.

Lab 7: Retrieve your chromoproteins by lysing the bacterial cells. We will use 2 separate purification strategies for your chromoproteins – affinity chromatography for the tagged chromoproteins and anion exchange chromatography (AEX) for the native chromoproteins.

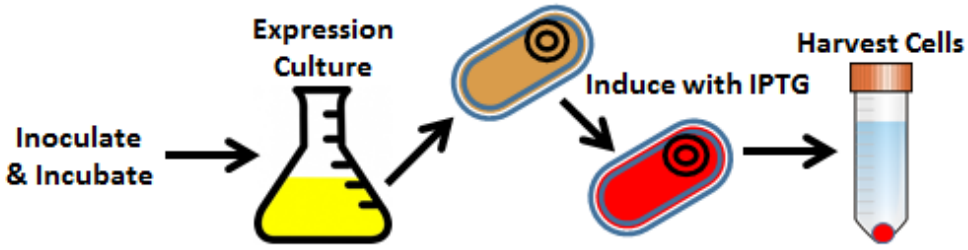
Lab 8: Finish purification and analyze the efficiency of the different purification techniques by running a PAGE gel, which separates all of the proteins into visible bands. If you only have one band, then your sample is 100% pure.

Lab 9: Recent advances in computer-based protein simulations have revolutionized the way that recombinant proteins are expressed and mutated. We will use 3 different programs to simulate protein structure – FoldIt, SwissPDB, and AutoDock.

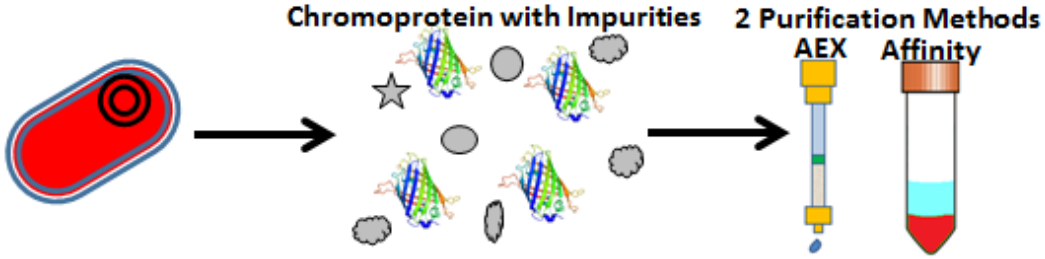
The overall goal of this module is to teach you how to optimize the conditions for bacterial fermentation/protein expression. Your lab report for this module will focus on the differences in the protein purification techniques. For example, which one was the easiest? The quickest? You will also include measurements of chromoprotein yield and purity in your reports to decide which method is the best choice.

Overview of Module 2 Experiments

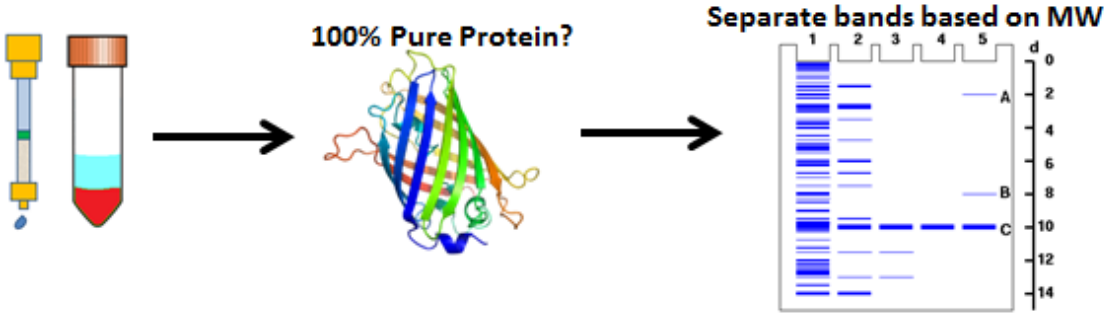
Lab 6: Grow E. coli and Activate Expression of the Chromoproteins



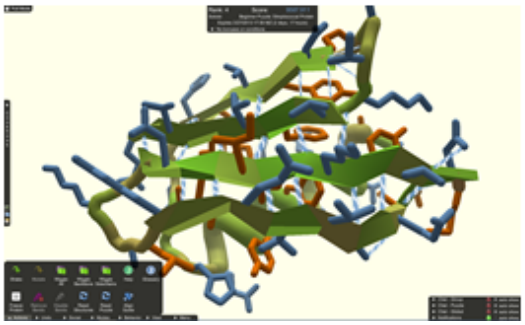
Lab 7: Lyse Cells and Begin Chromoprotein Purification

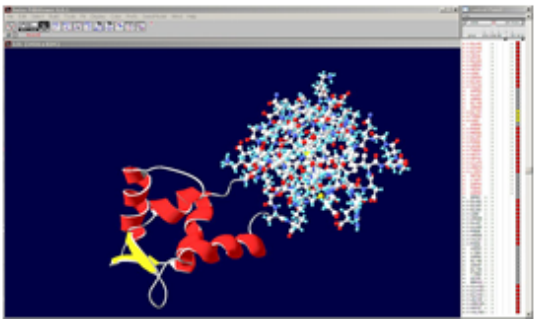


Lab 8: Finish Protein Purification and Analyze Proteins



Lab 9: Protein Simulation Software: FoldIt, SwissPDB, and AutoDock





Lab 6 – Bacterial Cell Culture and Protein Expression

Purpose: Grow up a large amount of BL21 *E. coli* that have your expression plasmid, then activate the expression of your gene by adding IPTG. Note: The instructor has already transformed BL21 with your plasmid.

Instructions:

Protein expression usually requires the following 4 steps, which are spread out over the course of 2-4 days:

1. Inoculation of a Starter Culture from a Plate
2. Inoculation of the Expression Culture from the Starter Culture
3. Induction of the Gene when the Culture reaches Stationary Phase (OD_{600} = maximum)
4. Incubation of the Induced Cells for Protein Expression, followed by Cell Harvest

Since we only have 3.5 hours today, I have started several staggered parallel identical fermentations to allow you to do all of the steps, as described below.

----------*Inoculation of a Starter Culture from a Plate* ----------

1. See Lab 4. We will not be doing this today.

---------- *Inoculation of the Expression Culture from the Starter Culture* ----------

1. Hold the expression culture up to the light and inspect it visually. If you can see dense cloudy swirls, then the culture is saturated/turbid and we can proceed. Otherwise, let the culture continue to grow.

2. **Prepare the expression culture in a sterile 1L baffled flask:**
    a. Mix 100 mL of water with 2.5 grams of LB powder in the flask, then autoclave (already done)
    b. Sterilize the bench and light a Bunsen burner
    c. Add 100 uL of ampicillin (100 mg/mL stock) to the media

3. Measure the absorbance of the starter culture at 600 nm (OD_{600}, should be 1-2 for a saturated culture).
    a. Transfer 1 mL of the starter culture to a cuvette, then dilute with media until $A_{600} < 1.0$.
    b. Measure the absorbance of your cuvette and another cuvette with sterile media.
    c. Subtract the absorbance of the sterile cuvette from your sample's absorbance.

4. Add enough volume of the starter culture to the expression culture to give it $OD_{600} = 0.1$.
    a. $C_1V_1 = C_2V_2$, where $C_1 = OD_{600}$ from Step 3, V_2 = expression culture final volume, and $C_2 = 0.1$.

5. Incubate the expression culture at 37°C and 225 rpm until its OD_{600} reaches a plateau (~1-2).
    a. Assuming a doubling time of ~20 min for *E. coli*, this should take about 2-4 hrs (with lag).

----------*Induction of the Expression Culture* ----------

1. Prepare a stock solution of 125 mM IPTG
    a. Mix 10 mL of ultrapure water with 300 mg of IPTG
    b. Pass the solution through a 0.22 um syringe filter and store at 4°C for up to 2 months

2. Add 400 uL of 125 mM IPTG stock to the flask to obtain a final concentration of 0.5 mM IPTG

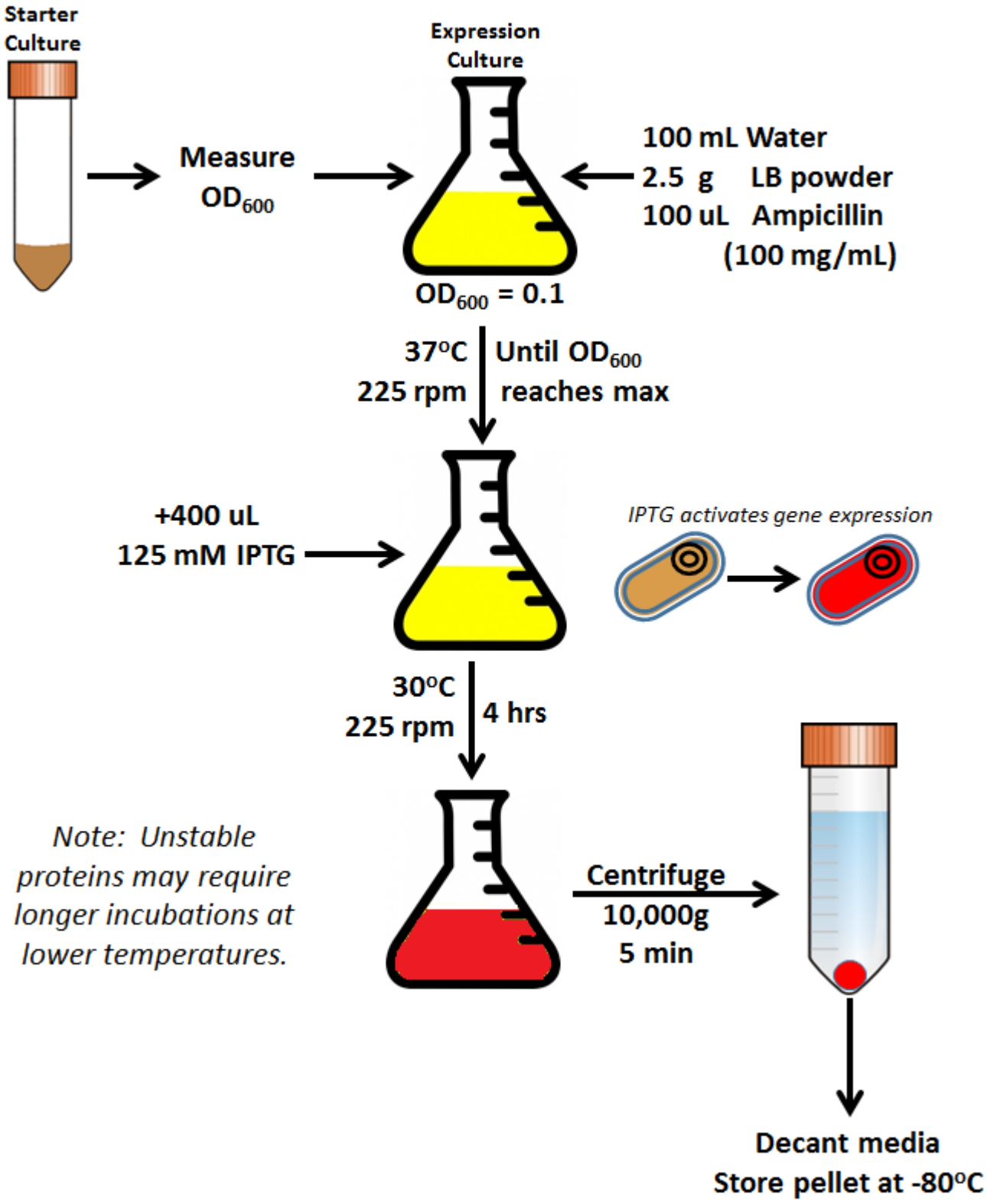
3. Incubate the culture at <u>30°C</u> and 225 rpm for 4 hours.

---------- *Cell Harvest and Storage* ----------

1. Decant your culture into separate 50 mL tubes – only put 40 mL in each tube or else it will leak.

2. Centrifuge the tubes at 10,000g for 5 minutes. Decant the supernatant and store the pellet at -80°C.

Protein Expression Visual Protocol



Lab 7a – Bacterial Cell Lysis and Clarification

Purpose: Extract your protein from the cells and remove as many solid debris as possible prior to purification.

Instructions:

Note: We will be conducting chemical lysis, but Dr. Elmer will also demonstrate sonication.
Note: Lysozyme will degrade chitin beads, so it must not be added to the sample with the tagged protein.

