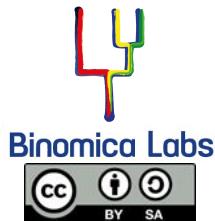




# Monthly Methods #2

## Molecular Techniques v1.0



### Aim of the Experiment

You'll learn how to amplify, clone, and sequence a key pigmentation gene from plants as a "Hello World" introduction to various molecular biology techniques.

### Background

The reading, writing, and editing of DNA has been one of humanity's greatest achievements. This technology allows us to literally shape the landscape we live in and give better agency over our environment and ourselves. In this experiment we will be covering many of the tools and techniques required to effectively manipulate DNA and in the process will learn a bit about the flowchart of processes required therein.

The gene we are targeting for this experiment is known as **Chalcone Synthase D**, a key element in the biosynthetic pathway for the production of various chemical pigments in flowers. The gene in question has some regions that are highly conserved (meaning that the sequence is very similar across multiple species) but also has an **intron** which varies in both length and composition from plant to plant. Hidden in this region tend to be **transposons** or "jumping genes" which we will cover at a later time but, for now, know they are pivotal in the patterning of flowers and thus are of great interest. From a broader standpoint, the gene is 1945 base pairs long, which is a nice easily amplifiable size for routine PCR and it's found in almost all flowering plants that express pigment in their petals.



For this experiment we will be using a commercial **cultivar** of *Petunia x hybrida* known as the Supertunia variety. These are hardy, fast growing, disease resistant petunias which flower constantly and give reliable pigmentation. Many of the current commercial varieties of petunia have yet to be sequenced in any meaningful way and some have never been analyzed in any sense. We are going to walk through the entire process of isolating this specific gene of interest from initial concept all the way to successful cloning and sequencing of this gene. The primers we will design may be functional in most petunias and any data generated from this experiment will be attributed to you and published in our annual data book. So without further ado, let's begin!

## Reagents

- Q5 DNA Polymerase with Q5 Buffer
- dNTP Mix
- Taq Polymerase Master Mix x5
- Distilled Water
- SacI Restriction Enzyme
- XbaI Restriction Enzyme
- Cutsmart Buffer
- Primers (PH-CHSD-WIDE, PH-CHSD-DET, PH-CHSD-SacI/XbaI, M13)
- pUC19 Cloning Vector
- Agarose
- SOC Media
- GelGreen DNA Stain
- Purple Loading Dye
- 2-log DNA Ladder
- TAE 50x Buffer
- LB Agar Media
- LB Liquid Media
- Ampicillin
- *E. coli* cells (any plasmid production optimized strain will suffice)



## Consumables

- Plant Genomic DNA Isolation Kit
- Bacterial MiniPrep Kit
- Qubit Fluorometer dsDNA Broad Range Kit
- Supertunia Royal Velvet (or any pigmented petunia)
- Sterile 1.5 mL Microcentrifuge Tubes
- Sterile 0.2 mL PCR Tubes
- Sterile 15 mL Tubes
- Sterile 50mL Tubes
- Sterile Toothpicks
- 100 mm Sterile Petri Dishes
- Pipette Tips (10 µL, 200 µL, 1000 µL)
- Microcentrifuge pestles
- Scalpel Blades (#11 or #10)

## Equipment

- Incubator-Shaker
- 125 mL Erlenmeyer Flasks
- 250 mL Erlenmeyer Flasks
- 1.5 mL, 0.2 mL, 15 mL Centrifuge Tube Racks
- Scalpel Handle (for #10 or #11 blades)
- Autoclave
- Microcentrifuge
- Blood Centrifuge (standard 15 mL fixed angle rotor)
- PCR Machine
- Incubator
- Microwave
- Gel Electrophoresis System
- DNA Blue Light Transilluminator
- Pipettes (2 µL, 20 µL, 200 µL, 1000 µL)
- Fridge/Freezer
- Laminar Flow Hood



## Procedure

**1.** Before we can do anything in the wet lab, we need to plan everything out. The first step is to learn a little bit about the gene we are looking for. We don't want to clone a gene just for the sake of it so here is some preliminary basic information on the gene in question.

[https://en.wikipedia.org/wiki/Chalcone\\_synthase](https://en.wikipedia.org/wiki/Chalcone_synthase)

The article goes into depth about this gene, which encodes an enzyme that, as the name entails, synthesizes chalcone from a specific chemical substrate.

The take home message is that this is the first gene in the biosynthesis pathway for flavonoids, a family of pigmentation molecules. It is the first dedicated step out of the phenylalanine pathway that begins the further modification of these molecules, which ultimately end up making all the colors that flowers can express.

**2.** Once we familiarize with the overall function and satisfy our curiosity with a few publications on the topic, the next step is to hunt down the sequence itself. This is done using the “Google of DNA” formally known as GenBank. This is a government sponsored system under the NCBI (National Center for Biotechnology Information) and is freely available to access. It houses one of the largest repositories of genetic information in the world and has the ability, through a system called BLAST (Basic Local Alignment Search Tool), to query every bit of DNA in said database for hits matching, or similar to, the sequence you provide. Link here:

<https://www.ncbi.nlm.nih.gov/genbank/>

NCBI Resources ▾ How To ▾ Sign in to NCBI

GenBank Nucleotide Search

GenBank GenBank Submit Genomes WGS Metagenomes TPA TSA INSDC Other

**GenBank Overview**

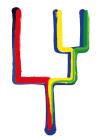
**What is GenBank?**

GenBank® is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences (*Nucleic Acids Research*, 2013 Jan; 41(D1):D36-42). GenBank is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Nucleotide Archive (ENA), and GenBank at NCBI. These three organizations exchange data on a daily basis.

A GenBank release occurs every two months and is available from the [ftp](#) site. The [release notes](#) for the current version of GenBank provide detailed information about the release and notifications of upcoming changes to GenBank. Release notes for [previous GenBank releases](#) are also available. GenBank growth statistics for both the traditional GenBank divisions and the WGS division are available from each release. GenBank growth [statistics](#) for both the traditional GenBank divisions and the WGS division are available from each release.

**GenBank Resources**

[GenBank Home](#)  
[Submission Types](#)  
[Submission Tools](#)  
[Search GenBank](#)  
[Update GenBank Records](#)



**3.** In the Nucleotide search bar, type in “petunia x hybrida chalcone synthase” and then scroll until you see a linear DNA entry with the name chsD in the name.

[P.hybrida chsD gene for chalcone synthase](#)

13. 2,948 bp linear DNA

Accession: X14593.1 GI: 20528

[GenBank](#) [FASTA](#) [Graphics](#)

**4.** Clicking on this brings you to the main entry page for this gene. It contains all the pertinent information regarding the original scientific article this gene was cited in, as well as the sequence and some annotations to denote what part of the sequence is the coding sequence (CDS), 5' and 3' UTR, and other regulatory elements outside of just the sequence of the gene which encodes for the actual protein.

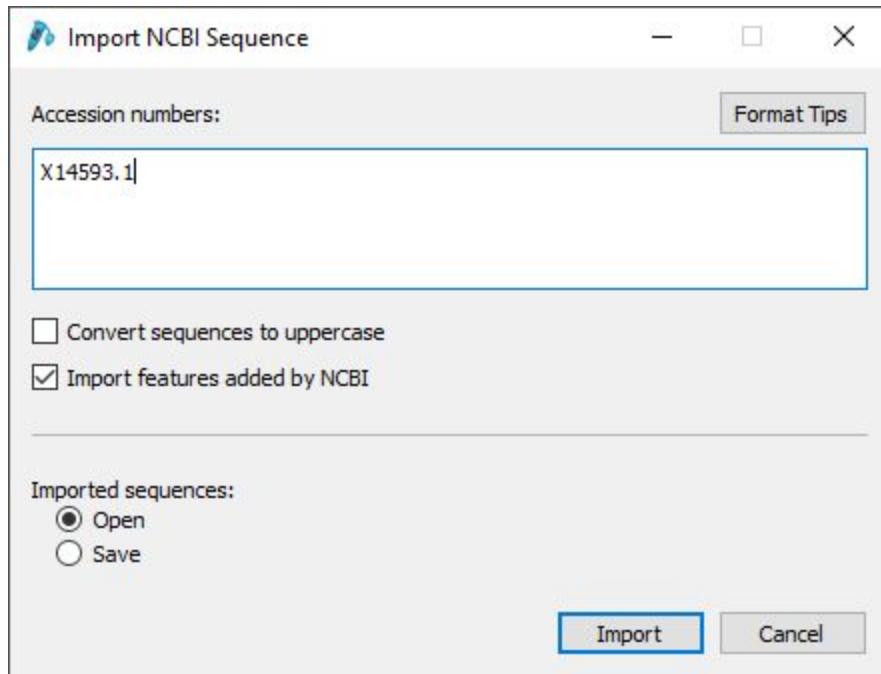
**5.** The most important part of this entire page, aside from the *actual* sequence of the gene, is the locus number, in this case it's **X14593.1**. This allows for easy importation of GenBank related data into a number of bioinformatics software.

## P.hybrida chsD gene for chalcone synthase

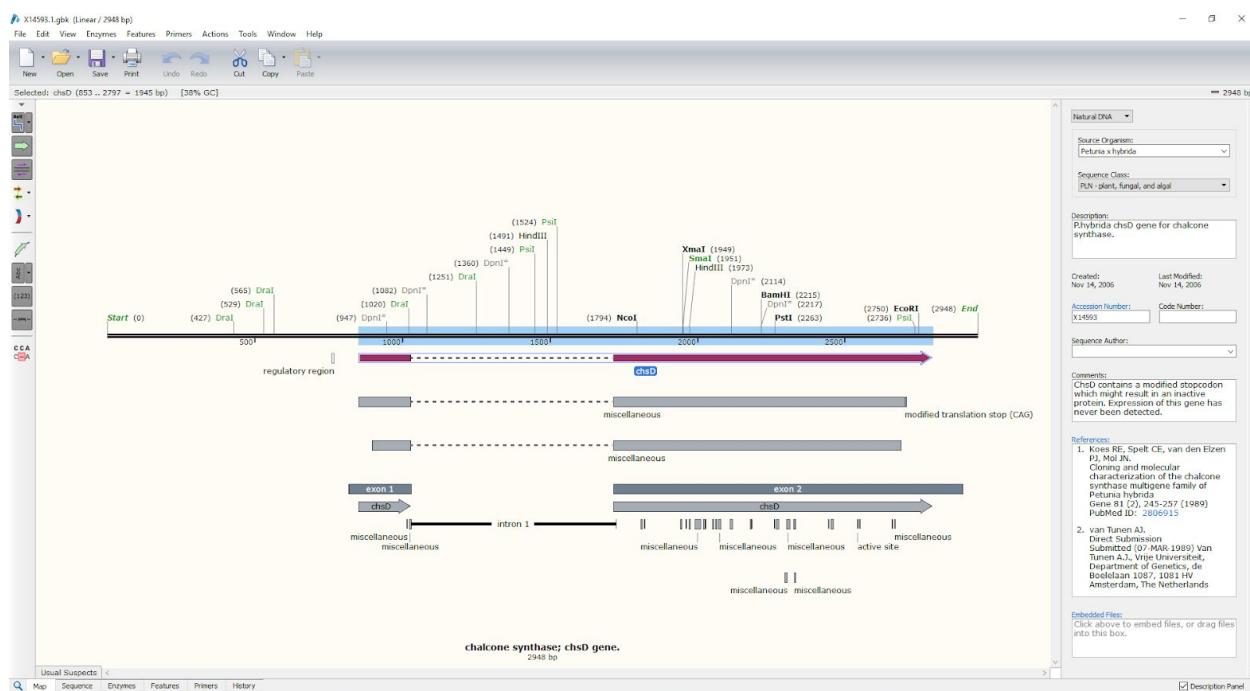
GenBank: X14593.1

[FASTA](#) [Graphics](#)

**6.** I have a personal preference for SnapGene. It is a great plasmid and DNA visualization tool, especially for routine assembly of plasmid constructs. You can download a 30 day trial of the full suite or use their free SnapGene Viewer to read and visualize said DNA. Click on the import button and select NCBI Sequence. Type in the locus number for the Petunia CHSD gene from above.



7. Now we have the entire gene sequence annotated and ready for us to play with. The dark red arrow is the gene itself; in the image below I clicked on it, which highlighted the sequence both on the Map tab AND the Sequence tab (found at the bottom left of the window). Familiarize yourself with the SnapGene layout. Press buttons, click on the tabs, explore the user interface. We'll be using this tool often. There are also video links in the Help menu to assist in navigating the program.





**8.** Now that we have the sequence and a means of interacting with it, let's start designing primers to amplify this sequence from petunia tissue. For this we'll be using a piece of software known as Primer3Plus. Link here:

<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>

Highlight the entire sequence (not just the CDS) in SnapGene and copy/paste it into the Primer3Plus window.

**9.** Go back to the SnapGene window and copy JUST the coding sequence into your clipboard. Then in the browser window, press Ctrl + F to bring up the search bar and paste in the coding sequence. The web browser will highlight the region in your whole sequence where the coding sequence is. At the edges of the highlighted sequence, apply a [ at the beginning and a ] at the end such that the coding sequence is flanked by brackets. This tells Primer3 what you wish to target when choosing primers.



10. Click on the General Settings tab and ensure the values in the text boxes match the ones seen in the image below.

**Primer3Plus**  
pick primers from a DNA sequence

[Primer3Manager](#) [Help](#)  
[About](#) [Source Code](#)

**Task:**  Select primer pairs to detect the given template sequence. Optionally targets and included/excluded regions can be specified.