1. **Begin thawing your cell pellets at room temperature.**

2. **Resuspend each cell pellet by adding 5 mL of 20 mM Tris (pH 8.0) and vortexing.**

3. **Combine all of the pellets that originated from the same culture (e.g. all CBD:I:aeBlue cells)**

4. **Prepare the following separate lysis buffers for the tagged and native chromoprotein**s:
    a. DNase I stock = 50% glycerol, 20 mM Tris, pH 8.0, 2 mM $MgCl_2$, 1 mg/mL
    b. Benzamidine and Lysozyme stocks were prepared in 20 mM Tris Buffer, pH 8.0

Reagent	Stock Concentration	Final Concentration	Lysis Buffer for Tagged Proteins	Lysis Buffer for Native Proteins
20 mM Tris Buffer	*Up to a final total volume of 50 mL*		mL	mL
Benzamidine HCl	**500 mM**	**5 mM**	mL	mL
Triton X-100	**100%**	**1%**	mL	mL
DNase I	**1 mg/mL**	**20 ug/mL**	mL	mL
CelLytic Reagent	**10X**	**1X**	mL	
Lysozyme	**10 mg/mL**	**100 ug/mL**		mL
		Total:	50 mL	50 mL

5. **Add lysis buffer to the cell suspension in a 1:1 v/v ratio (e.g. Add 25 mL of lysis buffer to 25 mL of cells)**
    a. Add the lysis buffer with lysozyme ONLY to the cells with native chromoprotein
    b. Add the lysis buffer with CelLytic ONLY to the cells with the tagged chromoprotein

6. **Put the tubes on the rocker for 1 hour while lysis proceeds.**

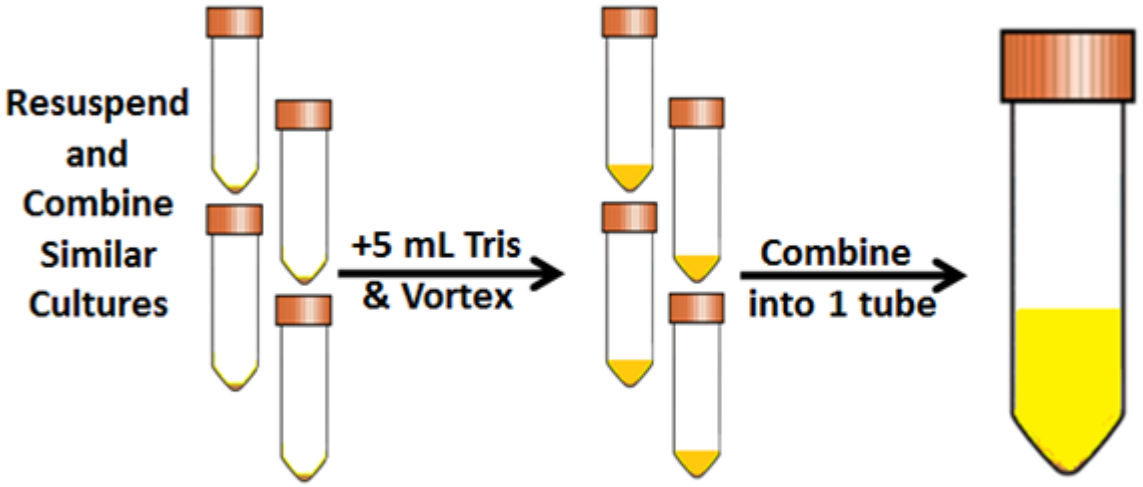
7. **Balance your tubes with water and centrifuge them at 18,000g for 10 minutes.**

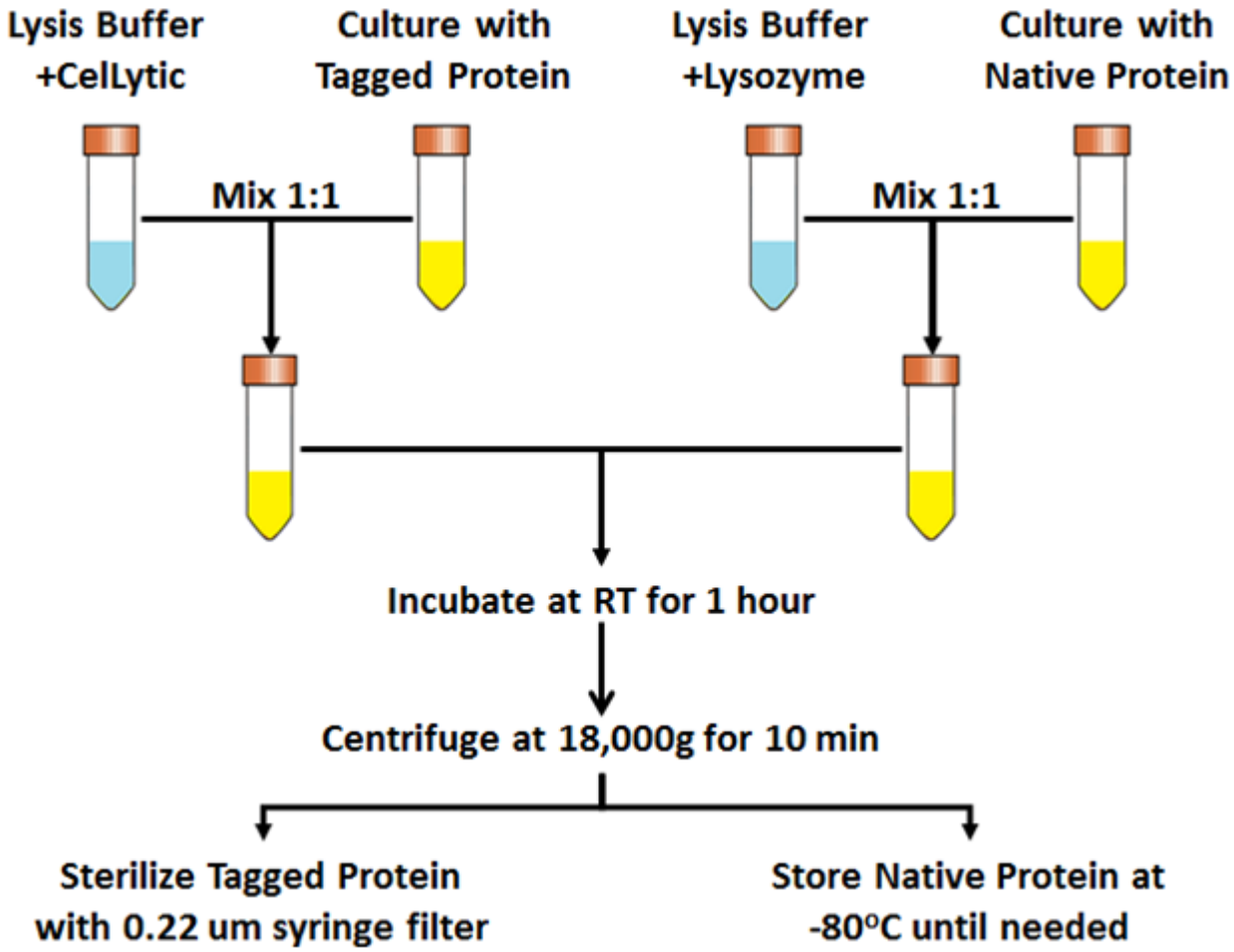
8. **Collect the supernatant and discard the cell pellets**
    a. Before you throw them away, compare the size of the cell pellets and the color of the supernatant solutions. Did one method appear to work better than the other? Mention this in your report.
    b. Record the absorbance spectrum of the cell lysates from 300-500 nm.

9. **Immediately pass the lysate with tagged protein through a 0.22 um filter and proceed to Lab 7b.**

10. **Label the lysate with the native protein and store it at -80°C until next time.**

Cell Lysis and Clarification Visual Protocol





Lab 7b – Protein Purification: Affinity Chromatography

Purpose: Purify your tagged chromoprotein from all of the other bacterial proteins in the lysate from Lab 7a.

Instructions:

1.) You will begin with a 50 mL tube with 4 mL of chitin resin in 20% EtOH

2.) Transfer the resin to the gravity column and let the beads settle for 1-2 minutes

3.) Open the drain valve, let the liquid level sink to the top of the bead bed, and close the drain valve.

4.) Slowly add 5 mL of Column Buffer A and then let it drain, but don't let the beads get dry.

a. Column Buffer A:
   i. Water 1.00 L
   ii. 20 mM Tris 2.42 g
   iii. 500 mM NaCl 29.22 g
   iv. 1 mM EDTA 0.29 g
   v. Titrate to **pH 8.0**
b. Repeat Step 4 until you have passed 50 mL of Column Buffer A through the bead bed.
c. Save a 1 mL sample of the column flow thru for Lab 8c.

5.) Add your tagged protein lysate to the column and mix thoroughly. Let the beads sit for 5 minutes.

a. Save a 1 mL sample of the crude lysate for Labs 8b/c.
b. If your lysate volume exceeds the column volume, drain the column and add more lysate.

6.) Open the drain valve and remove as much lysate as possible.

7.) **Slowly add 5 mL of Column Buffer B and then let it drain, but don't let the beads get dry**.

a. Column Buffer B:
   i. Water 1.00 L
   ii. 20 mM Tris 2.42 g
   iii. 1 mM EDTA 0.29 g
   iv. Titrate to **pH 6.2** (at T = 4°C)
b. Repeat Step 8 until you have passed 50 mL of Column Buffer B through the bead bed.

8.) Leave the beads at 4°C until next lab. During this time, the intein will cleave and release your protein.

9.) Take your beads out of the fridge and drain out as much liquid as possible.

10.) Add 1 mL of Column Buffer B to the beads and let that drain out into a separate tube.

a. Repeat Step 12 in separate tubes until the effluent is clear.

11.) Clean the beads (i.e. remove the CBD tag) by adding 30 mL of 0.3M NaOH. Incubate at RT for 30 min

a. Open the valve and save a 1 mL sample, then rinse the beads with another 70 mL of 0.3M NaOH.
b. Finish cleaning the column by rinsing with 50 mL water, followed by 50 mL Buffer A. Store at 4C.

12.) Concentrate the purified chromoprotein with a centrifugal filter (MWCO = 10 kDa, 3,000g for 10 min)

a. Record the total volume of sample you have: _____

13.) Measure the absorbance spectrum of the samples on the plate reader from 300-700 nm.

a. Add 100 uL of your sample to a 96 well plate (pathlength = 0.29 cm)
b. Record the absorbance from 300 to 700 nm in 2 nm intervals
c. If the absorbances are 0.1-1.0, save the spectrum. If not, dilute the sample and try again.
d. Keep track of the dilutions you use and correct the final absorbances as necessary.

Lab 8a – Protein Purification: Anion Exchange Chromatography

Purpose: Purify the native chromoprotein from all of the other bacterial proteins in the lysate from Lab 7a.

Instructions:

Before you begin, make sure a pressure alarm is set at ____ MPa and carefully inspect the unit.

1. **Check on your chitin beads – has the intein cleaved yet?**

2. **You will begin with a column of anion exchange resin stored in 1M NaOH, 2M NaCl.**

3. **Equilibrate the column with 20 mM Tris, pH ____ at ____ mL/min**
    a. Continue until the conductivity of the column effluent steadily reads _____ mS/cm.

4. **Load the sample onto the column using the sample loop (Dr. Elmer will do this step).**
    a. Save a 1 mL sample of the crude lysate for Lab 9.

5. **Wash the column with 20 mM Tris, pH ____ at ____ mL/min**
    a. Continue until the A_{280} of the effluent peaks and then decreases back to a minimum (____).
    b. Collect a 500 uL sample of the effluent when the A_{280} peaks.

6. **Begin running a gradient of NaCl from 0 mM to 1 M at a flow rate of ____ mL/min for ____ min.**
    a. During this gradient step, several impurities will elute and be detected as peaks in A_{280}.
    b. Collect samples of each impurity and collect your colorful protein as it elutes.
    c. Record where your protein eluted during the gradient on the chromatogram.

7. **Clean the column by rinsing it with 1M NaOH, 2M NaCl**
    a. Continue until the conductivity of the column effluent steadily reads ____ mS/cm.

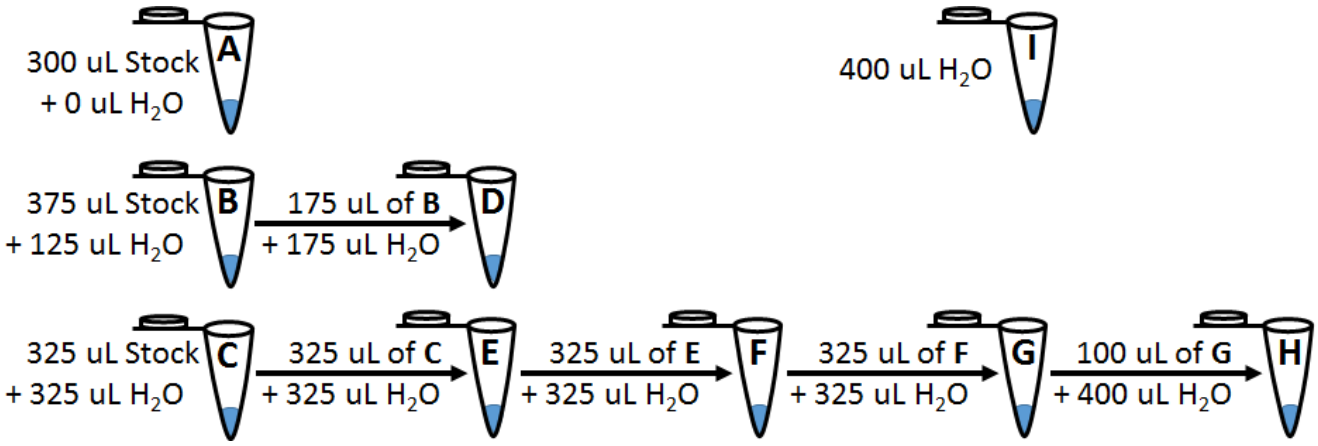
8. **Concentrate your chromoprotein with a centrifugal spin filter (10,000 kDa MWCO, 3,000g for 10 min).**
    a. Record the total volume of your sample: _____

9. **Measure the absorbance spectrum of the samples on the plate reader from 300-700 nm.**
    a. Add 100 uL of your sample to a 96 well plate (pathlength = 0.29 cm)
    b. Record the absorbance from 300 to 700 nm in 2 nm intervals
    c. If the absorbances are 0.1-1.0, save the spectrum. If not, dilute the sample and try again.
    d. Keep track of the dilutions you use and correct the final absorbances as necessary.

Lab 8b - BCA Protein Concentration Assay

1. **Begin by preparing BSA standards using the supplied 2 mg/mL BSA solution as follows:**

Vial	Water (uL)	Volume of BSA (uL)	Final BSA Conc. (ug/mL)
A	0	300 from Stock	2000
B	125	375 from Stock	1500
C	325	325 from Stock	1000
D	175	175 from Vial B	750
E	325	325 from Vial C	500
F	325	325 from Vial E	250
G	325	325 from Vial F	125
H	400	100 from Vial G	25
I	400	0	0



2. **Also prepare serial dilutions of your samples: 1x, 5x, 25x, and 125x.**

3. **Transfer 10 uL of the standard solutions and your samples to wells on a clear 96 well plate**

4. **Calculate how much working reagent (WR) you will need as follows:**
   - WR = (190 uL) x (2*samples+18)

5. **Prepare WR by mixing 50 parts solution A with 1 part solution B**
   - Ex: For 12.5 mL WR, mix: 12.5 mL solution A + 250 uL solution B

6. **Using the multichannel pipette, add 190 uL of WR to each well**

7. **Cover the plate and incubate at 37°C for 40 min**

8. **Measure the absorbance of each well at 562 nm**

9. **Prepare a standard curve using the standards (y-axis = Concentration, x-axis = absorbance)**
   - The data should be linear, such that you can fit it to y = mx + b
   - Once you calculate m and b, you can use this equation to estimate sample protein concentrations.

Lab 8c – Protein Characterization: PolyAcrylamide Gel Electrophoresis (PAGE)

Purpose: Determine the purity of the chromoprotein samples obtained by affinity and AEX chromatography.

---***Preparing a PAGE Gel***---

1. **Assemble the glass plates and test them for leaks with water. Fill the plates entirely and wait 5 min.**
2. **Meanwhile, mix the ingredients for the resolving gel listed below. Try not to introduce any bubbles!**
3. **Pipette the ~3 mL of resolving gel mixture into the plates. Add an additional 1 mL of water on top.**
4. **Let the gel solidify for 20-30 minutes – look for a clear line between the gel/water layers.**
5. **Prepare the stacking gel mixture and add it on top of the resolving gel layer. (remove water first!)**
6. **Add the well comb and submerge the gel in a tub of water – give it 20-30 minutes to solidify.**
7. **The finished gel can be stored in water for a few days.**

Test for Leaks

If the gel cast leaks:

-Check glass for chips
-Reassemble the cast
-Replace bottom gasket
-Use a different cast!

Cast Resolving Gel (12%)

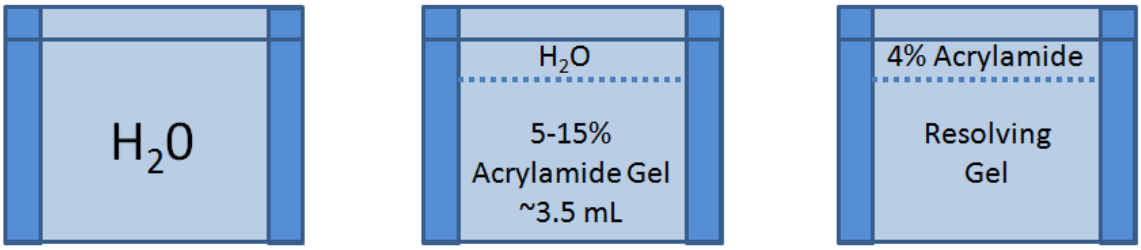
separates the proteins

1.7 mL	H_2O
4.0 mL	30% Acrylamide Mix
1.25 mL	Resolving Buffer
50 uL	SDS
25 uL	APS
2.5 uL	TEMED

Cast Stacking Gel (4%)

removes salts

1.83 mL	H_2O
0.39 mL	30% Acrylamide Mix
0.75 mL	Resolving Buffer
30 uL	SDS
15 uL	APS
3 uL	TEMED



---***Running a PAGE Gel***---

1. **Prepare samples by mixing them 1:1 with Laemmli buffer and incubating them at 95°C for 5 min.**
2. **Submerge the gel(s) in 1xPAGE running buffer and remove the well comb.**
3. **Load 10 uL of the following samples into each of the wells:**
   a. Lane 1 = MW Ladder
   b. Lane 2 = Native Chromoprotein Lysate
   c. Lane 3 = AEX-purified Chromoprotein
   d. Lane 4 = Tagged Chromoprotein Lysate
   e. Lane 5 = Chitin-purified Chromoprotein
   f. Lanes 6-10 = same as Lanes 1-5 but for the next group
4. **Run the gel(s) in two phases:**
   a. Salt separation – Since your AEX-purified sample may contain high amounts of salt, you must begin by running the gel at 40V for 15 minutes. This allows the salt to migrate ahead of the proteins in the stacking gel. If you skip this step, the salt will interfere with protein migration.
   b. Protein separation – Run the gel at 115V for 1 hour to separate the proteins in the resolving gel. Stop the electric current when the blue dye band reaches the bottom of the gel.
5. **Stain the gel overnight in Coomassie Blue stain solution on the rocker.**
6. **Destain the gel for ≥2 hrs in 20% EtOH/70% Acetic Acid and take a picture for your lab report.**

Additional Tips:

1.) Recipe for 10X PAGE running buffer:
   a. Water 1.0 L
   b. Tris base 60.4 gm
   c. Glycine 288 gm
   d. SDS 20 gm

2.) Prepare 1X running buffer by diluting with milliQ water. Running buffer may be reused until it becomes viscous (shake it and look for excessive bubble formation)

3.) APS is 100 mg/mL in water.

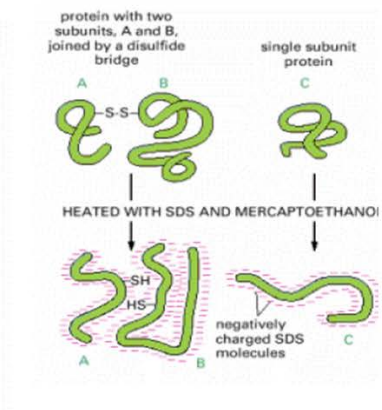
PAGE gel troubleshooting:

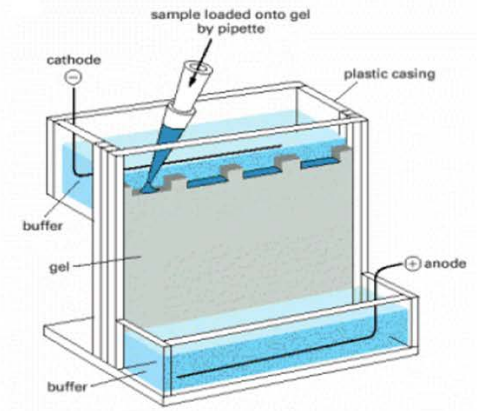
If the gel does not solidify:
- Make the gel solution quickly and try not to add bubbles to it when mixing
- Give the gel more time to solidify – sometimes it takes almost an hour
- Make fresh APS – it can go bad over time and some people recommend making fresh APS daily
- Degas the acrylamide solution to eliminate any dissolved oxygen

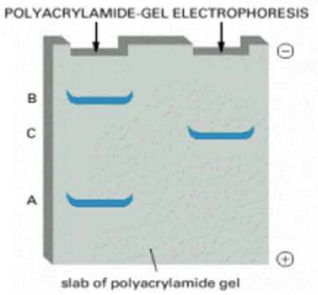
If bubbles form in the stacking gel:
- Make sure the comb you're using isn't broken – damaged combs allow oxygen into the gel
- Submerge the entire gel in water to prevent any air at all from contacting the gel
- This may cause the resolving gel to shrink a bit

If the bands do not stain properly:
- Run the gels again with a higher protein concentration
- Try staining your gel for a longer period of time (1-2 days)
- Destain the gel for a shorter period of time (1-2 hours)
- Try a new stain – use silver stain if a higher sensitivity is required

If the bands are not straight (e.g. wavy):
- Run the gel at a lower voltage for a longer time
- Make sure the gel is not overheating – put it in a cold room if necessary
- Desalt your samples if they have high salt concentrations
- Try denaturing the samples at 95C for longer periods of time 5-10 minutes







Lab 9a – Protein Structural Simulations: FoldIt

The goal of this exercise is to develop your intuition for protein structures by letting you play games with them.

Student Instructions:

1. Create a Foldit account
    a. http://fold.it/portal/user/register
    b. Choose a username that I can recognize (ex: JSmith or JohnSmith)

2. Join the group for our class:
    a. http://fold.it/portal/node/2003238
    b. Click "Request Membership" for the group or click "Join"

3. Important note: Please be courteous to all other FoldIt users. Your homework assignment only requires you to finish the training puzzles that are done individually, but more advanced puzzles may have chat features with other users. If you are disrespectful to other users, you may be banned and I will be unable to grade your assignment.

4. Your assignment is to complete all 32 of the training modules. You do not have to turn anything in – I will be able to track your progress once you have joined our group.
    a. Download the FoldIt program: http://fold.it/portal/ (download options on right side)
    b. We will complete some of the modules in class, but the rest are up to you.
    c. You will have to finish the remaining modules as homework by 3/27 at 1:30 pm (Exam 2)
    d. This assignment will count as 20% of your Exam 2 grade.

FoldIt Tips

1.) Always shake before you wiggle.
2.) Never be too proud to reset the structure.
3.) You can always press Ctrl + Z to undo your last action.
4.) Tips are always available in the upper right hand corner.
5.) Sometimes removing bands and doing one more wiggle boosts your score.

FoldIt Commands

- Shake – randomly moves individual sidechains
- Wiggle – randomly moves the backbone, side chains, or both
- Freeze – keeps one or more regions still while other regions are moved or wiggled
- Rubber bands – tells the program to keep two regions close together (shift, left click, drag)
- Cut – cuts the peptide bond (right click, Cut)
- Tweak – changes the orientation of alpha helices or beta strands (right click, tweak)
- Rebuild – randomly changes the conformation of a selected region (right click, rebuild)
- Mutate – randomly changes the selected sidechains to other sidechains
- Move – Left click on a segment. Right click the crosshairs to translate, left click arrows to rotate.

Lab 9b – Protein Structural Simulations: Homology Modeling in SwissPDB Viewer

Instructions for Exercise 1: Exploring the Structure of GFP

Begin by downloading the Swiss PDB Viewer:

1. Go to: http://spdbv.vital-it.ch/disclaim.html ….and agree to the terms and conditions
2. Select the appropriate download link for your operating system
3. Save the zip file and open it (Right click, select "Open with…", select Windows Explorer)
4. Copy and paste the folder (SPDBV_4.10_PC) out of the zip file
5. Open the SPDBV folder and double click on spdbv.exe to open the program

Import and Analyze the protein:

1. Begin by importing the PDB file for GFP (PDB ID: 1EMA)
   i. ***Option 1:*** Go to: http://www.rcsb.org/pdb/home/home.do, search for 1EMA in the top right, and download the gz file (not the FASTA or .txt files)
   ii. ***Option 2:*** In Deep View, click File → Import, then type in 1EMA in the PDB ID field and click "PDB file" under "Grab from server"

2. Use the **Tool Bar** to play around with how the protein structure is displayed.
   i. Adjust the display settings to make the protein easier to see.
      1. To thicken the bonds, click Display → Render in Solid 3D
      2. Change background color: Prefs → 3D Rendering → Background
      3. Change atom/side chain color: Color → CPK (or other choices…)
         4. For CPK: Note that C = white, O = red, N = blue, S = yellow

5. Go to the **Control Panel** to select specific amino acids and change display properties
   a. ***Can you find the fluorophore? Which residues are interact with it?***
      6. The fluorophore is hidden inside the beta barrel, but we can expose it by hiding the surrounding residues.
      7. Click Wind → Control Panel
      8. Scroll down until you find the fluorophore, which is labeled CRO66
      9. Click on CRO66, press "Alt" and "1", then click the "-" in the show column
         a. Now only the chromophore is visible
      10. Click on CRO66 again, then click Select → Neighbors of Selected Residues
      11. Click: Add to Selection groups that are within 5.000 A of the picked, then OK
         a. Now all of the residues that interact with the chromophore are visible
      12. If you wanted to mutate GFP to change its fluorescence properties, these residues would be a good place to start

3. Display a **Ramachandran Plot** to check protein stability and stresses.
   i. Are there any seemingly unstable residues in the structure? What are they?
      1. Click anywhere in the Control Panel and hit Ctrl + A
      2. Click Wind → Ramachandran Plot (or Ctrl + R)

Exercise 2: Structural Homology Modeling of a Protein with an Unknown Structure

In this exercise, we will predict the structure of one of the chromoproteins – aeBlue. No 3D structures are available for aeBlue, so we will have to use homology modeling to estimate its structure based on its amino sequence (shown below):

MASLVKKDMCIKMTMEGTVNGHHFKCVGEGEGKPFEGTQVEKIRITEGGPLPFAYDILAP
CCMYGSKTFIKHVSGIPDYFKESFPEGFTWERTQIFEDGGYLTIHQDTSLQGNNFIFKVN
VIGANFPANGPVMQKKTAGWEPCVEMLYPRDGVLCGQSLMALKCTDGNHLTSHLRTTYRS
RKPSNAVNMPEFHFGDHRIEILKAEQGKFYEQYESAVARYCEAAPSKLGHH

Predict the structure of this protein with SWISS-MODEL:

1. Go to: http://swissmodel.expasy.org/ . Click on "Start Modeling"
2. Copy the sequence above into the blank field and click "Search for Templates"
    a. This search may take a while….you may want to skip ahead in the video.
3. Examine the templates that appear – ***what do the matches have in common?***
4. Click on the Templates Tab and select the first entry (95.59% identity) and any other one
5. Click on the Sequence Similarity tab to see a visualization of how similar the templates are to the input sequence. Our sequence is a red dot and the homolog we selected is a large blue circle.
    a. You want a dot that is as close to the red dot as possible
6. Click on the Alignment tab to see how well the sequences align – gray letters are variations.
7. Click the "Build Models" button….this may take a few minutes
8. A lot of information will appear…here are the highlights:
    a. GMQE is a structural quality score – it estimates the accuracy of the structure and should be as high (close to 1) as possible.
    b. Model-Template Alignment shows the structural features that have been mimicked from the homolog onto our sequence (alpha helices, beta sheets, etc.)
9. Save the alignment files by clicking the save button at the top of the page
10. Extract the PDB file and open it with Swiss PDB viewer
11. Voila! Now we have a pretty good structure and we didn't even have to do crystallography.
12. Validate the model by checking the Ramachandran plot – ***Are there any stray dots?***
13. Check for clashes: Menu → Select → Residues Making Clashes - ***Are there any clashes?***
14. Check for unsatisfied H-Bonds: Menu → Select → Sidechains Lacking proper H-Bonds

Compare the predicted structure of your protein to the structure of the homolog:

1. Go back to the Templates tab and select the homolog you used to obtain this structure
2. Click the red More tab and click "Selected Templates to Deep View"
3. Extract the PDB file and open it in Swiss PDB viewer (along with your modeled structure)
4. Highlight differences in the models: Color → Layer
5. Can you spot any significant differences between the homolog and predicted structure?