Main    General Settings    Advanced Settings    Internal Oligo    Penalty Weights    Sequence Quality

Product Size Ranges

Primer Size Min:  Opt:  Max:   
Primer Tm Min:  Opt:  Max:  Max Tm Difference:   
Primer GC% Min:  Opt:  Max:  Fix the  prime end of the primer  
Concentration of monovalent cations:  Annealing Oligo Concentration:   
Concentration of divalent cations:  Concentration of dNTPs:

Mispriming/Repeat Library:

**Load and Save**  
Please select special settings here:  (use Activate Settings button to load the selected settings)  
To upload or save a settings file from your local computer, choose here:  
 No file chosen   

These settings allow for longer primers. This is needed for higher specificity when amplifying genes from genomic DNA since the probability of a primer sequence having more one binding site in the entire genome of an organism decreases the longer your primer is. More specificity, less chance of secondary amplification of sequences that are somewhat similar to your desired sequence. More on this notion in a later document but for now just know longer sequences are more ideal. Once you have the settings changed, click the green Pick Primers button.



**11.** Primer3 has now generated several potential pairs where the top most pair, shown in the picture below, is the most ideal candidate for PCR.

**Primer3Plus**  
pick primers from a DNA sequence

[Primer3Manager](#) [Help](#)  
[About](#) [Source Code](#)

< Back

Pair 1:

Left Primer 1: **Primer\_F**  
Sequence: tcattcaacgaatgctaattactga  
Start: 223 Length: 25 bp Tm: 60.0 °C GC: 32.0 % ANY: 7.0 SELF: 2.0

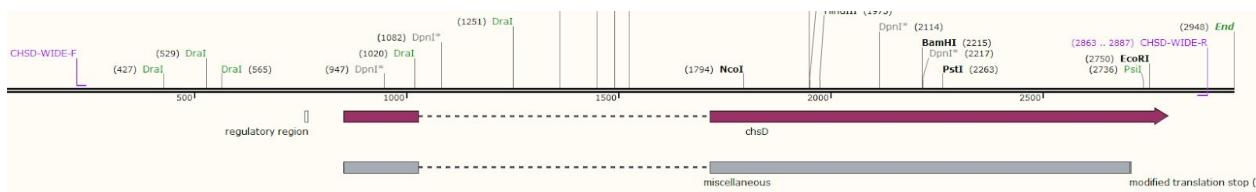
Right Primer 1: **Primer\_R**  
Sequence: tgaacaccccattactcctaaataaa  
Start: 2887 Length: 25 bp Tm: 60.0 °C GC: 36.0 % ANY: 3.0 SELF: 2.0

Product Size: 2665 bp Pair Any: 4.0 Pair End: 3.0

[Send to Primer3Manager](#) [Reset Form](#)

1	ttaagtacct	cacacaaaata	tgacattgcg	tgtgaggcat	acaataaaatt
51	ttctcgccga	ccggggccgg	actaggctag	gggattcaact	tattgacaaa
101	tgccttcctt	tttatgcttc	cctccaagga	aaaattgacc	taagcataga
151	ggcaaccaaa	actgagttca	acacaacatg	agagccaaca	acttttagaaa
201	gcctcaagtc	gggcgcctct	tttcattcaa	cgaatgctaa	ttactgaaaa
251	actacaagac	aattttatct	tcttcttgtc	caacgatgca	gtcaaataagt
301	tatcaccata	gtgtaccttc	gtttttgtcc	aaactaccta	cgttagaagt
351	tcaaaatttt	gttaggagaa	tactgcaata	tggagagtag	aagatgcaaa

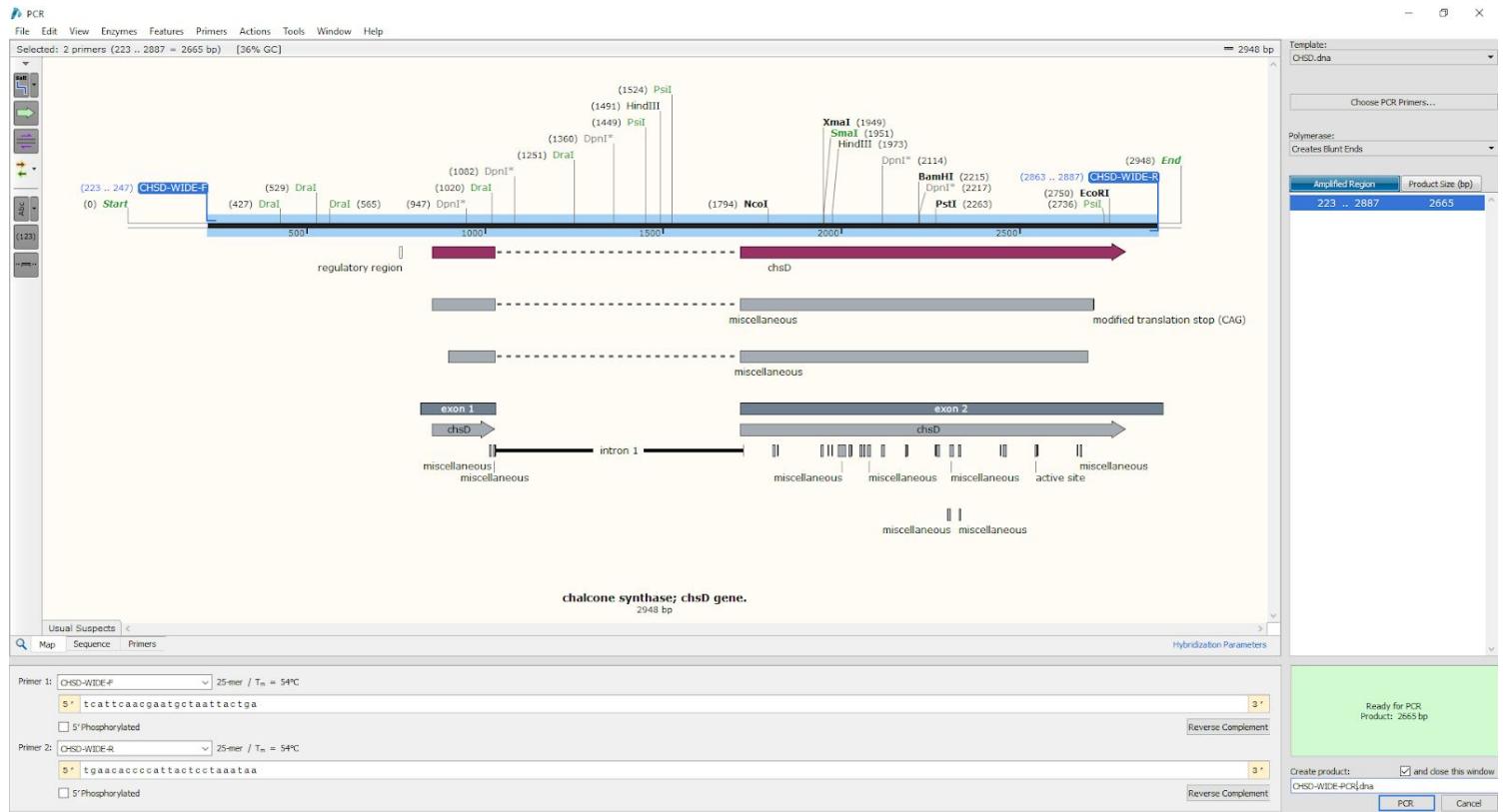
**12.** Copy the Primer\_F sequence into your clipboard. Open SnapGene, click on the Primer tab on the top, click Add Primer, and paste the sequence in. Name the primer “PH-CHSD-WIDE-F”. Click the “Add Primer to Template” button. This should add the primer onto the sequence and will annotate the binding site of this primer with a purple line on the sequence. Repeat the process for the Primer\_R sequence, add it to the template with the name PH-CHSD-WIDE-R. If all goes well, the result should look like this:



Note the forward primer is on top of the DNA sequence (long black parallel line) and the reverse primer is on the bottom. Next step is to simulate the PCR reaction we are going to run with these primers.



**13.** Click the Actions tab and click PCR. Now we are in a new window where we can specify, via the drop down box on the bottom left, the two primers we wish to use. The program will highlight the region to be amplified and all you need to do is rename the resulting DNA with CHSD-WIDE-PCR in the bottom right hand side. If all goes according to plan, your screen should look like this:



Click the PCR button and this will generate a new DNA file with your amplified construct exactly as it designed.

**14.** Now we have our first designed primer pair. Congrats! For your convenience here is the sequence. I've tested these, so, if your primers are different, consider using the following sequences for this experiment:

PH-CHSD-WIDE-F  
PH-CHSD-WDIE-R

**caa agt caa atg gtt cat cta aag tg**  
**ccc cat tac tcc taa ata ata gtt aca aac**

A PCR product by itself can't be cloned into a plasmid as is. This is because it lacks the **phosphorylated** ends needed to glue the sequence to the plasmid, in a process known as **ligation**. To make our gene of interest compatible with our plasmid, we



need to add a special sequence to each end of our gene, which contains the binding site for enzymes that can cut AND phosphorylate the gene. These enzymes, called restriction endonucleases, (and each enzyme, more or less) have a very specific and unique sequence they bind to and cut. Some cut blunt straight down through both strands of DNA, others cut in a zig-zag pattern making a shape called a **sticky end**. These ends that can only be ligated by things cut with the same enzyme. Having two sticky ends each cut by a different enzyme ensure that the gene will be ligated in the same orientation in the plasmid as desired. We will go into this in more depth in a bit. But for now, lets actually make some primers to add these adapters to the gene.

**15.** Going back to the Primer3Plus website, copy and paste the new PCR product sequence you simulated into the website text box. On the top left in the section marked “Task:” change it to “cloning”. This will force Primer3Plus to consider the ENTIRE sequence you entered as the target and only make primers that fit the constraints we want, instead of just picking primers within the sequence that are a best fit like we did earlier. Now we are going to add the adapters like I mentioned earlier.

For the beginning of the gene sequence, formally known as the 5' end (pronounced “five prime”), we are going to add the SacI restriction site. To the end of our gene (3' or “three prime end”) we are going to add the XbaI site. These two sites are present in the common and easily obtainable pUC19 plasmid. Commercially available plasmids used in molecular biology research have regions of their DNA known as **multiple cloning sites** (MCS) for short. An MCS is simply a stretch of DNA that contains several restriction enzyme cut sites in a row. These are normally selected for enzymes that are reliable, easily accessible, and, more often than not, whatever was available at the time the plasmid was built. pUC19 was made during a time where only a handful of enzymes were known. I'd like to cover the history of plasmids in a later publication which will go into the depth of the logic, methods, and limitations of that time period that led to the final design of such a venerable plasmid.

Getting back on topic, let's add the sites. To do so, we first need to know the sequence of the restriction site. We'll hunt down these sequences manually now, and I'll explain how to automate the addition later on. So let's scurry over to the New England Biolabs website and search for the sequence there.

Go to [www.NEB.com](http://www.NEB.com) and, in the search bar in the top right corner of the website, type in SacI. Click on the first hit you see. Note if you are purchasing from NEB, try



to see if there is a high fidelity version of the enzyme you wish to use. This is denoted by the name of the enzyme plus “HF”. It’s the same exact enzyme with the same DNA recognition sequence but it’s been modified to increase the fidelity of the enzyme. This means it is less likely to cut any other sequence except for the one it needs to. Once you find the sequences, write them down and keep them handy for later use. As a cheat sheet, here’s a snippet from the website:

## SacI-HF®



Non HF Version: **SacI**

5'... GAGCTC... 3'  
3'... CTCGAG... 5'

## XbaI



5'... TCTAGA... 3'  
3'... AGATCT... 5'

Going back to the Primer3Plus website, add the SacI sequence to the 5' end of your gene, namely GAGCTC and ensure it's written in all caps so you can see the cut site from the rest of the gene which is in lowercase. To the 3' end add the XbaI site (TCTAGA).

We now have the needed restriction enzyme adapter sites...there is an issue here. These enzymes need to attach to the sequence physically to cut the strand. The size of the enzyme needs to be taken into consideration since most cutters need a few extra bases to the left and right of the enzyme as a kind of landing pad. Luckily, NEB has written a whole article on this matter and have created a list of commonly used enzymes and how many bases they need after the recognition sequence for the enzymes to cut efficiently. This can be found here:

<https://www.neb.com/tools-and-resources/usage-guidelines/cleavage-close-to-the-end-of-dna-fragments>

All you need to do is simply search for your cutter in the list and see how many bases are needed. The list is fairly straight forward but do not forget to note if your cutter is the high fidelity “HF” variant or not because it may or may not change the landing pad requirements for said enzyme.



	1 bp	2 bp	3 bp	4 bp	5 bp
SacI	-	++	+++	+++	+++
SacI-HF®	-	+	+++	+++	+++

According to this chart, we need at least 3 bases after the edge side of the recognition sequence. This means that there needs to be 3 bases to the LEFT of the recognition site if the cutter is on the 5' end of the gene and 3 bases to the RIGHT if the cut site is on the 3' end.

	1 bp	2 bp	3 bp	4 bp	5 bp
XbaI	++	++	++	++	++

Note that XbaI only needs 1 base pair of landing pad and basically does the same amount in terms of efficient cutting regardless of how many bases you add. This is great for primer design since every base you add changes the melting point of the primer or contributes to hairpins or secondary structures so general rule of thumb is to use cutters that require the least amount of extra bases and ideally low in G's or C's since they contribute the most to the melting point of the primer. I'll go into melting point when we cover PCR but for now, know less is more when it comes to which cutter to choose and what bases to add as landing pads for the enzyme.