Assignment: You will be required to provide a structure for your chromoprotein in Lab Report 2. If the structure already exists, simply use SwissPDB to view it. However, if the structure is not available, you will need to follow the protocol above to predict the structure with homology modeling.

Guidelines for Lab Report 2

Synopsis

This report will be the same format as Lab Report 1, but with a focus on recombinant protein expression in *E. coli.*

The first two pages must include the following sections:

- **Title/Authors** – Include a descriptive and concise title, along with a list your group members
- **Abstract** – A summary that consists of 1-2 sentences for each of the following sections (≤100 words).
- **Introduction** – Describe the chitin binding domain, intein, and how they are both used to purify proteins. Also include advantages and disadvantages associated with the CBD and intein.
  - See figure 1 below - refer to this figure in your Introduction
- **Methods** – Explain the variables you tested to optimize protein yields (gene structure and purification method) and their importance.
  - DO NOT rewrite the protocols.
- **Results** – Describe the results obtained in Labs 6-9.
  - **Lab 6**: What fermentation conditions were used? Why?
  - **Lab 7**: Was one lysis method more efficient than the other?
  - **Labs 7/8**: Compare and contrast the following characteristics of the two purification methods:
    - Which one was faster? ….cheaper?
    - What was the purity obtained with each method? (PAGE)
    - What was the total protein and chromoprotein yield obtained with each method? (BCA)
  - **Lab 8:** Compare the absorbance spectra of your chromoprotein and a chromoprotein from another group – use these spectra to explain the colors of the different proteins.
  - **Labs 9**: Describe how you obtained a 3D structure for your chromoprotein
- **Conclusion** – Summarize your findings – indicate which purification method you prefer and why.

The last 1-2 pages will contain figures and citations. For this report, you must include the following figures:

- **Figure 1: Schematic of Chitin Binding Domain – Intein – Chromoprotein Fusion**
  - Make your own figure. DO NOT copy my illustrations from the notes.
  - Create and label an illustration that shows how the CBD, intein, and chromoprotein are linked.
- **Figure 2: Sample Purity (PAGE, Lab 8)**
  - Completely annotate the gel – label the ladder, each band, and the individual lanes
  - Indicate the observed MW of your chromoprotein
- **Figure 3: Chromoprotein Yield (Lab 8)**
  - Use a bar graph or table to display the total protein and chromoprotein yields obtained from each purification method (AEX and affinity).
- **Figure 4: UV-Visible Absorbance Spectra (Lab 8)**
  - Label your axes and the two absorbance spectra with the name of each chromoprotein.
- **Figure 5: 3D Structure of Chromoprotein**
  - Include a 3D structure for your chromoprotein from SwissPDBviewer. Label the N and C termini.
- **Include your references in the remaining space.**
- **Note:** Include captions for each figure. Each caption should include enough information for a well-educated/trained reader to understand its significance, but it should also be succinct.

Note: You do not need to take up the entire 4 pages. Just be sure to address all of the prompts listed above.

Your report will also be graded for spelling, grammar, and quality (e.g. logical and smooth flow, good organization, etc.). I take it as a personal offense when a paper is not spell checked prior to submission!

Lab Report 2 Rubric

Abstract			
Overall Quality: Briefly summarize the entire report.	1 *Incorrect Info*	2 *Sections Missing*	3 *Complete Summary*
Introduction			
Describe the CBD and intein	1	2	3
Advantages of the fusion tag	1	2	3
Disadvantages of fusion tag	1	2	3
Methods			
Which variables were tested?	1	2	3
Why are they important?	1	2	3
Results			
What fermentation conditions?	1	2	3
Compare lysis methods.	1	2	3
Which purification method was faster?	1	2	3
….cheaper?	1	2	3
Which method gave higher purity?	1	2	3
….higher yields?	1	2	3
Compare spectra of chromoproteins	1	2	3
Describe how you modeled the protein.	1	2	3
For all prompts above, 3 = correctly addressed, 2 = partially correct, 1 = incorrect, 0 = missing			
Conclusion			
Overall Quality: Draw conclusions based on the results	1 *Missing parts*	2 *Partially incorrect*	3 *Correct conclusions*
Figures			
1: Schematic of CBD-Intein-chromopro.	1	2	3
2: Sample Purity	1	2	3
3: Protein Yield	1	2	3
4: UV-Vis Spectra	1	2	3
5 – 3D Structure of Protein	1	2	3
3 = Perfect resolution/annotation/caption, 2 = labels or caption missing, 1 = no labels/caption			
References			
Overall Quality: Appropriate references to support text	1 *1 reference*	2 *2 references*	3 *≥3 references*

Spelling and Grammar

10 = no spelling or grammar errors, -1 pt for every error

Negative pts: -	0	1	2	3	4	5	6	7	8	9	10

Organization and Flow

10 = Logical order and proper segues between sections, -1 pt for every instructor comment

Negative pts: -	0	1	2	3	4	5	6	7	8	9	10

Total = /83

The instructor reserves the right to modify this rubric at any time.

Module 3: Mammalian Cell Culture Techniques

Introduction

Unfortunately, while making recombinant proteins in E. coli is relatively easy, it is not always feasible for some proteins. For example, some pharmaceutical proteins (e.g. antibodies) require complex post-translational modifications that can only be performed in eukaryotic cells. In addition, bacterial cell lysate also contains toxic substances (e.g. endotoxin) that can be extremely harmful if not properly removed. Consequently, many people have transitioned to using insect, animal, or even human cells to produce proteins that are as safe and effective as possible. The purpose of this module is to teach you all of the various techniques used to culture animal or human cells in the laboratory. Once you know how to culture the cells, we will then use CRISPR/Cas9 to integrate the gene for a red fluorescent protein (mCherry) into their genome for high-level recombinant protein expression. An overview of each lab is shown below:

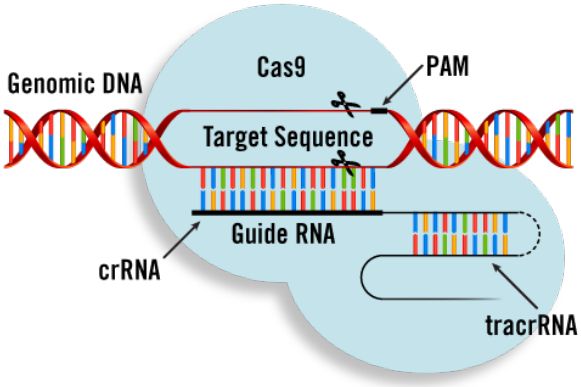
Lab 10: This lab is an introduction to basic animal cell culture techniques. You will learn how to start animal cell cultures from cryogenic stocks, maintain those cultures for several weeks, and freeze them again for storage.

Lab 11: Adding a gene to a eukaryotic genome actually requires two different plasmids – one that expresses a guide RNA and Cas9 to cut the genome, and a second plasmid that inserts your gene into the cut site. In this lab, you will use a novel technique called "Oligo Annealing Cloning" to prepare the Cas9/gRNA plasmid.

Lab 12: In this lab, you will transfect the cells with your plasmids to insert the gene into their genome. Positive transfectants (cells that received the gene) will then be selected with puromycin.

Lab 13: If the transfection was a success, then the cells should be fluorescent. You will use fluorescent microscopy to look for GFP expression (indicates that Cas9 successfully expressed) and mCherry expression (indicates successful delivery of the knock in plasmid). We will also use a fluorescent dye (Hoechst 33342) to stain the nucleus and select for positively integrated cells with puromycin.

Lab 14: The focus of this last lab will be on large scale animal cell culture in rocking bioreactors. This is the type of technology we would use to grow up our transfected cells to produce large amounts of mCherry.



Lab 10 – Basic Animal Cell Culture Techniques

Purpose: Today you will learn all of the basic skills used to grow animal/human cells in the lab, including:

1.) Aseptically prepare media and other cell culture solutions
2.) Start a fresh cell culture from a stock of cryogenically frozen cells
3.) Maintain a cell culture and/or transfer cells between flasks
4.) Freeze excess cells for future use

Instructions:

---------- ***Preparing Phosphate Buffered Saline (PBS)*** ----------

1. **Mix the following ingredients in a 500 mL bottle:**
   a. MilliQ water 500 mL
   b. NaCl 4.00 g
   c. KCl 0.10 g
   d. Na_2HPO_4 0.72 g
   e. KH_2PO_4 0.12 g

2. **The pH should already be ~7.4, but check it with a pH meter to be sure and titrate if necessary**

3. **You may make a 10x PBS solution, but keep in mind that these stocks are prone to contamination**

4. **Sterilize PBS with an autoclave (121°C for 20 minutes) or 0.2 μm bottle filter**

5. **PBS may be stored at room temperature or in the fridge (Note: PBS may crystallize at cold temps)**

---------- ***Preparing RPMI Media*** ----------

1. **Prepare RPMI media by mixing the following reagents:**
   a. Water 500 mL
   b. RPMI powder 5.20 g
   c. Sodium bicarbonate 1.85 g
   d. HEPES 2.98 g
   e. Titrate to pH 7.4

2. **Thaw aliquots of fetal bovine serum (50 mL) and penicillin/streptomycin (5 mL) stocks**

3. **Prepare supplemented RPMI media by adding 10% FBS and 1% Pen/Strep**

4. **Filter the media solution using a 0.2 μm bottle filter**

5. **RPMI media may be stored in the fridge until it expires (about 6 months or less)**

---------- ***Thawing Animal Cells*** ----------

1. **Remove the cells from the -80°C freezer or liquid nitrogen dewar**

2. **Immediately transfer the cells to the 37°C water bath**

3. **While the cells are thawing, pipette 9 mL of warm media into a T75 flask**

4. **Transfer the thawed cells to the T-75 flask and label it (group name, cell line, date, & passage #)**

5. **Incubate the plate at 37°C for 2-7 days (check the flask each day to monitor cell growth)**

---------- ***Resuspending Adherent Cells*** ----------

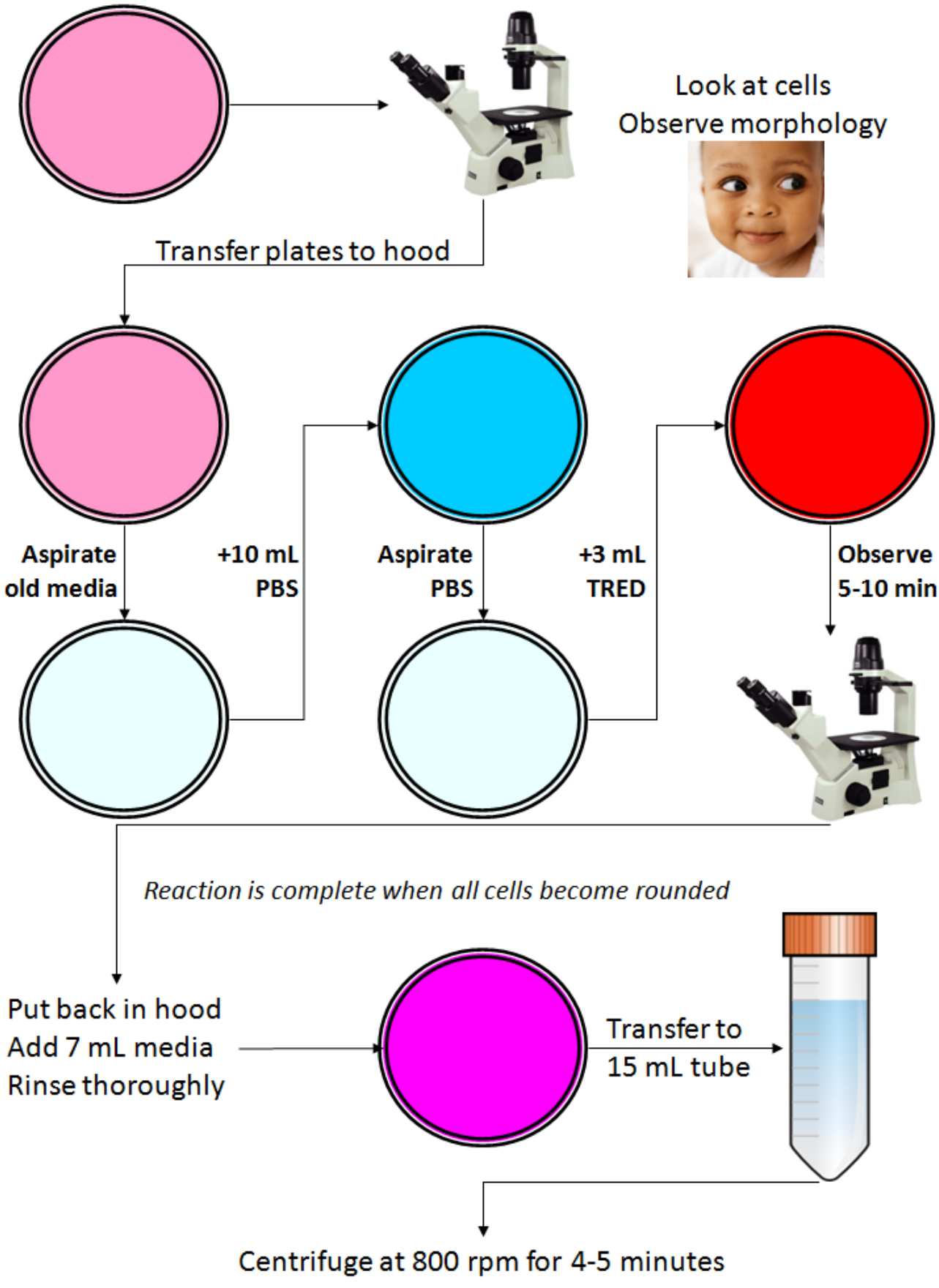
1. **Look at your cells under the microscope. What do they look like? Estimate their confluency.**
2. **Transfer the plate into to the hood. DO NOT spray the cell dish with EtOH**
3. **Aspirate the old media from the flask**
4. **Add 10 mL of sterile PBS onto the flask and swirl it to rinse the cells**
5. **Aspirate the PBS from the flask**
6. **Add 3 mL of TRED to the flask and swirl until evenly coated**
7. **Put the flask in the incubator at 37°C for 5-10 min**
    a. Watch the cell morphology change as the TRED detaches them from the plate
8. **Once the cells have detached, tilt the flask to collect the cells in one corner of the flask**
9. **Add an additional 7 mL of media to slough off the rest of the cells, while keeping the flask tilted.**
10. **Transfer the cell suspension to a 15 mL tube**
11. **Balance and centrifuge the tube(s) at 800 rpm for 4 min**
    a. Meanwhile, begin cleaning the hemocytometer with 70% ethanol. See steps 1-3 below.
12. **Transfer the tube back to the hood and aspirate as much of the media as possible**
13. **Add 10 mL of media to the cell pellet and resuspend the cells by carefully triturating**

---------- ***Counting Cells*** ----------

1. **Spray the hemocytometer with 75% EtOH**
2. **Dry it off carefully with a KimWipe**
3. **Let the hemocytometer air-dry for at least 1 minute**
4. **Put the cover slip on the hemocytometer**
5. **Transfer 10 µL of <u>recently</u> resuspended cells onto the hemocytometer**
6. **Count 4 squares in the hemocytometer field**
    a. There should be ~10-100 cells per square
    b. If there are more, dilute. If there are fewer, centrifuge and resuspend with less media
7. **Calculate the average number of cells in each square (N)**

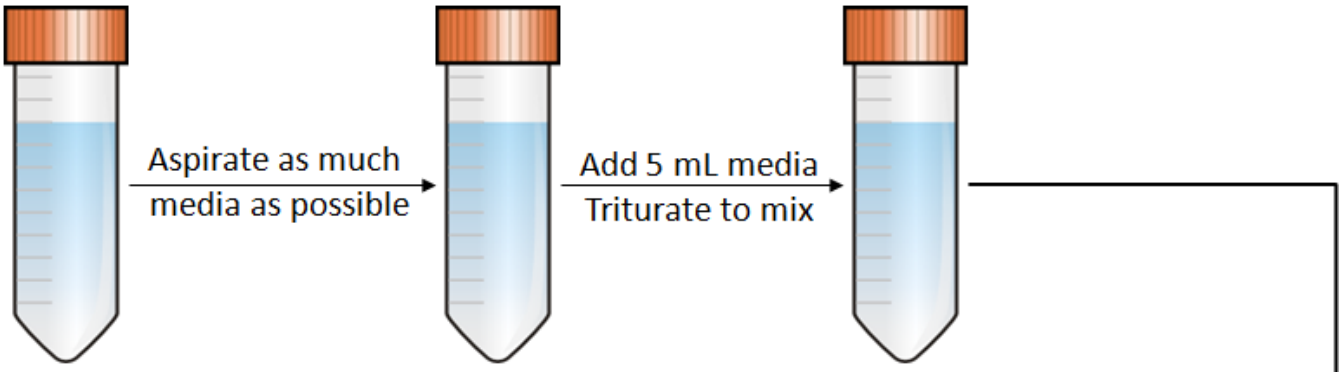
$$N_{ave} = \left(\frac{N_1 + N_2 + N_3 + N_4}{4}\right) x \frac{10^4}{mL}$$

Visual Protocol: Resuspending Cells

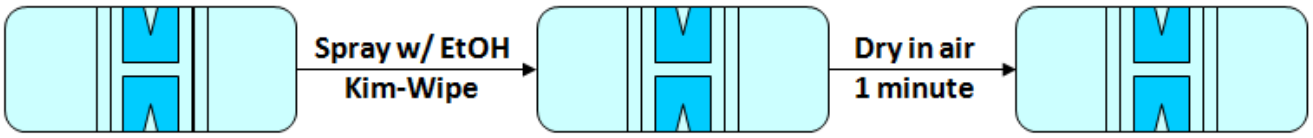


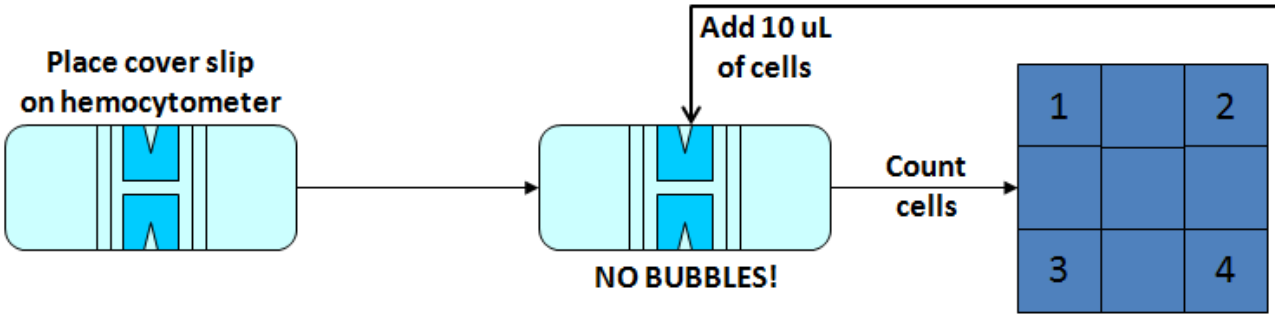
Visual Protocol Passaging Cells:

One person resuspends the cells:

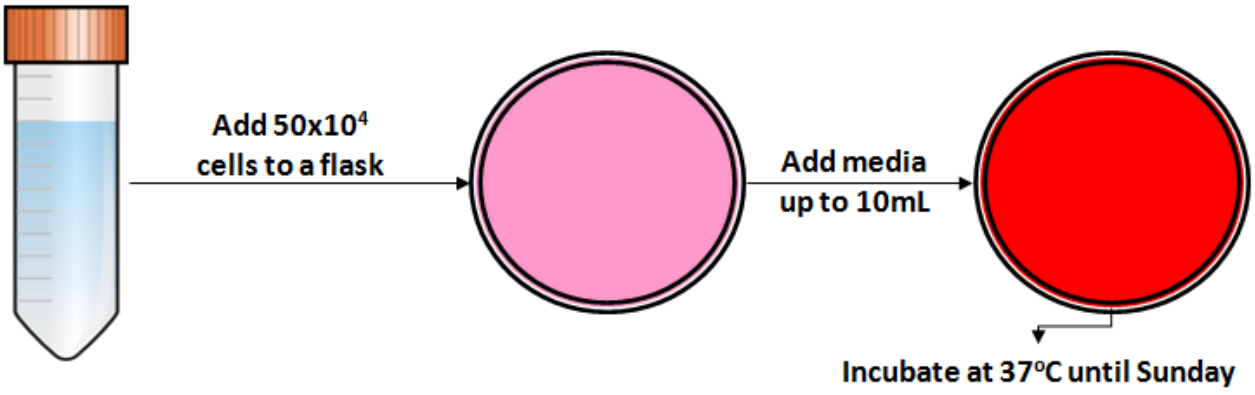


While another cleans off the Hemocytometer:





$$Cells = \frac{N_{ave} x 10^4}{mL} = \left(\frac{N_1 + N_2 + N_3 + N_4 + N_5}{5} \right) \frac{10^4}{mL}$$



---------- *Passaging Cells to a New Flask* ----------

8. **Calculate the volume of cell suspension (V_{cell}) needed to transfer 50 x 10^4 cells to a new T-75 flask.**

9. **Briefly resuspend the cells by trituration – many of them will have settled during counting**

10. **Transfer "V_{cell}" of cell suspension to a new T-75 flask**

11. **Label the flasks with group name, cell line, and passage number**

12. **Incubate the plates at 37°C until the next lab period**

---------- *Freezing Cells for Cryogenic Storage* ----------

1.) **We will save any unused cells for next year by freezing them with DMSO**

2.) **Dilute or concentrate your cell suspension to the appropriate cell concentration for freezing/thawing**
   a. It is best to have 100-200 x 10^4 cells/mL, but a little bit less may work sometimes
   b. If your cell concentration is higher than this range, dilute it with media to make more stocks
   c. If your cell concentration is too low, centrifuge the cells and resuspend in a lower volume

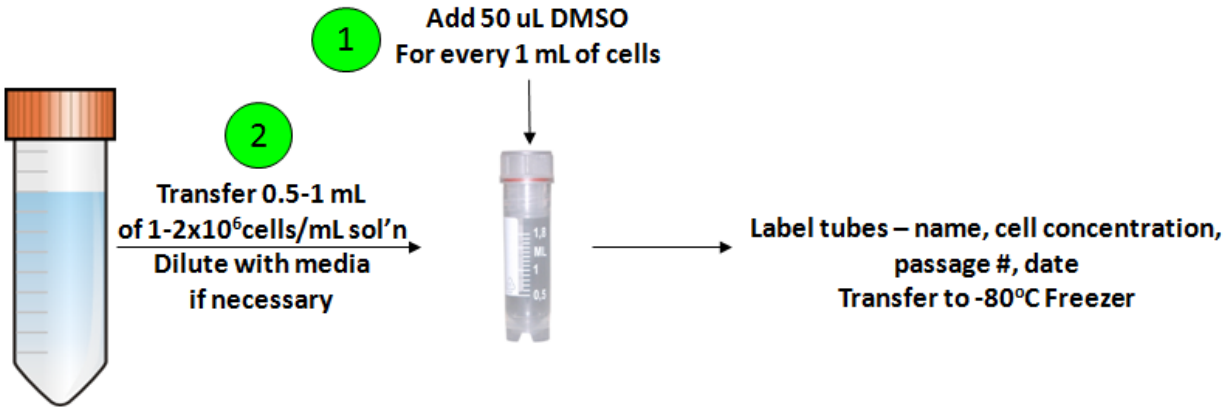
3.) **Add 100 uL of DMSO to a new cryovial (final concentration of DMSO will be 10%)**

4.) **Transfer 900 uL of cell suspension to the cryovial and mix well by pipetting**

5.) **Label the tube with Cell Line name, concentration, date, and passage number**

6.) **Incubate the tubes at RT for 15 min, then transfer the cells into the -80°C freezer**
   a. The incubation gives the DMSO enough time to enter the cells.
   b. Ideally, cells should be cooled at a rate of -1°C/min

Freezing Cells with DMSO:



Lab 11 – Oligo Annealing Cloning

Purpose: Insert a new gRNA sequence into the Cas9 knock out plasmid that tells Cas9 where to cut the genome.