On your screen you should now have Primer3Plus open with your PCR product sequence pasted in and the SacI recognition site added (IN ALL CAPS) to the 5' end and the XbaI site to the 3' end. You now need to add 3 or so bases on the 5' end BEFORE the SacI site and at least 1 base AFTER the XbaI site. Try a few combinations of bases, add **curly brackets** {...} to the beginning and end of your sequence and then click the “Pick Primers” button. Keep trying until you get back a pair of primers with no flags (no orange error messages beneath the primer sequence). If you get an error saying there is too high a self complementarity, you want to add more G's or C's such that the primer shortens in length and thus avoiding the 3' region of that primer that you cannot change since it's inside the sequence you are trying to amplify. Primer design is a bit of an art and there is a lot of conversation around how to properly design primers. It varies from person to person and this is my particular way of making them. It may not be what everyone recommends as the “proper way” of designing primers but in the literal thousands of primers I've designed, I have yet to have a pair that has failed due to poor design. If you feel like skipping ahead, try adding **a t a** to the 5' end and **t a t** to the



3' end. These landing pad bases should produce primers with no warning flags.

The final primer sequence for the cloning pair is as follows:

PH-CHSD-SacI-F **ata**GAGCTC~~ccaa~~agtcaa~~atgg~~tt

PH-CHSD-XbaI-R **tat**TCTAGA~~cccc~~attactcctaaa

The red letters are the landing pad, capitalized and bolded letters is the recognition site, and the lowercase letters is the start of the gene in question. Add these to your CHSD-WIDE-PCR.dna file using the Primers > Add Primer function.

So far we've produced two pairs of primers. One pair to amplify out the gene itself using the most optimal primers possible, where the PCR product is LARGER than the gene itself and our gene of interest is nested within this product. The second pair of primers will be used to add the adapter site to the sequence using the PCR product of the first primer pair as the template. When adding adapter sequences, we remove the amount of bases that bind to the gene of interest to make space for the recognition site so the specificity, the uniqueness of the binding site with respect to the rest of the organism's genome, decreases. To mitigate this we do a process called **nested PCR** where we amplify a region containing our gene of interest and then use that PCR product as the template to do a second PCR to avoid the lack of specificity of the cloning primers using just the genomic dna.

**16.** Next step is to make one last pair of primers known as detection primers. These are primers INSIDE the gene of interest that yield a short 300-600 bp PCR product using borderline ideal sequences. We'll use these primers as a positive control to ensure the gene we are looking for is actually present. A fragment around 500 bp is ideal for Taq Polymerase to amplify and 25 bp at 60°C is the optimal length and annealing temperature for primers in general so we are going to let Primer3Plus pick the best pair given these constraints. Somewhere in the gene are two regions of 25 bp that are almost perfect for PCR. To make these, simply copy and paste just the CDS of the gene we are trying to amplify into the sequence window of Primer3Plus and change make sure the "Task:" section is set to "detection". In the General Settings section, ensure the "Product Size Ranges" parameter is set to "300-500" to ensure it only picks primers which yield PCR products between 300 and 500 base pairs in length. Click Pick Primers and you should get some nice candidates. Here is the primer pair I generated and used:

PH-CHSD-DET-F taa tca taa ttt acc ctc aat cct att ttt

PH-CHSD-DET-R ctc tgt taa gtg cat gta tct ctt aat



Add these primers to your PCR product file as well. We have now designed all the primers we need for this experiment. Next step: ordering primers!

**17.** Thermo-Fisher offers a promotional product known as Value Oligos which are priced at a flat rate of \$5 per primer up to 40 base pairs. They give you a discount if you order 25 primers or more. I tend to use this supplier for my primers but I am always shopping around for the best price and convenience. So far, they have yet to fail me in terms of primer quality but they are by no means the cheapest source of oligos. Do ask for quotes and shop around. Develop a relationship with your supplier representative and never settle for the asking price for any bit of biotech reagent or equipment purchase. Regardless of which supplier you end up using, make sure you add the M13 primer pairs to your order as well. They will come in handy in all of your cloning endeavors and just a good primer pair to have around. Here is the sequence:

**M13-F** GTA AAA CGA CGG CCA GT

**M13-R** CAG GAA ACA GCT ATG AC

**18.** While we wait for primers to arrive, let's simulate the actual cloning process so that we know our design makes logical sense and the experiment will yield the construct we desire. Let's start first by simulating a PCR using the PH-CHSD-SacI-F and PH-CHSD-XbaI-R primers. Name the PCR product CHSD-SacI-XbaI-PCR. Make sure you save your work often!



**PCR**

File Edit View Enzymes Features Primers Actions Tools Window Help

Selected: 2 primers (706 .. 2882 = 2177 bp) [37% GC]

Template: P x hybrids chsD gene for chalcone synthase.dna

Polymerase: Create Blunt ends

Amplified Region Product Size (bp)

706 .. 2882 2194

Start (0) 500 1000 1500 2000 2500 End (2948)

(706 .. 731) PH-CHSD-WIDE-F (706 .. 720) PH-CHSD-SaI-F (828) DraI (947) DpnI<sup>+</sup> (1082) DpnI<sup>+</sup> (1020) DraI (947) DpnI<sup>+</sup> (1409 .. 1438) PH-CHSD-DET-F (1251) DraI (1360) DpnI<sup>+</sup> (1449) PstI (1730 .. 1789) PH-CHSD-DET-R (1491) MspI<sup>+</sup> (1442) PstI HindIII (1979) DpnI<sup>+</sup> (2114) BamHI (2215) DpnI<sup>+</sup> (2217) PstI (2263) PstI (2736) EcoRI (2790) P-H-CHSD-XbaI-R (2882 .. 2881) P-H-CHSD-XbaI-R (2888 .. 2882) End (2948)

regulatory region chsD modified translation stop (CAO)

miscellaneous miscellaneous

exon 1 chsD exon 2 chsD active site

intron 1 miscellaneous miscellaneous miscellaneous miscellaneous

miscellaneous miscellaneous

chalcone synthase: chsD gene. 2948 bp

Usual Suspects Map Sequence Primers Hybridization Parameters

Primer 1: PH-CHSD-SaI-F 24 mer / T<sub>m</sub> = 43°C  
5' ataaGACTccaaagtcaaatggtt  
3' Phosphorylated  
Primer 2: PH-CHSD-XbaI-R 25 mer / T<sub>m</sub> = 49°C  
5' tattCTAGaccccatatcttctaaa  
3' Phosphorylated

Ready for PCR Product: 2194 bp

Create product:  and close this window  
chsD-SaI-XbaI-PCR.dna  
PCR Cancel

**19.** Let's import the pUC19 vector so we have it ready for us to use. Go to File > Import > SnapGene Online Resources. Type in “pUC19” and click “Import”.

Save to Collection

Save to Main Collection Ctrl+Alt+Shift+S

**Import**

Export

Batch Convert File Format...

Page Setup... Ctrl+Shift+P

Print... Ctrl+P

Exit Ctrl+Q

NCBI Sequence... Ctrl+Shift+O

Addgene Plasmid...

**SnapGene Online Sequence...**

Clone Manager Primer Collection...

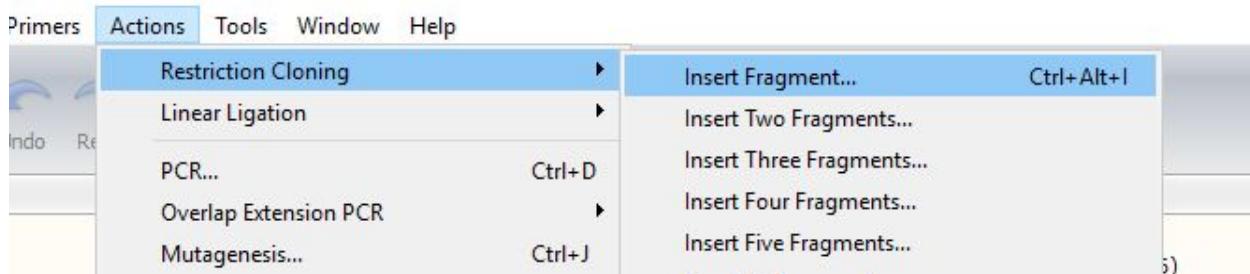
Vector NTI® Database...

Vector NTI® Oligo Archive...

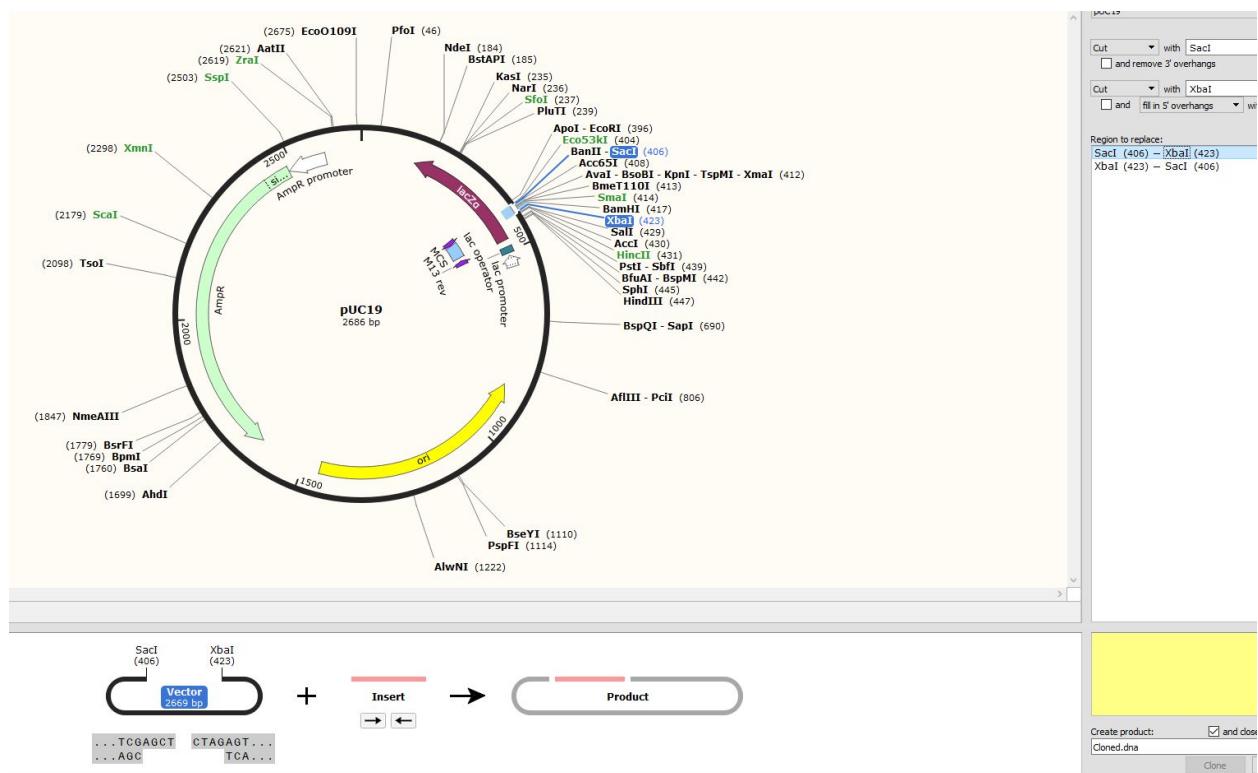
(795)  
(753)  
PH-CHSD-DET-I  
(664) DpnI<sup>+</sup>  
SaI



**20.** Lets simulate the cloning now that we have all the parts ready. Go to Actions > Restriction Cloning > Insert Fragment



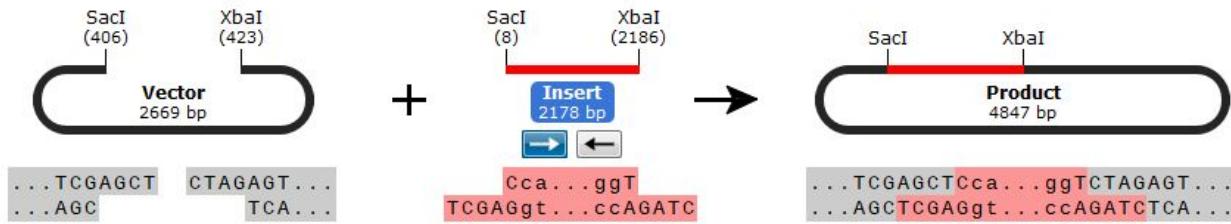
Now, in the top right corner's where it says "Cut with", type in SacI in the first box and XbaI in the second box. This is telling SnapGene that we wish to open up the pUC19 plasmid using the cutters we mentioned. Note the blue highlighted region on the plasmid map. This denotes the region in the vector we are going to replace.