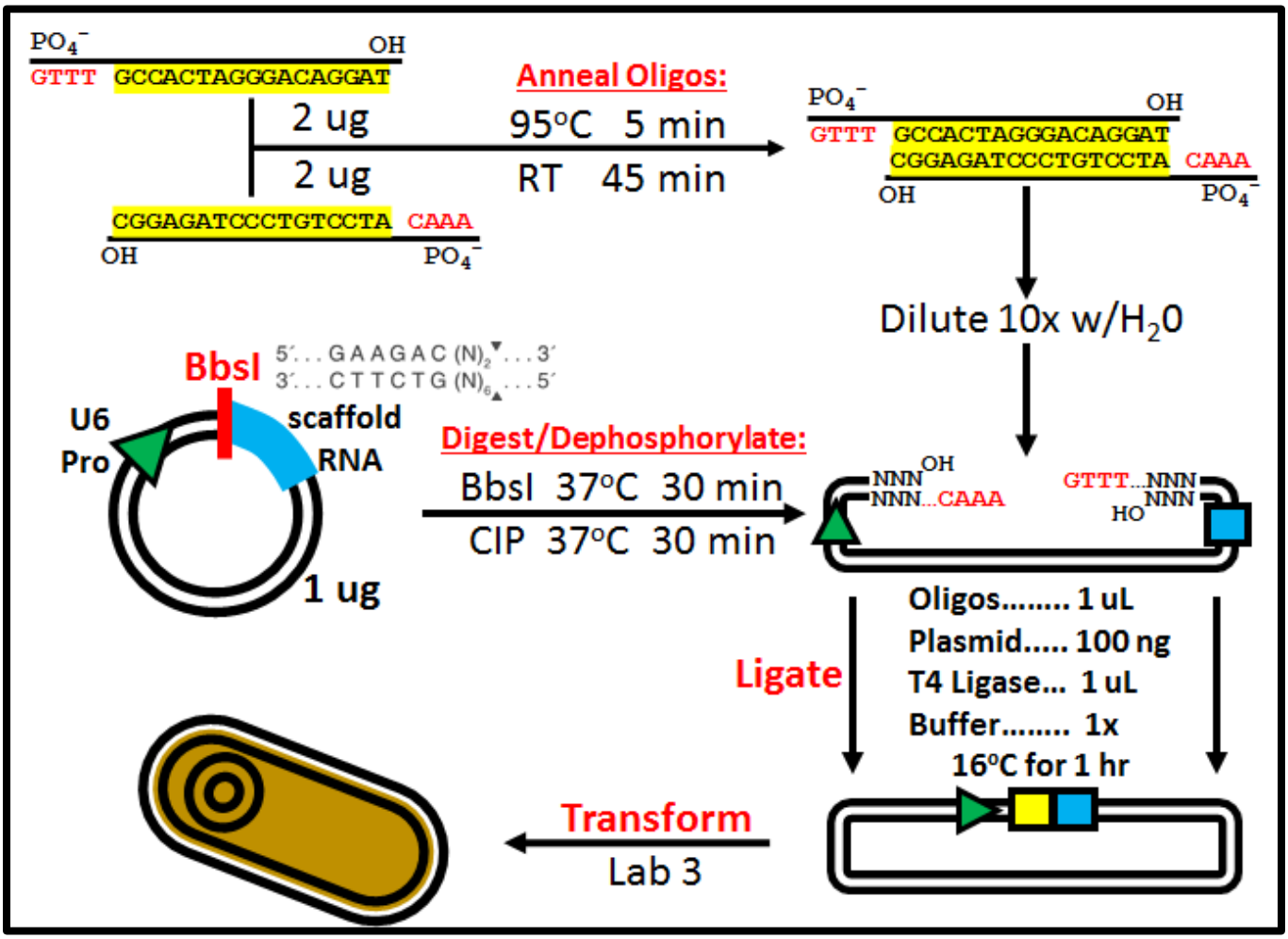
Introduction:

The Cas9 knock out plasmid contains the following genetic elements:

1) Bacterial replication elements: beta lactamase gene (ampicillin resistance) and origin of replication
2) Cas9-GFP Cassette: CAG promoter driving stoichiometric expression of Cas9 and GFP with a 2A linker
3) gRNA Cassette: U6 promoter (for RNA Pol III) driving transcription of gRNA + scaffoldRNA for Cas9

All of these elements remain constant between Cas9 experiments, except the gRNA sequence, which determines where Cas9 will cut the genome. The gRNA sequence (**17**-20 bp) can be inserted by cutting the plasmid with the restriction enzyme BbsI. The linearized plasmid is then left with sticky ends with 4 bp overhangs that can be utilized to insert the gRNA in a sequence and orientation-specific manner (see virtual protocol). Since gRNA sequences are so short, they can easily be prepared from short, synthetic, complimentary oligonucleotides using a technique called oligo annealing. In this technique, two complimentary oligos are mixed in equimolar concentrations, heated to 95°C, then cooled to room temperature to form a duplex containing the gRNA sequence flanked by sticking ends that are complimentary to the BbsI cut site. Mixing the annealed oligos and linearized plasmid DNA then allows them to hydrogen bond, while a DNA ligase seals the phosphodiester backbone to produce the desired plasmid. The plasmid is then transformed into bacteria for storage and replication.

Note: Since we are using one restriction enzyme, the plasmid must be dephosphorylated to prevent self-ligation.



Instructions:

1) The instructor has already prepared the BbsI-digested linearized plasmid for you:

……Plasmid……NNNNNN – 3′ OH 5′ w/o PO_4^- - **GTTT** NNNNNN……Plasmid……
……Plasmid……NNNNNN **CAAA** - 5′ w/o PO_4^- 3′OH - NNNNNN……Plasmid……

Reagents	[Stock]	Required Amount	Volumes
Plasmid DNA		**1 ug**	*1 µL*
HF Buffer	**10x**	**1x**	*µL*
Water	*Add up to a final total volume of 50 µL*		*µL*
BbsI-HF Enzyme	**20 U/uL**	**1 U**	*1 µL*
		Total:	*20 µL*

a. Mix the reagents well, then incubate at 37°C for 30 min
b. Add 1 uL of Calf Intestinal Phosphatase (CIP) to remove 5′ PO_4^-, then incubate at 37°C for 30 min
c. Confirm digestion on a 1% agarose gel, then extract the **linearized dephosphorylated** plasmid

2) You will begin by mixing the **phosphorylated** oligos together to form the duplex:

5′PO_4^- – **GTTT** **GCCACTAGGGACAGGAT** – 3′OH
3′OH – **CGGAGATCCCTGTCCTA** **CAAA** – 5′PO_4^-

Reagents	[Stock]	Required Amount	Volumes
Phosphorylated Sense Oligo	**100 ng/µL**	**2 µg**	*µL*
Phosphorylated Antisense Oligo	**100 ng/µL**	**2 µg**	*µL*
		Total:	*µL*

a. Heat the mixture to 95C for 5 minutes, then slowly cool to room temperature for 45 minutes
b. Dilute the annealed oligos 10-fold with water (e.g. 40 uL oligos + 360 uL water)

3) Mix the annealed oligos, linearized plasmid backbone, and T4 DNA ligase to seal the fragments together.

……Plasmid……NNNNNN **GTTT** **GCCACTAGGGACAGGAT** **GTTT** NNNNNN……Plasmid……
……Plasmid……NNNNNN **CAAA** **CGGAGATCCCTGTCCTA** **CAAA** NNNNNN……Plasmid……

Reagents	[Stock]	Required Amount	Volumes
Annealed Oligos	**---**	**---**	*1 µL*
Digested Plasmid		**100 ng**	*µL*
T4 Ligase Buffer	**2x**	**1x**	*µL*
Water	*Add up to a final total volume of 20 µL*		*µL*
T4 DNA Ligase	*Concentrations not provided*		*1 µL*
		Total:	*20 µL*

a. Incubate the ligation reaction at 16°C for 1 hour

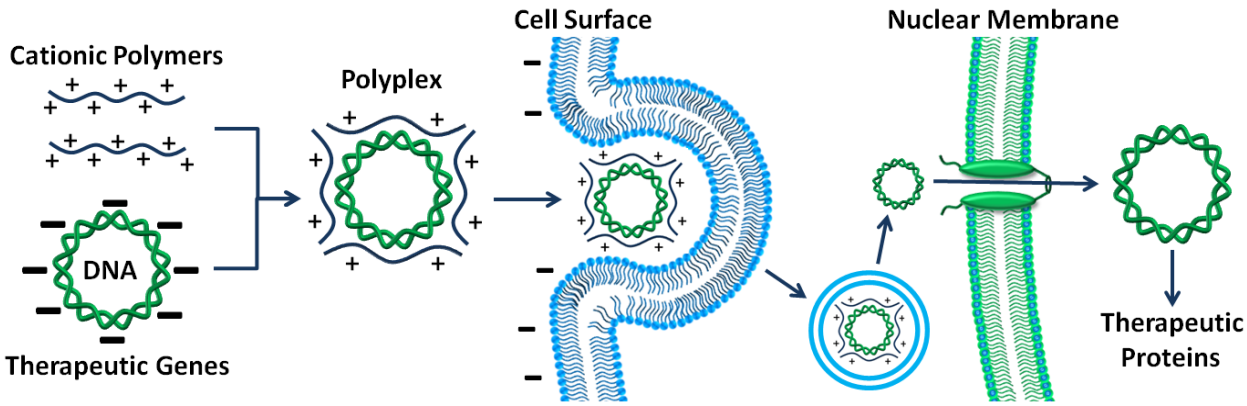
4) Transform DH5α *E. coli* with 5 uL of the ligation reaction mixture as described in Lab 3.

Lab 12 – Animal Cell Transfection

Purpose: Deliver the knock out and knock in plasmids to nuclei of the host cells to facilitate genomic integration

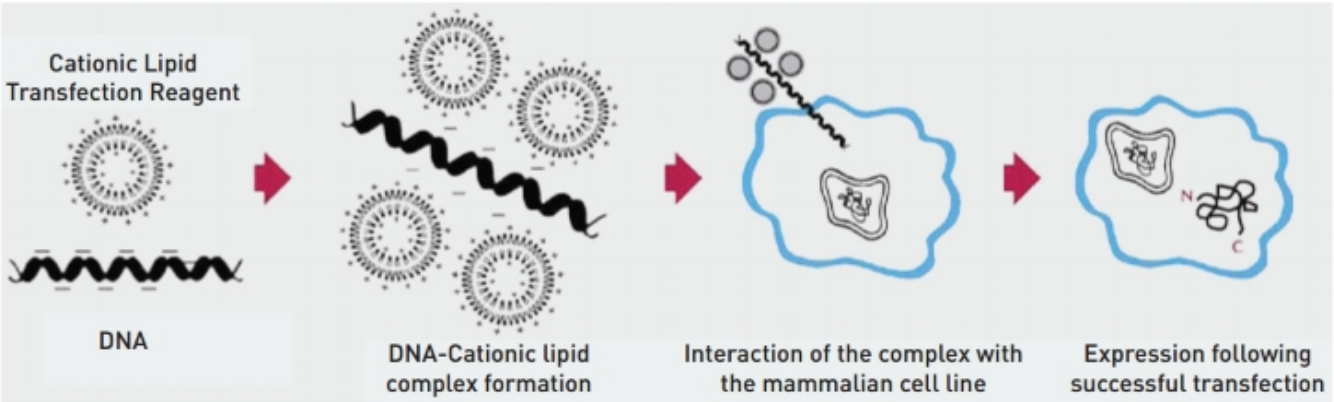
Introduction:

While transformation of *E. coli* is fairly easy and straightforward, transfection of mammalian cells is much more challenging because animal cells have several physical barriers (e.g. cellular, endosomal, and nuclear membrane) that block the entry of foreign DNA. In addition, cells also express a variety of receptors and enzymes that detect foreign DNA and then respond by suppressing expression of the foreign genes. However, development of effective transfection methods could potentially provide a cure for hundreds of genetic diseases (e.g. cancer).



Many different delivery vehicles for DNA have been developed, including viruses, polymers and lipids. While viruses (e.g. adenovirus) are definitely the most efficient, they are also expensive and require more time to prepare. In contrast, cationic polymers (e.g. polyethyleneimine, PEI) are much cheaper, but they are far less effective and can be quite toxic. Fortunately, cationic lipids represent a good compromise between these two options – they are affordable, provide a moderately high efficiency, and have minimal toxicity.

In this lab, you will use the cationic lipid Lipofectamine 3000 from Thermo Fisher to simultaneously deliver the knock in and knock out plasmids. Due to its ampiphillic nature, Lipofectamine forms positively charged liposomes that resemble the phospholipid bilayer of cells. Those liposomes then associate with negatively charged DNA to form a "lipoplex" with a net positive charge that can easily bind to the negatively charged cell membrane. At that point, the liposomes may directly fuse to the cell membrane or be taken up by endosomes and fuse to the endosomal membrane. Either way, the DNA cargo is released into the cytoplasm and then must wait for mitosis to occur before it can enter the nucleus.



https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/transfection-basics/gene-delivery-technologies/cationic-lipid-mediated-delivery/how-cationic-lipid-mediated-transfection-works.html

1.) Begin by preparing a master mix of DNA solution. Mix well.

Reagents	[Stock]	Required Amount	Volumes
Knock Out Plasmid		**750 ng**	*μL*
Knock In Plasmid		**750 ng**	*μL*
Opti-MEM Medium	*Add to a final total volume of 100 μL*		*μL*
P3000 Reagent	*Add 2 uL per 1 ug of DNA*		*μL*
		Total:	*75 μL*

2.) Prepare three different solutions of Lipofectamine (0x, 1x, and 2x). Mix well.

Reagents	0x Control	1x Solution	2x Solution
Opti-MEM Medium	*25 μL*	*25 μL*	*25 μL*
Lipofectamine 3000	*0 μL*	*1.5 μL*	*3 μL*

3.) Add 25 uL of the diluted DNA solution to each lipofectamine tube (0x, 1x, 2x). Mix well.

4.) Incubate the tube at room temperature for 15 minutes.

5.) Transfer the full 50 uL from each tube into their own separate wells on a 24 well plate.
   a. One of these wells will be used for nuclear staining with Hoechst 33342 in Lab 13.

6.) Incubate the transfected cells at 37C for 2-7 days, then test for GFP and mCherry expression.
   a. No media change is necessary when using Lipofectamine, but is necessary for PEI.

Lab 13 –Fluorescent Microscopy and Cell Selection

Purpose: Observe positively transfected fluorescent cells and then select for them with puromycin.