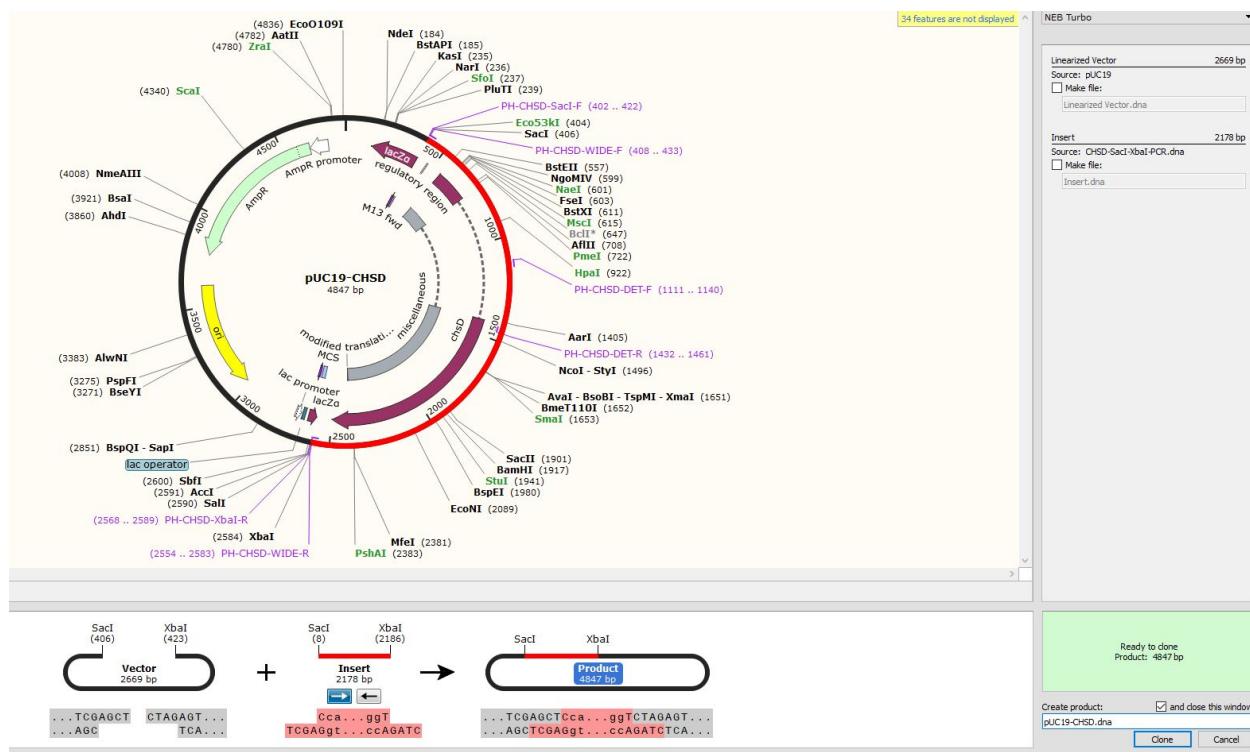


Next, click on the red “Insert” bar on the bottom to select what fragment of DNA will be inserted. For us it will be the CHSD-SacI-XbaI-PCR fragment. Fill in the “Cut with” region with the same cutters, SacI and XbaI.

Note the visual logic of the sticky ends that have been cut by the SacI and XbaI sites in the picture below. If you click on the Product area, you'll see the final product and hopefully will help you understand what just happened.

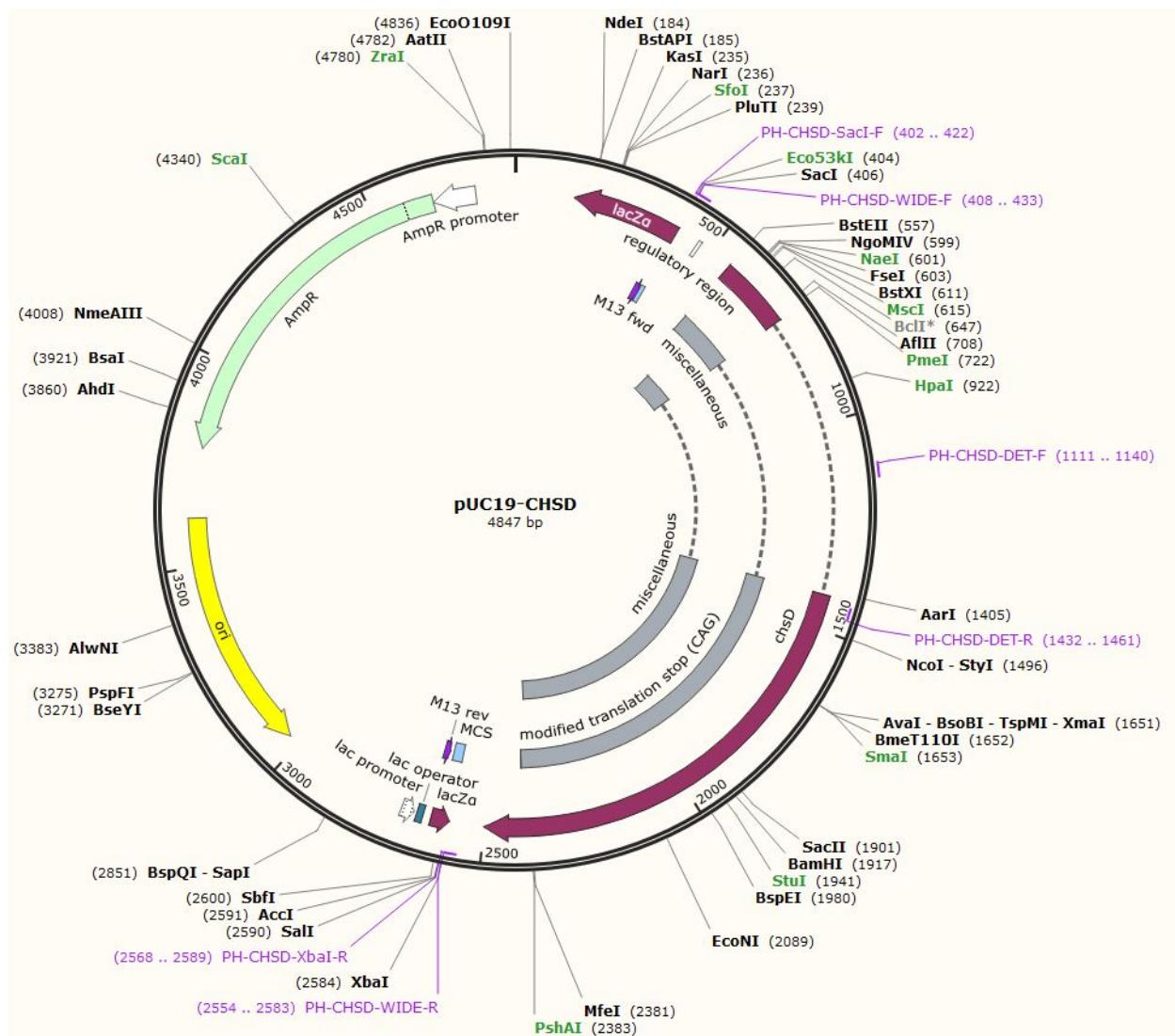


The cut pieces fit together like a jigsaw puzzle where the pieces only fit when they join with DNA cut by the same enzyme.



Name the new construct pUC19-CHSD and press the “Clone” button. We’ve now completed the simulation of the exact experiment we are going to run in the wet lab shortly. Take a moment to review what was just done and how the final plasmid looks like. Note the SacI and XbaI sites are still there (denoted by the bolded enzyme name). The ligation repairs the cut site so it can be used again to remove this gene or add other genes before or after ours. This is the heart of molecular cloning and the cornerstone of biotechnology.

Here is our final vector map. Congratulations, you simulate your first biotech experiment! If you click on the History tab at the bottom left, you can see the exact steps taken to construct this plasmid. Print that page out along with this map for your records. Makes for neat stickers too!



**21.** By now, your primers should have arrived and we are ready to begin the actual physical construction of this plasmid. Let's keep our momentum going and proceed with the laboratory work!

The first thing we need to do once we make it to the wetlab is ensure we have all the materials to proceed. You can get by one day at a time but ideally you'd like all the reagents and equipment ready from the beginning. Take some time to go over what has been said, as well as all of the steps beyond this point, to get a sense of what is required of you in terms of time, reagents, equipment, and other resources. It's always good to be prepared.



After we do roll call and inventory, the next step would be to streak some *E. coli* cells onto LB-Agar media so we can start the process of making **competent cells**. Having fresh cells is crucial in efficient cloning and is also a good exercise to warm you back up to the routine of labwork, in case this is either your first time in a lab setting, or you've been out of the lab for a while and feel rusty. Let's take our time and go at whatever pace you feel comfortable. I'll do my best to state when a good stopping point is, in case the day has to be cut short or planned weeks ahead of time. Okay, back on topic!

Take a sterile inoculation loop and sample from whatever form of *E. coli* stock you have in hand. This can be a colony plate, a liquid culture, an agar stab, glycerol stock, etc. Regardless of from where, take a sample of this cell stock and streak it across the petri dish. You can follow the formal way of streaking a non-antibiotic plate via this link:

<https://homepages.wmich.edu/~rossbach/bios312/LabProcedures/Streak%20plate%20procedure.html>

The objective of this practice is to isolate individual colonies (clusters of genetically identical cells, originating from a single cell, separated from a culture by spreading them across a Petri dish). Theoretically, a single colony contains genetically identical cells, therefore will most likely behave predictably in terms of transformation efficiency, growth, etc. You want to eliminate as many variables from an experiment as possible, so having near identical genotype is ideal. Colony isolation is one way to ensure this.

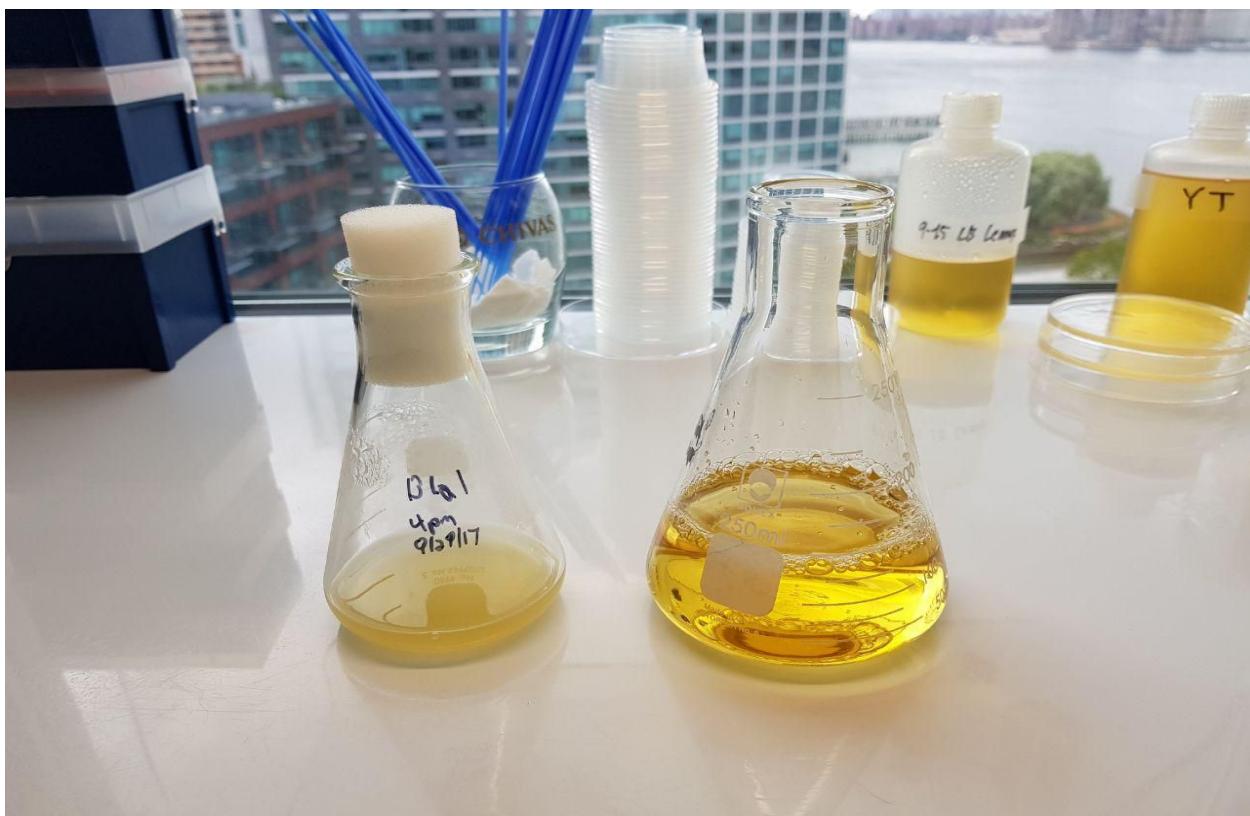
Incubate the cells upside down overnight at 37°C. Overnight is anywhere from 16-20 hours typically. If you use fast growing cells like NEB Turbos, you can get workable colonies in as little as 12 hours.