Analysis of Transfected Cells

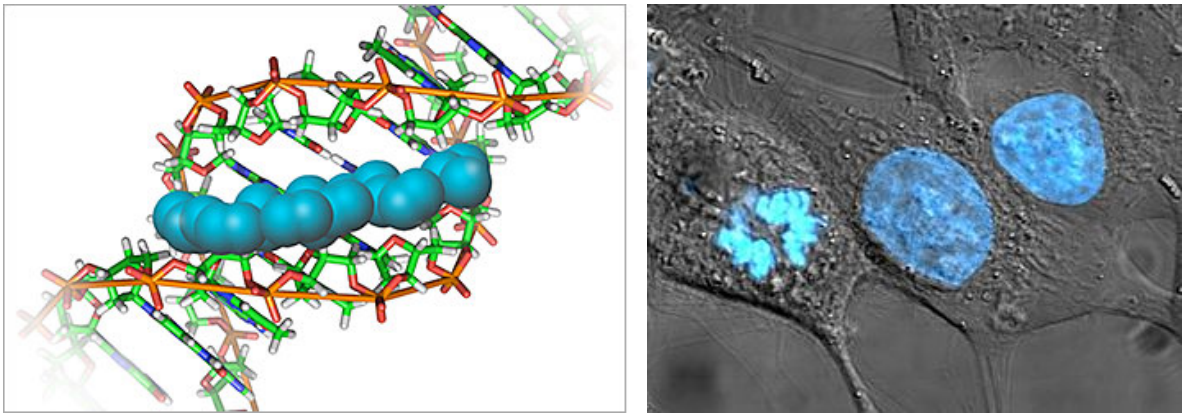
By now (t = 7 days post transfection), the cells you transfected last week should have had plenty of time to take up the plasmid and start expressing the genes (Cas9, GFP, mCherry, and the Puromycin resistance gene). Today, you will check the cells for transgene expression/fluorescence and then select for the positive transfectants by adding puromycin. Specifically, you will be looking for the following types of signals:

- **Green fluorescence** – indicates GFP expression, which can only occur if Cas9 was also expressed
  - Recall that Cas9 and GFP were linked in a single gene by the self-cleaving 2A peptide
- **Red fluorescence** – indicates delivery of the knock-in plasmid, but does not confirm integration
  - The promoter on the knock-in plasmid will express mCherry before and after integration

Consequently, your goal is to find at least a few cells that fluoresce both green and red (indicating that both plasmids were successfully delivered and Cas9 was definitely expressed). The only remaining question is whether or not the mCherry gene was successfully integrated into the AAVS1 site in the genome. We address this question by adding puromycin, which will kill any cells that did not integrate the puromycin resistance gene (and the mCherry cassette), since that gene only expresses when integrated into the genome (by taking advantage of an existing host promoter).

Nuclear Staining with Hoescht

As an additional exercise, we will also stain our cells with the fluorescent dye Hoechst 33342. Like EtBr, this dye intercalates DNA and gives a strong fluorescent signal (but it emits blue light). This DNA-dependent fluorescence allows the dye to selectively mark the nuclei of cells. In addition, it can also be used to detect mitosis in cells, since the DNA condenses into visible chromatin structures during cell division.



Left: Hoescht 33342 intercalating DNA. Right: Cell nuclei fluorescing after staining with Hoescht 33342. Note the differences in fluorescence - the cell on the left is undergoing mitosis while the others are in G phase.

WARNING: The fluorescent light source for the microscope generates UV light. The system is built to prevent UV light from escaping, but you must wear safety glasses that block UV when not looking in the eyepieces.

---------- ***Hoechst 33342 Staining Prep*** ----------

1.) You will only stain one of the wells (0x Lipofectamine) from Lab 12. DO NOT stain all of them!
2.) Remove the media from the cells and replace it with 150 uL of PBS
3.) Add 1 drop of the NucBlue Live Ready Probes Reagent (Hoechst 33342 dye) to the well.
    a. Immediately cover the plate, since the reagent is light sensitive
4.) Let the plate sit for a minute at room temperature
5.) Bring the plate to the fluorescent microscope in Dr. Comolli's lab

---------- ***Fluorescent Microscopy (GFP, mCherry, and Hoechst)*** ----------

7.) Prepare the microscope by turning everything on
    a. Turn on the computer, modem, and white light source
    b. Only turn on the fluorescent light source if you will be doing fluorescence.
        i. The bulb has a limited life time and must warm up for 5 min and cool down for 30 min
    c. Open the Nikon NIS-Elements software

8.) Begin by focusing the cells with the white light using the eyepiece (rotate right side knob to "EYE")
    a. Make sure the power buttons on the light source and microscope are on.
    b. Adjust the white light intensity knob (left side) to an acceptable setting.
    c. Focus the cells by turning the rough (bigger) and fine (smaller) focus knobs on the side
    d. Rotate the right side knob to "SIDE" to see the image on the computer screen

9.) Once the cells are focused, switch to fluorescence mode to find fluorescent cells.
    a. Turn on the fluorescent light source if you haven't already, then wait 5 minutes for full power
    b. Turn off the white light source on the microscope (white button, lower left side)
    c. Open the aperture (swiveling lever on right side under the stage; O = Open, C = Closed)
    d. Rotate the light cube carousel until the appropriate filters for your fluorophore are aligned:

Fluorophore	Excitation maximum	Emission maximum	Position	Excitation Filter	Dichroic Mirror Cutoff	Emission Filter
Hoechst 33342	360 nm	460 nm	1	340-380 nm	<400 nm	435-485 nm
GFP	488 nm	509 nm	2	465-495 nm	<505 nm	515-555 nm
mCherry	587 nm	610 nm	3	528-553 nm	<565 nm	590-650 nm

10.) Capture images of the cells (turn the right side knob to "SIDE" if it is not already there)
    a. White light image – close the aperture, turn on the white light, and focus the image
    b. Fluorescent images – open the aperture, then use the appropriate light cube for each image
    c. Do not move the cells at all while taking these pictures
    d. Overlay the images in powerpoint or GIMP to create a figure for your lab report

11.)TURN EVERYTHING OFF AND COVER THE MICROSCOPE WHEN YOU ARE DONE!

---------- ***Selecting for Positively Integrated Cells with Puromycin*** ----------

12.)Select for positively integrated cells by adding 0.5 ug/mL puromycin (Sigma, P7255) to the cells.

13.) Culture the cells for an additional 1-2 weeks. Add new media (with puromycin) every few days.

14.) If any cells remain after selection, sequence their genomes to confirm genomic integration.
    a. Red fluorescence can help you eliminate negatives, but cannot definitively identify positives

Lab 14 – Large Scale Animal Cell Culture in a WAVE Bioreactor

No instructions for this lab – the instructor will simply demonstrate how the bioreactor works.

The WAVE bioreactor is the industry standard for expressing recombinant proteins in non-adherent CHO cells.

Notes/Observations

Guidelines for Lab Report 3

Synopsis

This report will be the same format as Lab Report 1, but with a focus on animal cell culture/protein expression.

The first two pages must include the following sections:

- **Title/Authors** – Include a descriptive and concise title, along with a list your group members
- **Abstract** – A summary that consists of 1-2 sentences for each of the following sections (≤100 words).
- **Introduction/Methods** – Since we did not vary many variables in our experiments (we were just hoping they would work!), the Intro and methods sections will be combined and emphasized in this report.
  - Begin by describing what Cas9 does and how it does it (hint: gRNA)
  - Next, describe how this phenomenon can be used to integrate a gene into the genome (hint: HDR)
  - Also give a detailed description of all of the reagents required for Cas9-mediated genomic editing. Be very specific when describing each of the genes and the inserted sequence.
  - Finally, explain the roles of the two fluorescent proteins and the puromycin resistance gene.
- **Results** – Describe the results obtained in Labs 11-13. (Labs 10 and 14 do not need to be addressed)
  - **Lab 11**: Did the oligo annealing experiment work? (i.e. did you get the desired plasmid?)
  - **Labs 12-13**: Interpret the results of your transfections using your microscopy images:
    - Did Cas9 express? How do you know?
    - Compare the results obtained with 1x and 2x Lipofectamine.
    - Estimate the transfection efficiency of each condition – What % of cells were transfected?
    - Was the mCherry gene delivered? …integrated into the genome? How do you know?
- **Synopsis** – Did the technique work? If not, provide potential problems and solutions.

The last 1-2 pages will contain figures and citations. For this report, you must include the following figures:

- **Figure 1: Schematic of Cas9-mediated Genome Editing**
  - Make your own figures. DO NOT copy my illustrations from the notes.
  - Show how Cas9 can be used to integrate a gene into the genome.
- **Figure 2: Oligo Annealing Cloning to Prepare the Knock-In Plasmid**
  - Show all of the steps in oligo annealing and provide a detailed map of the knock-in plasmid, including gRNA, Cas9, GFP, promoters, terminators, and other important genetic elements.
- **Figure 3: CPEC for the Production of Knock-In Plasmids**
  - We did not make our own Knock-In plasmid, but I want you to prepare a schematic that shows how you would use CPEC to prepare a new knock-in plasmid.
- **Figure 4: Images of Transfected Cells**
  - Include images that show GFP and mCherry fluorescence for 1x and 2x Lipofectamine.
- **Figure 5: Images of Stained Cells**
  - Include images that highlight nuclei by overlaying Hoechst fluorescence on a white light image.
- **Include your references in the remaining space.**
- **Note:** Include captions for each figure. Each caption should include enough information for a well-educated/trained reader to understand its significance, but it should also be succinct.

Note: You do not need to take up the entire 4 pages. Just be sure to address all of the prompts listed above.

Your report will also be graded for spelling, grammar, and quality (e.g. logical and smooth flow, good organization, etc.). I take it as a personal offense when a paper is not spell checked prior to submission!

Lab Report 3 Rubric

Abstract			
Overall Quality: Briefly summarize the entire report.	1 *Incorrect Info*	2 *Sections Missing*	3 *Complete Summary*
Introduction/Methods			
Describe Cas9 and explain what it does.	1	2	3
Explain Cas9-mediated genome integration	1	2	3
Detailed description of knock-out plasmid	1	2	3
Detailed description of knock-in plasmid	1	2	3
Roles of GFP, mCherry, and Puro	1	2	3
Results			
Did the oligo annealing experiment work?	1	2	3
Did Cas9 express?	1	2	3
Compare 1x and 2x Lipofectamine	1	2	3
Estimate transfection efficiency	1	2	3
Was the mCherry gene delivered?..integrated?	1	2	3
For all prompts above, 3 = correctly addressed, 2 = partially correct, 1 = incorrect, 0 = missing			
Conclusion			
Overall Quality: Did the experiments work? If not, why?	1 *Missing parts*	2 *Partially incorrect*	3 *Correct conclusions*
Figures			
1: Schematic of Cas9 genome editing	1	2	3
2: Oligo Annealing Cloning for gRNA	1	2	3
3: CPEC for Knock-In Plasmid	1	2	3
4: Images of Transfected Cells (GFP/mCherry)	1	2	3
5: Images of Stained Cells (Hoechst)	1	2	3
3 = Perfect resolution/annotation/caption, 2 = labels or caption missing, 1 = no labels/caption			
References			
Overall Quality: Appropriate references to support text	1 *1 reference*	2 *2 references*	3 *≥3 references*

Spelling and Grammar

10 = no spelling or grammar errors, -1 pt for every error

Negative pts: -	0	1	2	3	4	5	6	7	8	9	10

Organization and Flow

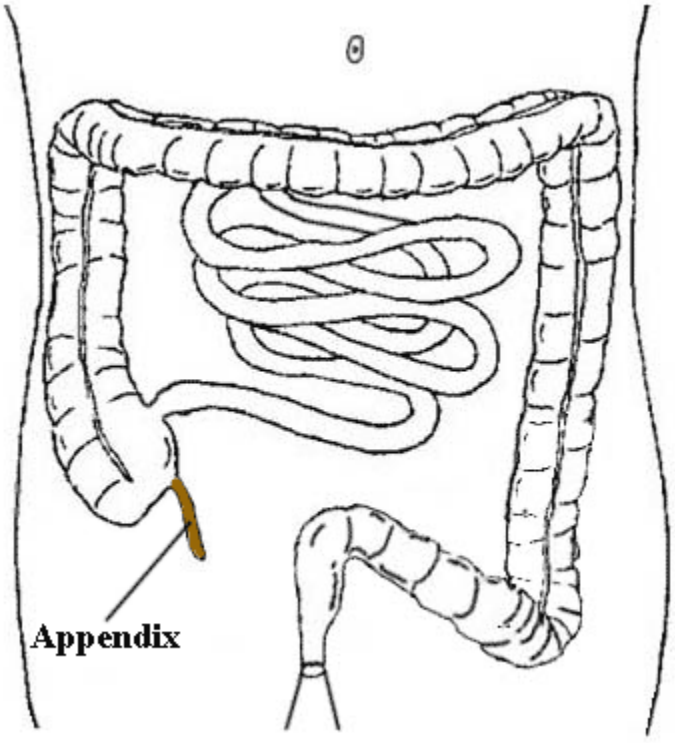
10 = Logical order and proper segues between sections, -1 pt for every instructor comment

Negative pts: -	0	1	2	3	4	5	6	7	8	9	10

Total = /74

The instructor reserves the right to modify this rubric at any time.