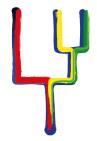
**22.** The following day, take your Petri dish out of the incubator and examine the colonies. Any contamination? Do you see weird colored cells? Colonies of pure *E. coli* cultures are beige uniformly shaped circles 1-2 mm wide (size depending on speed of cell growth, but key word is uniform). If all is well, dispense 25 mL of LB Liquid media into a sterile 125 mL Erlenmeyer flask under aseptic conditions (laminar flow hood, biosafety cabinet, dead air box, etc.). Using a sterile toothpick and gloved hands cleaned with isopropyl alcohol, select one well-isolated colony from the Petri dish and poke it with the toothpick. Drop the toothpick carrying the colony cells into



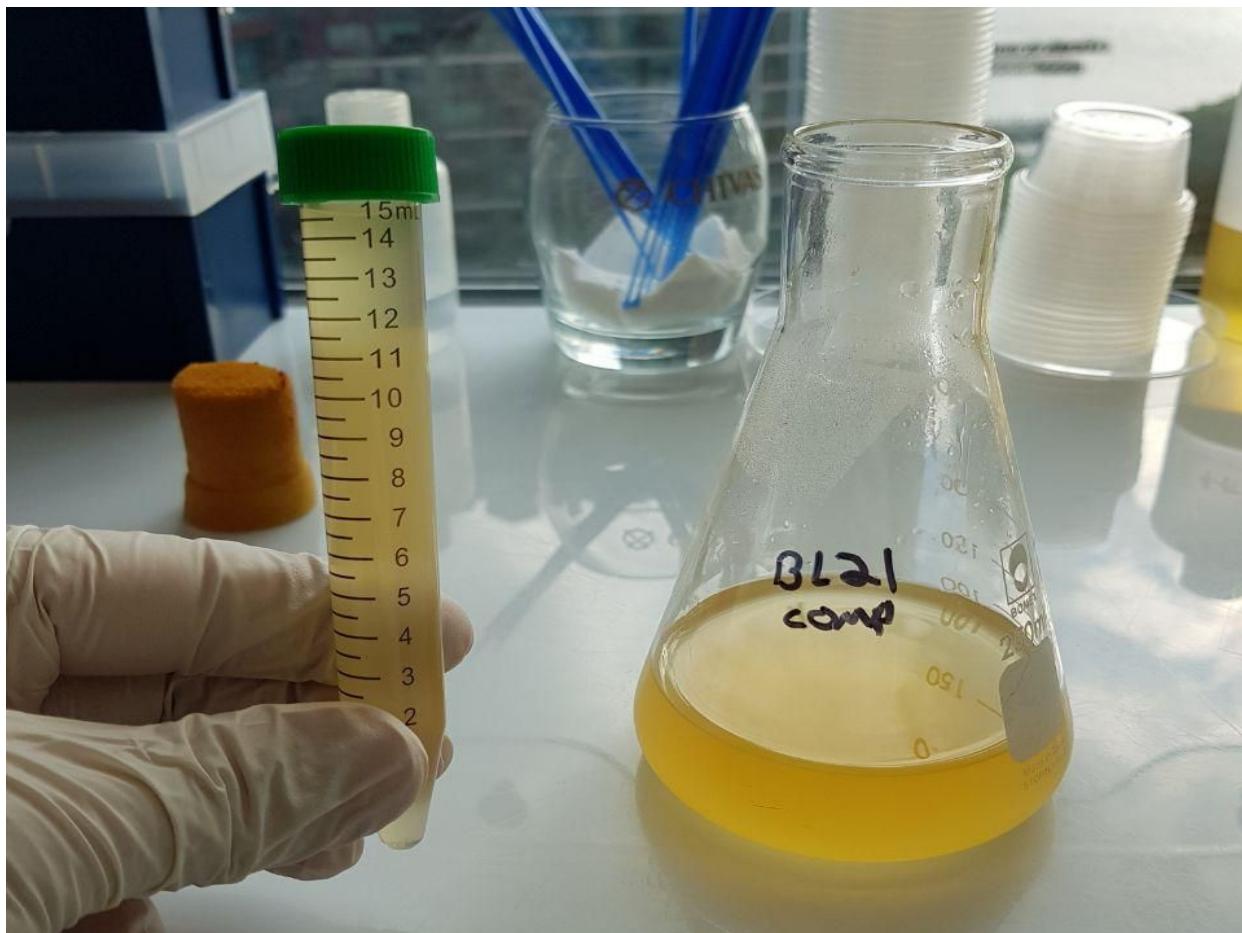
the media flask. Cap the flask with a foam stopper and place it in an incubator-shaker set to 37°C, 250 rpm and allow the flask to shake overnight.

**23.** The following day, prepare a sterile 250 mL Erlenmeyer flask filled with 125 mL of LB Liquid media. Take 1000 µL of the overnight culture and dispense it into the 250 mL flask. Place the inoculated 250 mL flask in the incubator-shaker set to 37°C and 250 rpm. Set a timer for 4 hours and allow the flask to shake until the timer expires. If you are using NEB Turbo cells, 4 hours is exactly enough time to reach an optical density of 0.5. This is read in a spectrophotometer set to 600 nm. If you do not have access to a spectrophotometer, optical density of 0.5 looks like runny egg yolk in terms of opacity. Do not exceed 6 hours regardless. If you feel specifically productive, you can take a 1 mL sample every hour until the 4 hour mark and, if it's still under 0.5, take a sample every 15 minutes and read it on a spectrophotometer. Plot the curve in Excel and determine the growth rate for the next time you make competent cells.





**24.** Once the cells reach an optical density of ~0.5, dispense the culture into 6-8 15 mL centrifuge tubes to filled to the brim.

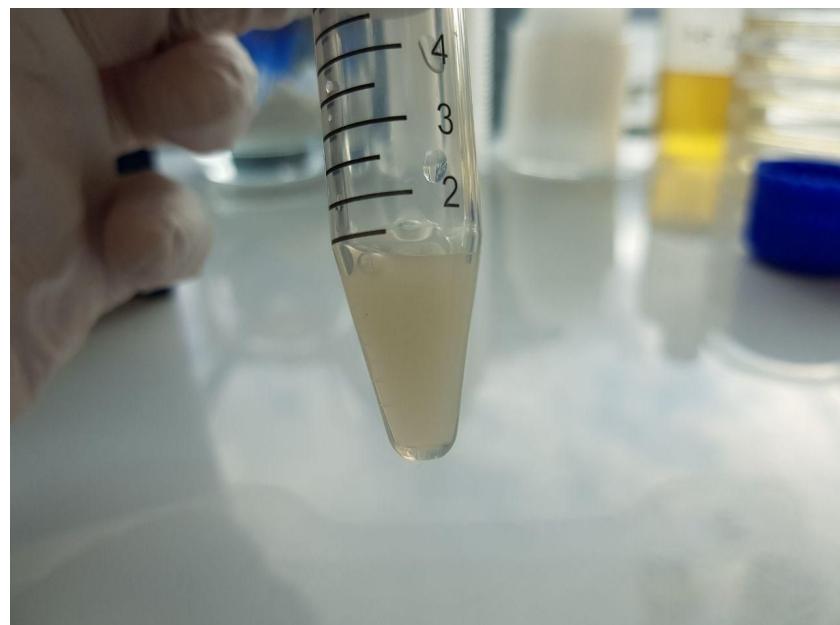


**25.** Spin down the cells in a fixed angle blood centrifuge at 3500 rpm or thereabouts for 10 minutes. Once the timer is done, remove the tubes slowly as to not disturb the pellet at the bottom of the tube. Dump out the liquid portion of each tube slowly into a waste beaker as to not disturb the pellets. Set the tubes on a rack.



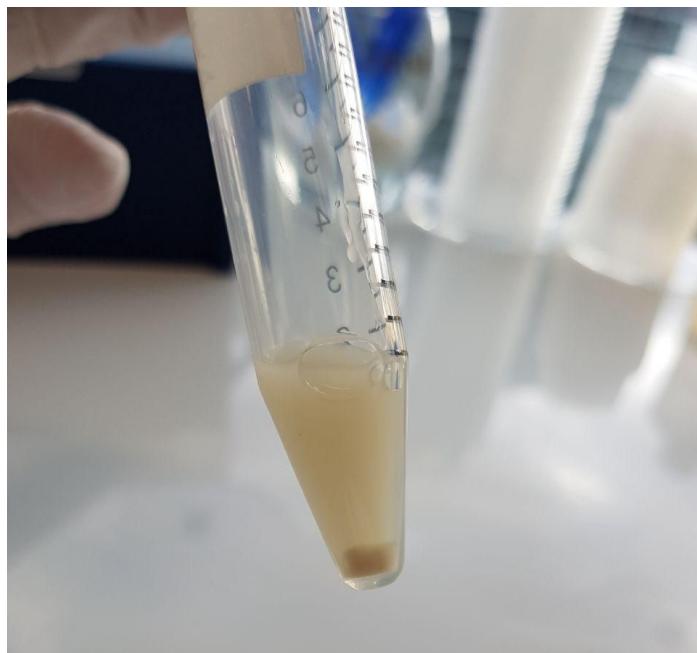
**26.** The next step you take here will be determined by the kind of competent cells you are making. If you are making **electrocompetent cells** for use in **electroporation**, you are going to resuspend the pellet of a single tube using 1 mL of sterile distilled water. If you are going to make **chemically competent cells** for use in **heat shock transformation**, you will resuspend the pellet using 1 mL of LB Liquid media. I'll include a protocol for both chemically competent and electrocompetent cell preparation, but, for this specific experiment, I will use electroporation as my method of transforming bacteria.

→ Resuspend the pellet by pipetting up and down until the pellet is fully dispersed into the solution.





Take that 1 mL of resuspended pellet and move it to another tube containing a pellet. Resuspend the pellet using this liquid until that pellet also disperses into solution.



Do so for all the remaining pellets until they are all concentrated into a single tube. To this tube, add sterile distilled water to the 15 mL mark. Cap the tube and invert several times to mix the contents. Spin the tube down for 10 minutes at 3500 rpm in a blood centrifuge. Be sure to use a counterbalance 15 mL tube filled with water to properly balance the centrifuge rotor.

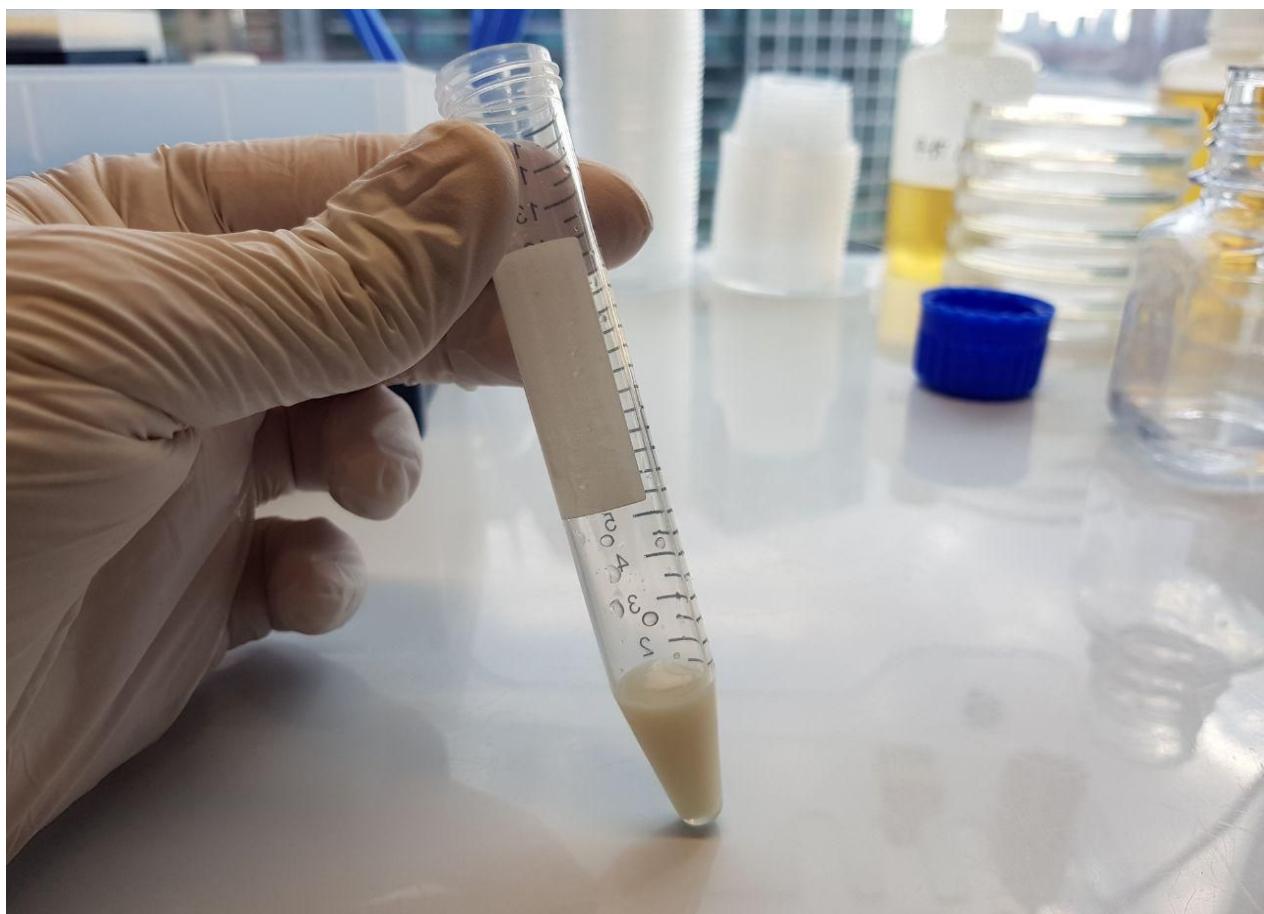


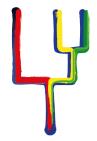


Discard the liquid portion of the bacterial tube, be mindful of the pellet.

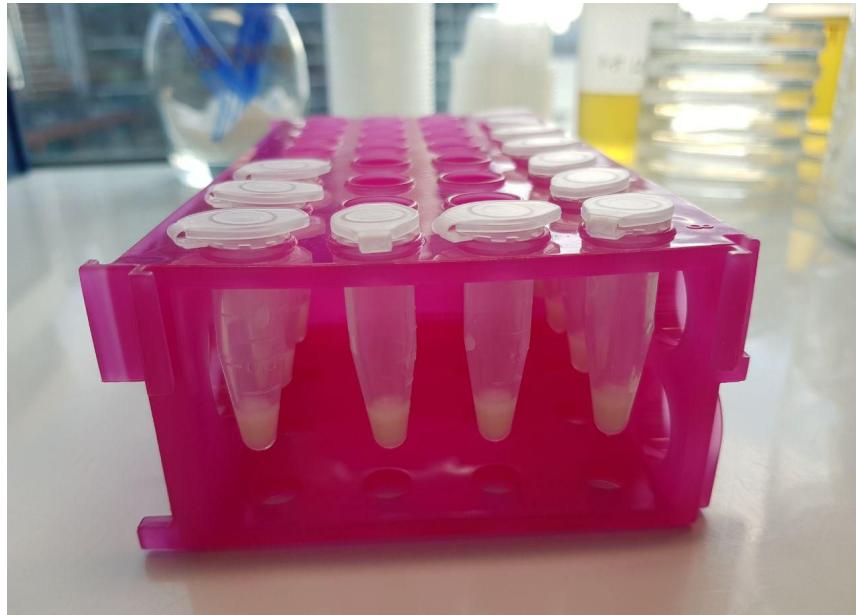
Resuspend pellet in 1 mL of sterile distilled water. Add sterile distilled water to the 15 mL mark, cap, invert to mix, and spin down again. This is the second of three washing steps. The objective of this task is to wash away the protein debris of the cells but more importantly the salts in the bacterial suspension. Electroporation works on the formation of a strong electromagnetic field, onto which the cells align and form pores in the membrane. Contrary to initial thoughts, we do not want there to be actual current flowing across the plates of the electroporation cuvette, thus the resistance of the liquid added to the cuvette must be very high. To reduce the conductivity of the bacteria suspension, 3 washes with distilled water will suffice.

Repeat the wash step one more time, for a total of three washes. Discard the liquid portion carefully, add 1 mL of sterile 10% Glycerol solution. Resuspend slowly, until the pellet is thoroughly dispersed in solution.





Distribute 100  $\mu$ L of this final bacterial solution into 10-12 sterile 1.5 mL microcentrifuge tubes. Label each tube cap with the cell line name and date.



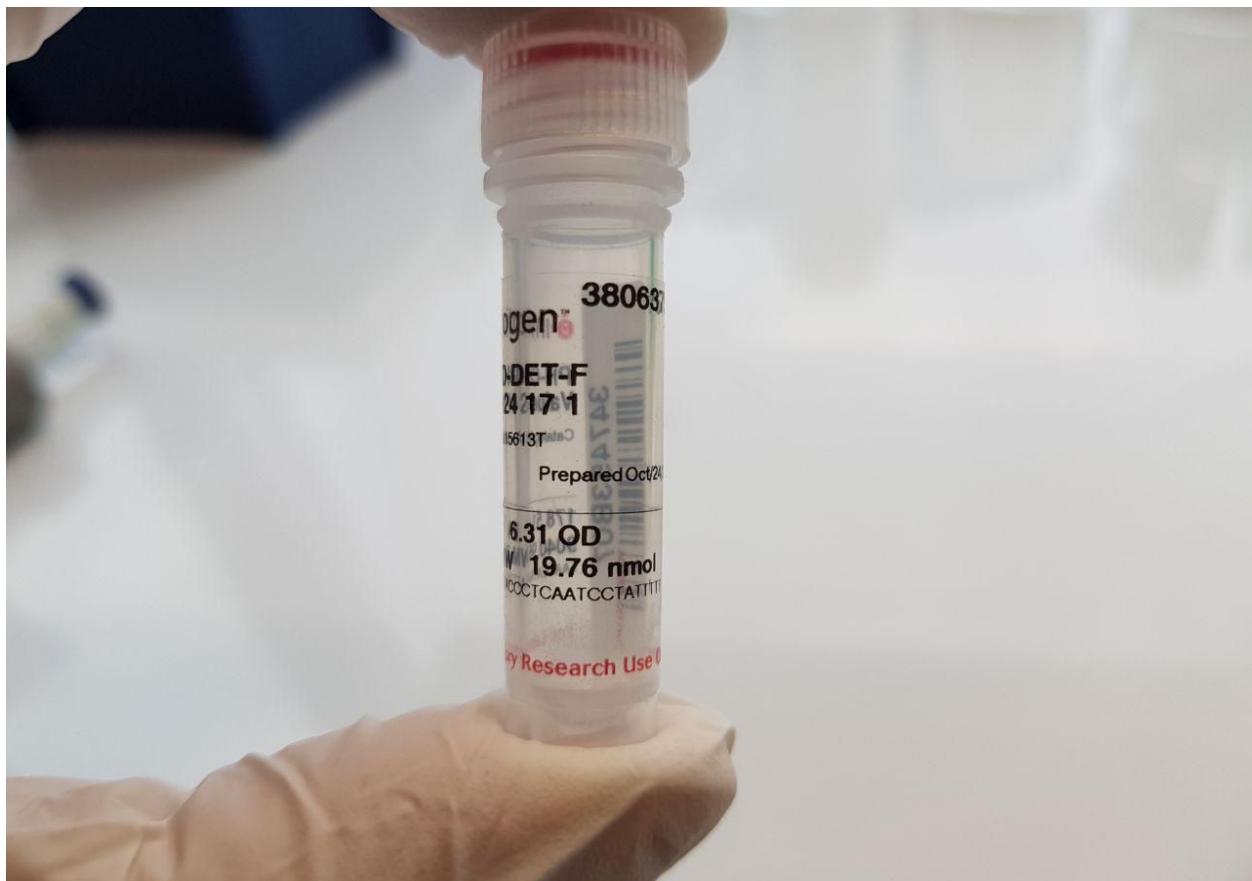
Place tubes in freezer until needed. Both electrocompetent and chemically competent cells last no longer than a month if stored at -20°C (standard freezer temperature).





**27.** Now that we've made competent cells, let's move on to some molecular work. First thing on the agenda is resuspending primers. Primers come dry in a tube as a thin film that is almost always stuck to the very bottom of the tube and impossible to see. Sometimes this film loosens from the walls of the tube during shipping and it may be flopping around in the tube. To ensure it's at the bottom, let's go ahead and give it a short 10 second spin in a microcentrifuge at maximum speed.

**28.** If you look at the primer tube, you will note a bunch of info on the tube label. The most important number here is the amount of nanomoles of DNA that is present in the tube. PCR is a VERY sensitive reaction, requiring stringent concentrations of reagents. The most important concentration to worry about in our case here is the primers. We want to normalize the concentration to 100 millimolar (mM) regardless of how much starting material we have to work with. Primers from Thermo-Fisher vary in the amount delivered between 10.0 and 30.0 nanomoles. In this case it's 19.76 nanomoles.





To figure out how much sterile distilled water we need to add to each tube, to make 100 mM concentrated stocks, we simply move the decimal place over once such that 19.76 nanomoles becomes 197.6 microliters of water. Add that exact amount to the tube and do the same for each subsequent primer tube. Allow the water to dissolve the primers for 5-10 minutes and then vortex 10 times in 1 second pulses and then one time for 10 seconds straight. Spin down the tubes for 10 seconds. Keep in freezer until needed. Now that you normalized your primers to the 100 mM stock concentration, we can move on to the next step.



**29.** We just made master stocks of each of our primer tubes and now we need to make our working stocks. These tubes will contain a final concentration of 10 mM of primer. To do this we simply thaw the primer master stock tubes from the freezer, add 180  $\mu$ L of sterile distilled water to a sterile microcentrifuge tube, label it with the primer that is about to be added, and then add 20  $\mu$ L of that primer from the master stocks to the tube. Vortex, spin down, freeze until needed. These primer tubes are now ready for PCR.





**30.** So far we've made media, made competent cells, and resuspended primers. We are now ready to begin extracting genomic DNA from our Petunia plant! Let's begin.

Select one happy, healthy petunia plant that is pigmented. Don't use white petunias, since it isn't certain that the chalcone synthase gene is absent or present but broken in some way. If you are really curious, include one white petunia plant and see if you get a positive signal from the later PCR reaction. You will need two samples either way so you can mix and match the plants in question. Either way remember to label tubes and don't lose track of the actual plant you took samples from since you may need to resample or include it into other protocols.



**31.** Collect two sets of 100 mg of leaf tissue from the youngest leaves on the plant. Plant cell count does not vary much between young and old leaves, since the cells just expand until mature rather than multiply through mitosis. All you would be doing when selecting a larger leaf vs a smaller one is diluting the amount of cells, thus the amount of genomic DNA we are able to obtain.



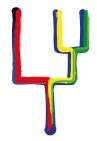


**32.** Label two microcentrifuge tubes with some identifier to denote it's petunia genomic DNA. Since both tubes come from the sample plant, you do not need to discriminate the two samples entirely. An arbitrary "Pet 1" and "Pet 2" would suffice. Add the 100 mg of each plant sample to the corresponding tubes.



**33.** Add 500  $\mu$ L of lysis buffer to the tube and using a sterile micropipette, begin to homogenize the leaf tissue until no large clumps form and the tissue is uniform in texture.





**34.** A continuous cycle of downwards pushes with a twist at the end is enough to thoroughly homogenize. Ensure no large pieces of tissue remain and no leaf tissue falls from the tube.

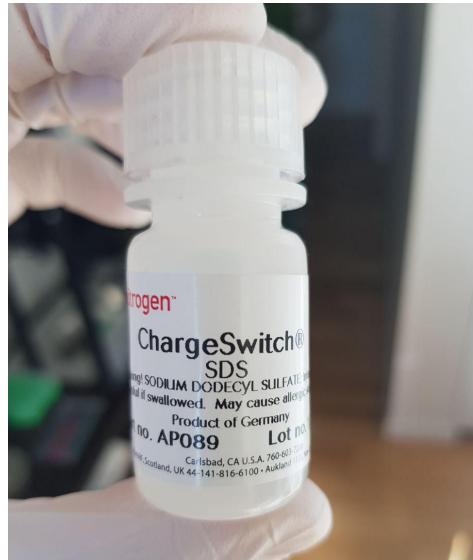


**35.** Once completely homogenized, add 2 µL of RNase from the fridge. This helps digest all the RNA that has been released from the ruptured cells which can inhibit sequencing, routine cloning, and even PCR in some cases. Add another 500 µL of the lysis buffer. Normally the kit asks for the addition of this buffer in it entirety but it wouldn't leave any room for the pestle, so we split the reaction during grinding.



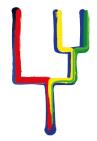


**36.** Now add 100 µL of 10% SDS to the homogenate, mix via inverting the tube several times and then allow the tube to incubate for 5 minutes.



**37.** Add 400 µL of Precipitation Buffer (N5) to the homogenate. Invert 10 times to mix thoroughly.





**38.** Spin down the homogenate tubes at maximum speed in a microcentrifuge for 5 minutes to produce a clear lysate.



**39.** Transfer only the liquid portion to a new labeled 1.5 mL microcentrifuge tube. Avoid the green pellet at all costs. We are now ready for the purification steps!





**40.** Add 100  $\mu$ L of 10% Detergent (D1) to the tube. During the next steps **DO NOT CLOSE THE CAP ON THE TUBE**. The volume will be close to the brim and you will lose reagent if you close the cap.



**41.** Resuspend a magnet bead tube by vortexing thoroughly. The magnetic beads will settle rather quickly and you'd want them kept in suspension to achieve a reliable concentration of beads.





**42.** Add 40  $\mu\text{L}$  of resuspended magnetic beads to each tube. These beads will begin to bind DNA to them due to the functionalized chemical groups on the surface of the iron nanoparticles that attract the strands.

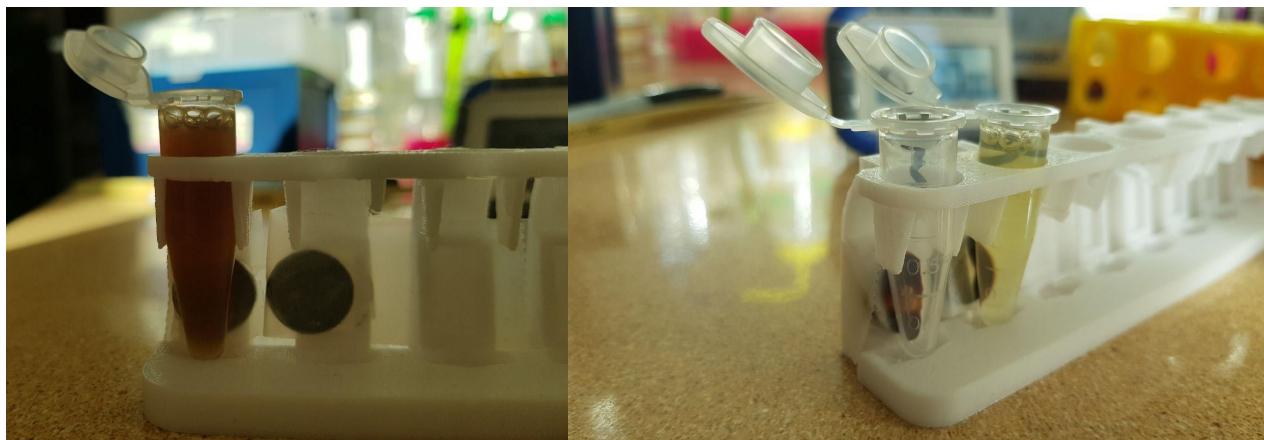


**43.** Gently, and I mean GENTLY, pipette up and down to mix the lysate with the magnetic beads using a 1000  $\mu\text{L}$  pipette set to 900  $\mu\text{L}$ . **AVOID MAKING BUBBLES AT ALL COSTS.**





**44.** Incubate for one minute at room temperature and then place the tubes into a magnetic tube rack. The commercial racks are a total rip off so you can either make a simple rack using a standard microcentrifuge tube rack, a magnet, and tape. I use a 3D printed rack from Thingiverse (<https://www.thingiverse.com/thing:79424>). The idea is to make something that holds a tube and one of the walls of the rack contains a magnet held close to the tube to hold onto the magnetic beads while you pipette liquids in and out of the tube. This allows you to wash away all the proteins, debris, and other junk but keep the DNA safely bound to the walls of the tube. Allow the tube to sit in the magnetic rack for 3-5 minutes, depending on the pull strength of the magnet. At this point, it's better to overshoot the time required to collect all of the magnetic beads against the wall of the tube. Since this is a critical step and we are not using the commercial magnetic beads, the time required to fully collect them will vary and best be determined empirically.