Appendix



Sequence of pCIX

Vector Description:

The CBD-Intein-X plasmid has a pBR322 origin with a low copy number, ampicillin resistance through a β-lactamase gene, and a T7 promoter/terminator that is regulated by a LacI repressor protein binding site. The vectors express a fusion protein with a chitin binding domain followed by a pH-inducible self-cleaving intein and the target protein. A ribosome binding site (**RBS**) is located 7 bp upstream of the start codon to enhance expression. These vectors were generously provided by Dr. David Wood, who optimized the intein gene.

The chromoprotein genes are cloned into the pCIX vector after the intein gene (….TGTACACAAC) and the Hind III restriction site (AAGCTT).

Sequence of pCIX:

CACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATA
CGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGGATCCAATAATTTTGTTTAACTTTAAG**AAG**
GAGATATACATATGACGACAAATCCTGGTGTATCCGCTTGGCAGGTCAACACAGCTTATACTGCGGGACAATTGGT
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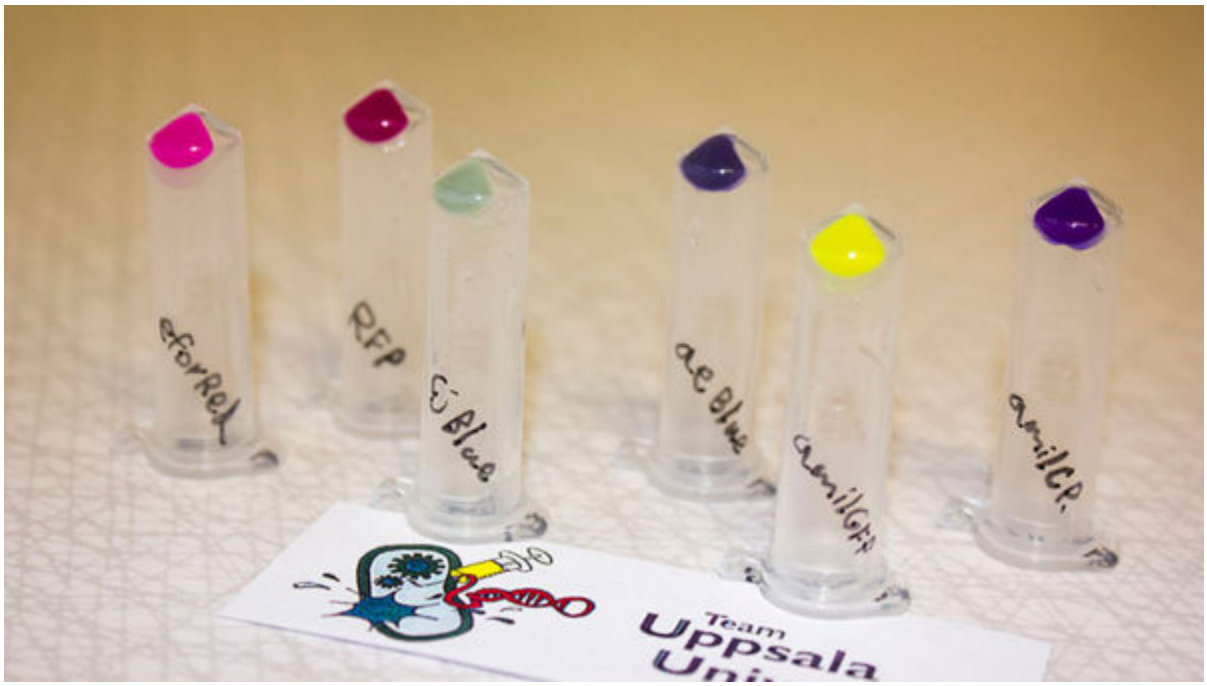
CG

Chromoprotein Gene DNA Sequences



iGEM12_Uppsala_University: The Uppsala chromoprotein collection and RFP. The image shows pellets of *E coli* expressing chromoproteins eforRed BBa_K592012, RFP BBa_E1010, cjBlue BBa_K592011, aeBlue BBa_K864401, amilGFP BBa_K592010 and amilCP BBa_K592009.

COLOR: aeBlue Gene Sequence from the anemone *Actinia equina* (699 bp, 231 AA): BBa_K1033929

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FLUORO: amilGFP Gene Sequence from the coral *Acropora millepora* (699 bp, 230 AA): BBa_K1033931

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COLOR: asPink Gene Sequence from the anemone *Anemonia sulcata* (702 bp, 231 AA): BBa_K1033927

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FLUORO: eforRed Gene sequence of the coral *Echinopora forskaliana* (687 bp): BBa_K592012

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COLOR: tsPurple Gene Sequence from Tinsel? (690 bp): BBa_K1033905

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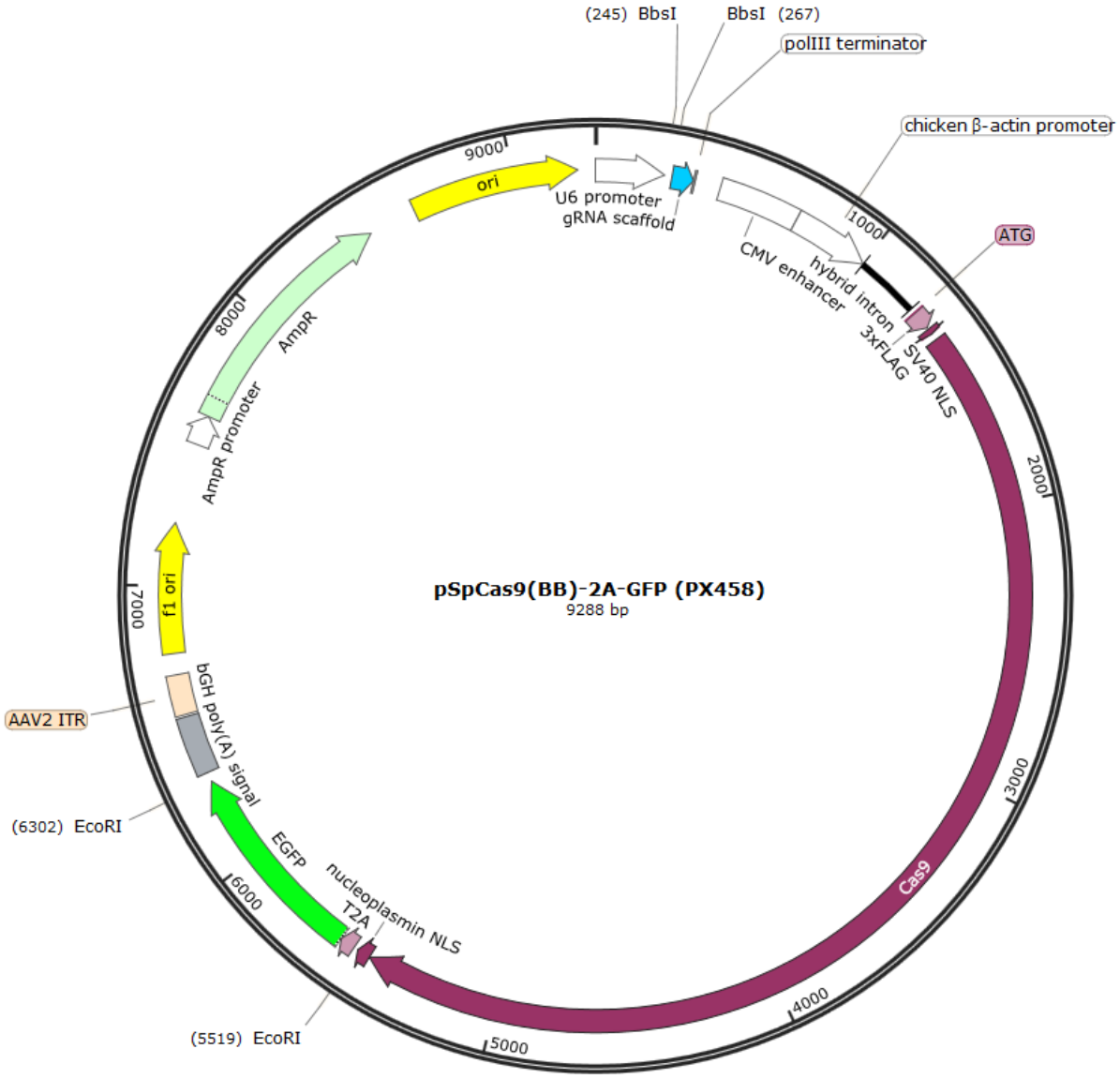
TAATAA

Sequence of Cas9 Knock Out Plasmid (without gRNA)

Knockout Plasmid: Cas9+gRNA (https://www.addgene.org/48138/)

- Backbone = AmpR + pBR322 ori (also has an f1 ori…)
- gRNA: u6 promoter for gRNA in BbsI cloning site, followed by scaffold and Pol III terminator
    - BbsI-HF = GAAGAC(N2)/CTTCTG(N6)
- Cas9: CAG (CMV/Actin/intron) promoter for 3Xflag-Cas9-NLS-T2A-EGFP-bGH terminator



Sequence of pSpCas9(BB)-2A-GFP (PX458)

Order of elements: U6 promoter – BbsI site (GAAGACNN) – scaffold RNA – terminator (TTTTT); CAG promoter – Cas9 – 2A – EGFP – bGH terminator; f1 ori; ampicillin resistance gene and promoter, Pbr322 ori

GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGT
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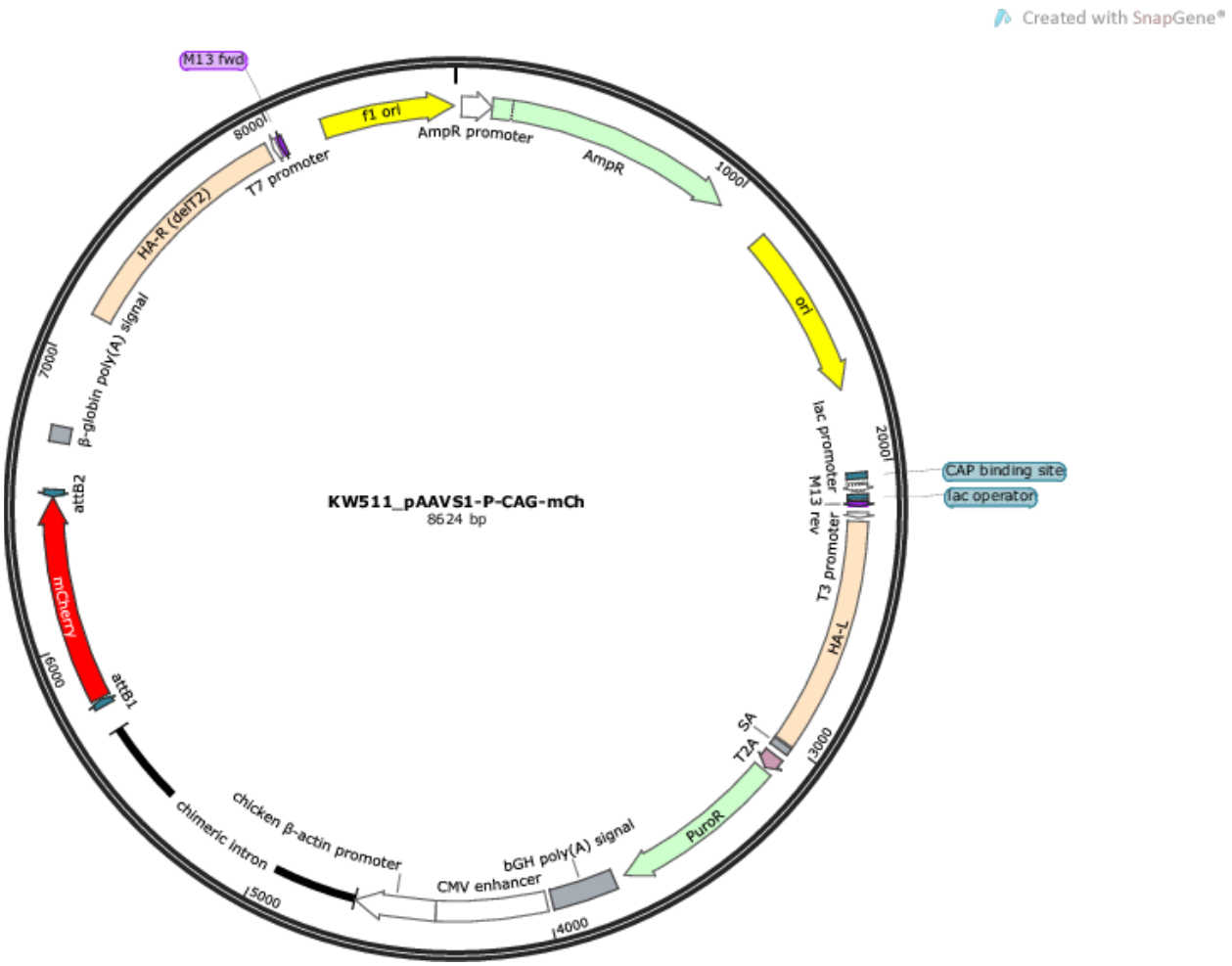
Sequence of Knock In Plasmid (targeting AAVS1 with Puro and mCherry)

Plasmid containing AAVS1 homology arms and a puromycin-mCherry insert

https://www.addgene.org/80492/



Sequence:

Order of Elements: Ampicillin resistance gene and promoter, pbr322 ori, LHA-2A linker – Puro gene – bGH terminator – CAG promoter – mCherry gene – rabbit beta globin terminator – RHA – f1 ori

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Sequence of Genomic Insertion Site – AAVS1

Color code = LHA – 5’G – 16 nt of gRNA – PAM (NGG) - RHA

ACCESSION S51329

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