**45.** Note the next step will require a bit of speed so be sure to read the step ahead of time and have your pipette and reagents in hand. Without removing the tube from the magnet, carefully pipette out the liquid portion. Be sure to not touch or disturb the clump of magnetic beads in the process. Quickly add 1 mL of Wash Buffer (W12) to each tube containing exposed magnetic beads. You don't want the beads to dry so be sure to add the wash buffer immediately after removing the liquid portion.





**46.** Using a 1000  $\mu\text{L}$  pipette set to 900  $\mu\text{L}$ , gently mix the magnetic beads in the wash buffer by pipetting up and down slowly 5 times. Do not overmix. If the magnetic beads are still clumped together, don't worry, this is okay.



**47.** Place the tube back on the magnet rack and allow 3-5 minutes for the beads to collect against the wall of the tube.





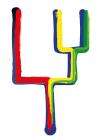
**48.** Repeat steps 45 through 47 to wash the magnetic beads once more. This ensures any and all lysis buffer, residual proteins, inhibitory chemicals, etc. are washed away leaving behind only magnetic beads and the DNA bound to it.

**49.** Once you remove the wash buffer, quickly add 100 µL of elution buffer. Remove the tube from the magnet rack. Pipette the magnetic beads up and down 15 to 30 times to fully resuspend the magnetic beads. Allow this reaction to incubate for 1 minute at room temperature.

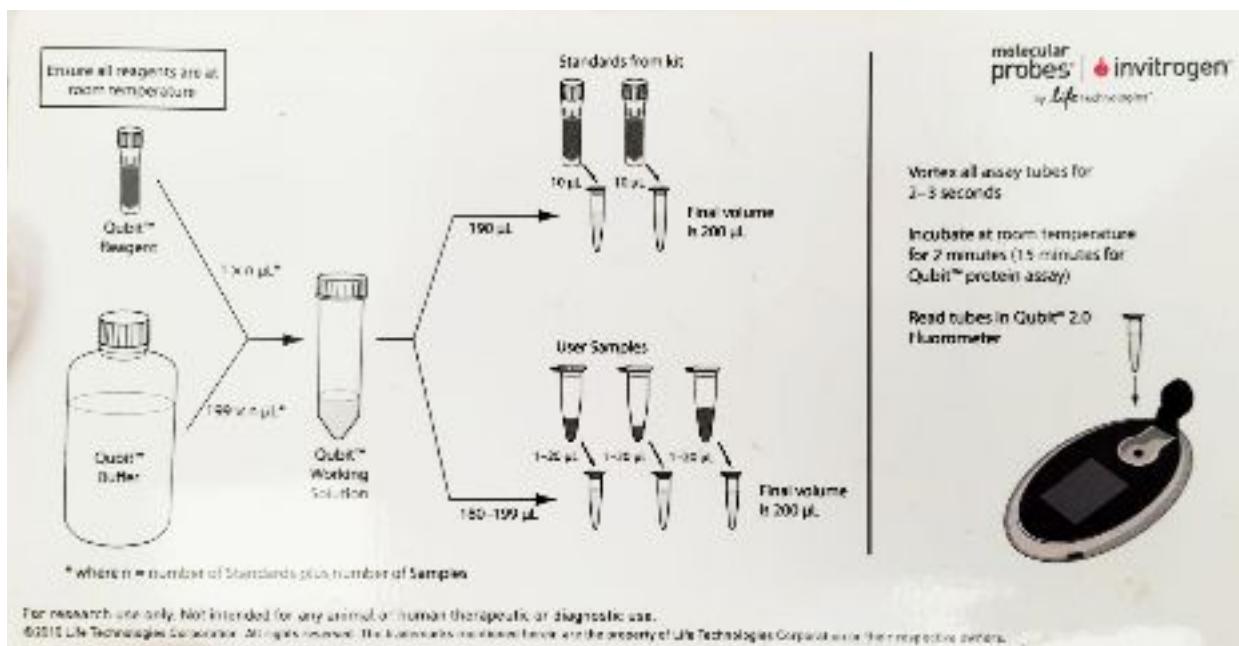


**50.** Apply the tube to the magnetic rack one last time. Allow the reaction to sit for at least 5 minutes to fully separate out all of the beads from the liquid portion. This time we will be keeping the liquid portion since it contains our genomic DNA of interest. While leaving the tube onto the magnetic rack, carefully remove the liquid once the magnetic beads completely collect against the wall. Dispense that liquid into a new tube. Label this tube with the name of the plant, the word “gDNA”, the date, and some kind of sample number or identifier in case you have multiple samples from the same plant. Now that we extracted the DNA, let’s see how much we have!





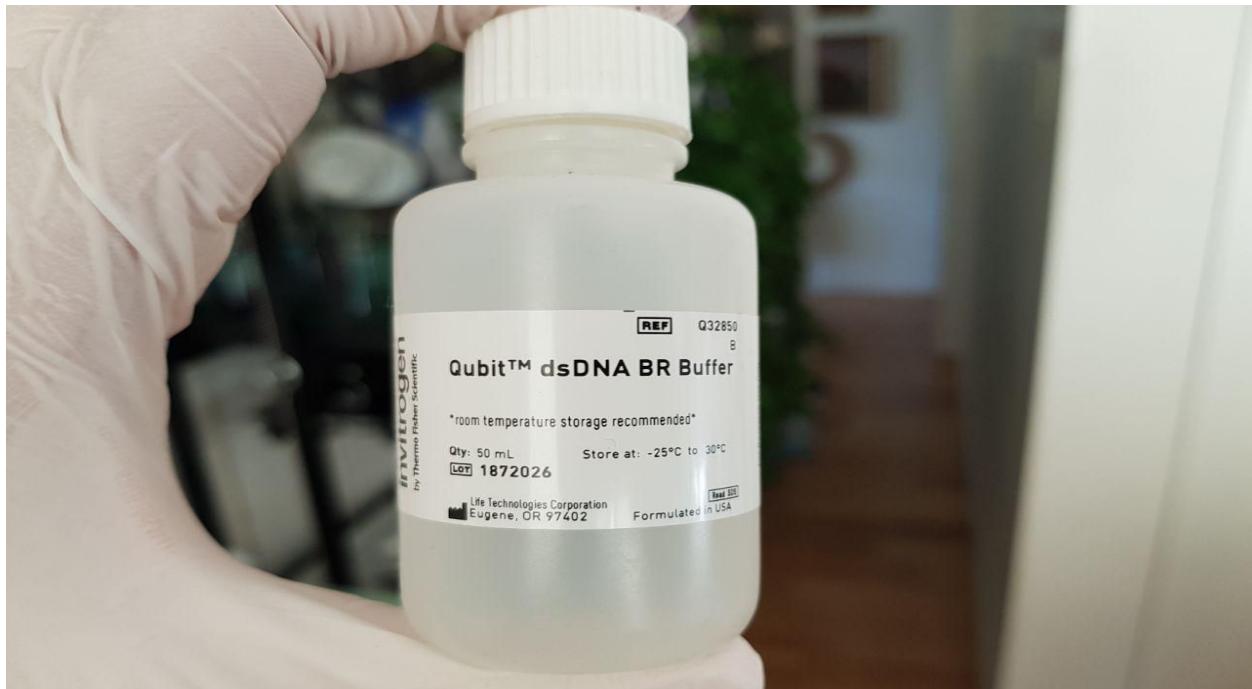
**51.** Gather the supplies needed for running the Qubit 2.0 Fluorometer. This includes the buffer, DNA dye, Low & High Standards, and the fancy clear tubes that come with the device that I have a sneaking suspicion is just thin walled 0.5 mL PCR tubes. Once you have all of the things in place, allow the standards to equilibrate to room temperature since they live in the fridge normally.



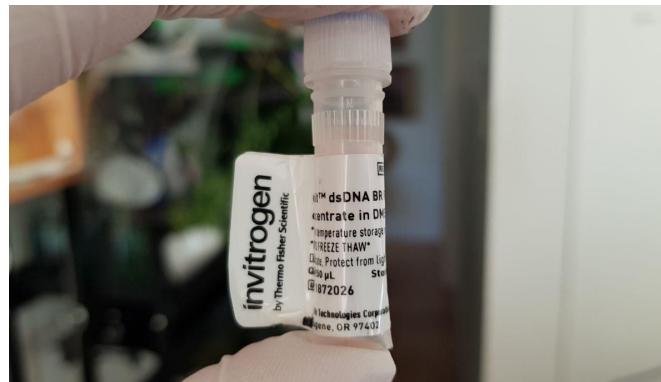
**52.** According to this handy dandy flowchart, we first calculate how many samples we will be needing for this experiment. In our case it would be the two petunia genomic DNA samples we just made, the low standard containing 0 ng/ $\mu$ L DNA, the high standard containing 100 ng/ $\mu$ L of DNA, and one extra reaction for the “devil’s cut”, an additional reaction to ensure any kind of routine pipetting error does not leave us lacking one whole reaction’s worth of volume. Our total amount of samples is 5 so we will need 1000  $\mu$ L of buffer (200  $\mu$ L per sample), 5  $\mu$ L of DNA dye, and 4 fancy tubes to hold each reaction.



**53.** To a sterile 1.5 mL microcentrifuge, add 1000 µL of the Qubit buffer. This has all the necessary chemicals at the right pH and concentration to promote the binding of the DNA dye to the DNA itself.



**54.** Now take out 5 µL from the tube containing the buffer and then replace the removed volume by adding 5 µL of the DNA stain to said tube. It's far simpler and at times more accurate to remove a volume from something than to pipette the exact amount, since larger pipettes are less precise than smaller ones. We want 5 µL of DNA dye IN 1000 µL of buffer so, instead of pipetting 995 µL, we simply remove 5 µL of the buffer and replace it with 5 µL of dye. Store DNA dye stock in dark afterwards.





**55.** Vortex the solution for a few seconds to evenly distribute the DNA dye in the buffer. The tube should now take an ever-so-slight pink hue. Avoid direct sunlight if possible.

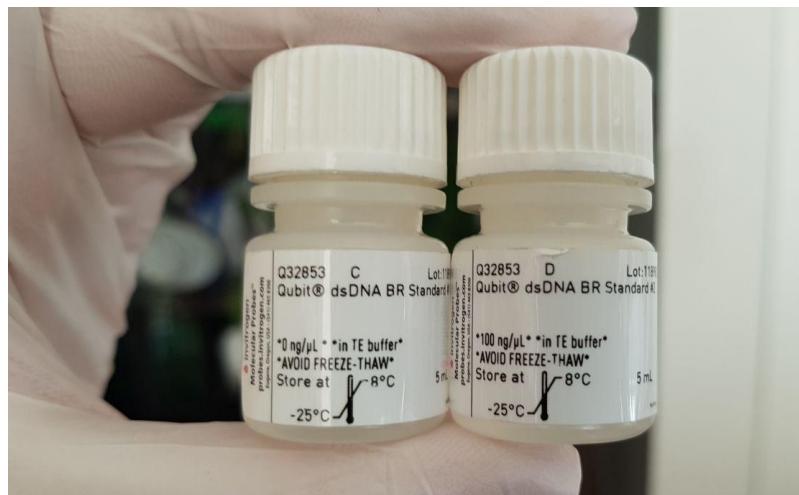


**56.** Distribute 190  $\mu$ L of the now complete working buffer we just prepared into each of the four labeled Qubit tubes. Make sure you do not write on the conical part of the tube wall, since this is where the Qubit fluorometer takes its readings.





**57.** Add 10 µL of each of the DNA standards to the corresponding tubes.

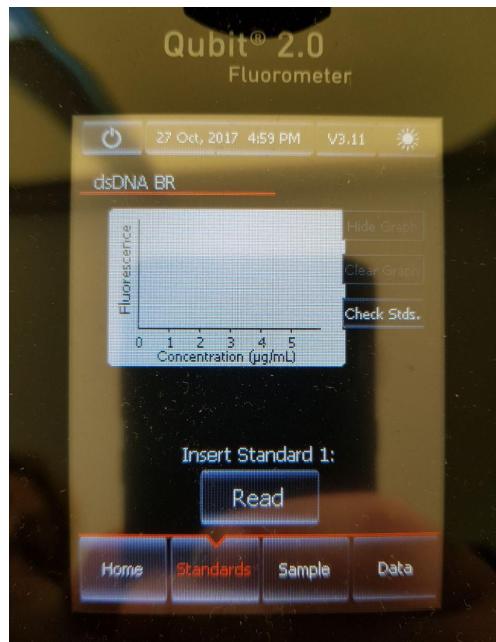


**58.** Add 10 µL of each of the Petunia Genomic DNA tubes to the corresponding Qubit tubes. Vortex all of the tubes and allow them to incubate at room temperature **AWAY FROM SUNLIGHT** for 5 minutes.

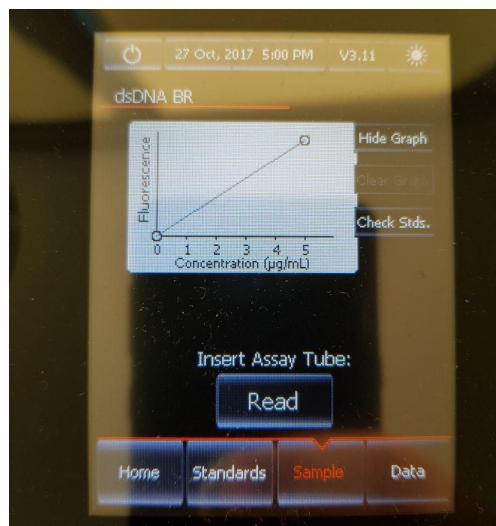




**59.** Turn on your Qubit 2.0 Fluorometer. Click on the DNA button. Select dsDNA Broad Range. It will then prompt you if you'd like to read new standards. Press yes and it will take you to this screen. Place the Low Standard into the qubit and press the Read button. A white dot should appear in the lower left corner of the graph.

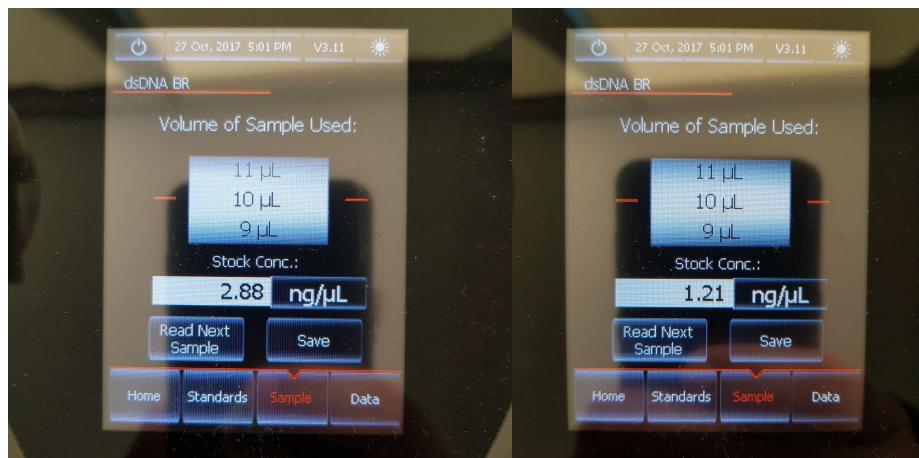


**60.** Add the High standard tube the Qubit and press the Read button. It should then add a dot to the upper right hand corner. The machine now knows how zero DNA and a LOT of DNA looks like in terms of fluorescence. Your machine is now calibrated and can accurately read your unknown samples of petunia genomic DNA.

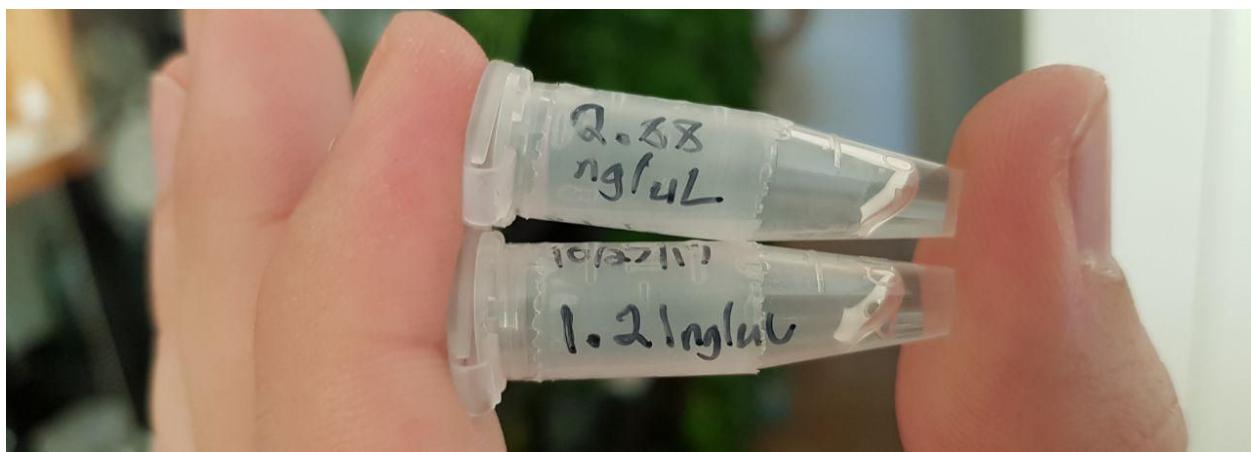




**61.** Add your petunia genomic DNA qubit tubes to the fluorometer and press the Read tube button. Then click on the calculate concentration button and switch the units to ng/ $\mu$ L (a more relevant concentration for our purposes). These were the results of my Purple and White petunia respectively.



**62.** Write down the values of your DNA quantification onto the corresponding tubes. We are now completely finished with the genomic DNA isolation, purification, and quantification steps. Congratulations! Store genomic DNA in freezer until needed.





**63.** Now that we finished all the preparatory steps, let's review what we are going to do next.

We are going to run a PCR (polymerase chain reaction) using a very special kind of DNA polymerase called Q5. This is an enzyme similar to the high fidelity PFU polymerase in that it has a fidelity rating (rate of error during DNA copying) of 289x better than conventional Taq Polymerase. This means that there is a very low chance of getting amplification errors (base pairs that are incorrectly added) during the PCR reaction of our gene of interest. We will be using our freshly isolated genomic DNA and our recently resuspended and diluted primer working stocks. Along with those, we will need our DNTP mix (free-floating As, Gs, Cs, and Ts needed during PCR), the Q5 reaction buffer, sterile distilled water, 0.2 mL PCR tubes, and a bucket of crushed ice or a fancy tube chiller block. Regardless of what source of ice you use, you are going to need to prepare all of the reagents in a cold setting since Q5 will amplify poorly if warmed with primers present. Let's amplify our gene!

**Protocol for a Routine PCR**

- Mix individual components prior to use.
- Assemble all reaction components on ice.

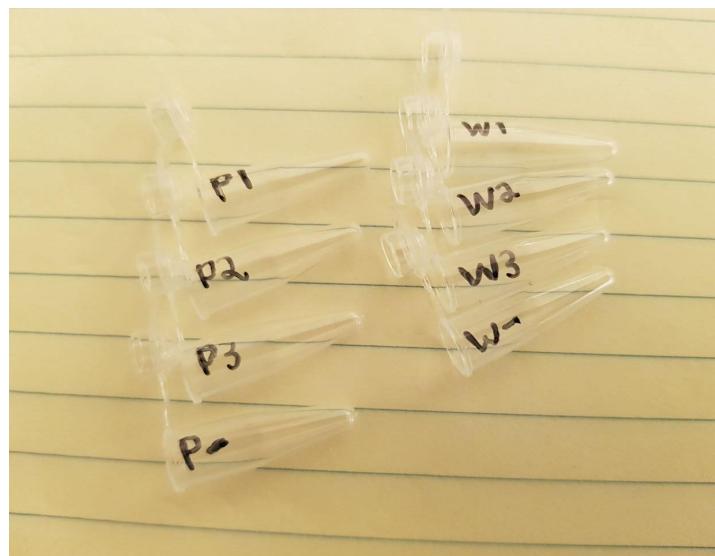
COMPONENTS	25 $\mu$ l RXN	50 $\mu$ l RXN	FINAL CONC.
5X Q5 Reaction Buffer	5 $\mu$ l	10 $\mu$ l	(1X)
10 mM dNTPs	0.5 $\mu$ l	1 $\mu$ l	200 $\mu$ M
10 $\mu$ M Forward Primer	1.25 $\mu$ l	2.5 $\mu$ l	0.5 $\mu$ M
10 $\mu$ M Reverse Primer	1.25 $\mu$ l	2.5 $\mu$ l	0.5 $\mu$ M
Template DNA	variable	variable	<1,000 ng
Q5 High-Fidelity DNA Polymerase	0.25 $\mu$ l	0.5 $\mu$ l	0.02 U/ $\mu$ l
5X Q5 High GC Enhancer (optional)	(5 $\mu$ l)	(10 $\mu$ l)	(1X)
Nuclease-Free Water	to 25 $\mu$ l	to 50 $\mu$ l	



**64.** Thaw the following primers and place them on your ice block:

PH-CHSD-DET-F  
PH-CHSD-DET-R  
PH-CHSD-WIDE-F  
PH-CHSD-WIDE-R  
PH-CHSD-SacI-F  
PH-CHSD-XbaI-R

Label four tubes for each genomic sample you prepared. In my case I had one white petunia and one purple petunia I wanted to test so I labeled the purple petunia DNA tubes as P1, P2, P3, P- and for the white I labeled them as W1, W2, W3, and W-. The P- and W- tubes will contain distilled water as a negative control for the genomic DNA. This ensures that any signal we get from the PCR reaction was not caused by contaminated water. Since all land plants have this gene, the risk for contamination is rather high. Good experimental design always has at least one negative control, and if possible a positive control. Since we are testing out the primers for the first time, and you have no means of acquiring a sequence confirmed sample of Chalcone Synthase D, we have to rely on the size of the resulting bands as our indicator of successful PCR.





**65.** Place the tubes on ice and to each tube, add 2  $\mu$ L of the corresponding DNA source to it. Pick up your tube and pipette while looking at the tube to ensure proper pipetting occurred. Small volumes like 2  $\mu$ L can sometimes come out poorly and pipetting small volumes into a dry tube can often result in error if not monitored properly.



**66.** Prepare a recipe for a master mix for the amount of Q5 reactions we desire. In this case it's 8 tubes worth. I was a little too paranoid and made a master mix for 10 reactions to be super certain I did not lose a sample due to pipetting error. Since Q5 polymerase is expensive, you do not need to do more than 1 extra reaction for every 10 reactions worth of PCR you plan on doing. We then look to the recipe for a single Q5 reaction and multiply the values for each by the total number of reactions we are preparing. In our case it's simple since we are multiplying our values by 10.

Link to recipe here:

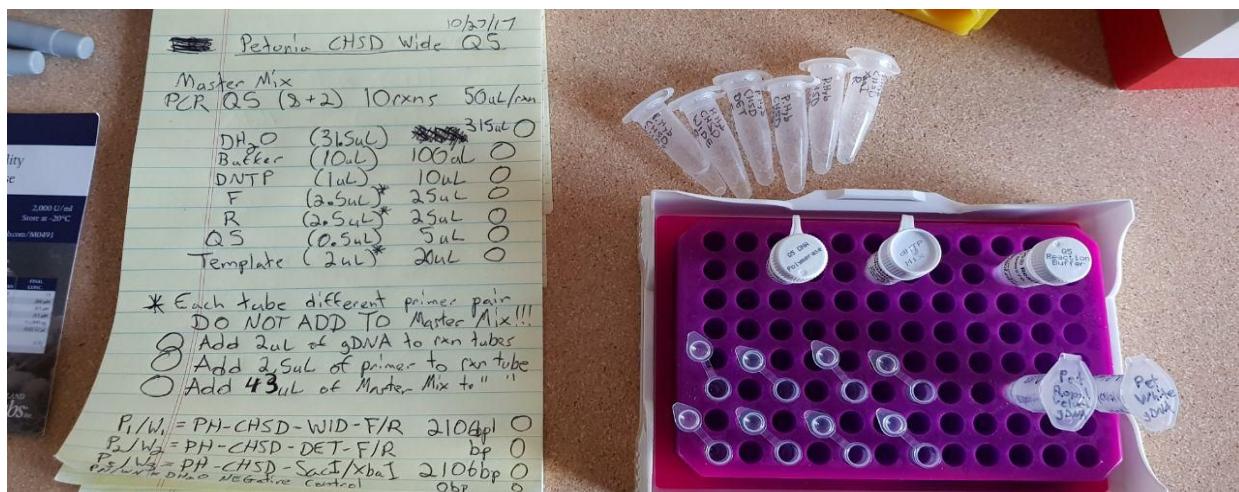
<https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491>



And here is a table for your convenience:

Component	Volume for 1 rxn	Volume for 10 rxns
DH <sub>2</sub> O	31.5 µL	315 µL
Q5 Buffer	10 µL	100 µL
DNTPs	1 µL	10 µL
Q5 DNA Polymerase <b>ADDED LAST</b>	0.5 µL	5 µL

This is for a total of 50 µL per reaction tube. Note the template DNA is already in the PCR tubes so when we dispense the master mix, we will be adding 43 µL of Master Mix to the tubes containing DNA so the resulting final volume is 50 µL. We split the template from the master mix, or any other component that is different. In this case we are also using different primers for each reaction so we leave out the primers from our master mix and pipette them into each tube instead. The purpose of a master mix is to create a uniform pool of commonly shared reagents such that the reactions have similar components for things that are similar (this avoids pipetting errors causing different results) and helps us better isolate the reagents that we are changing as our variables. In this case both the template DNA **AND** the primer pairs are different for each reaction tube. Here is my notepad explaining the steps I am about to take. Note that I added a circle at the end of each step. This is a checkbox and I try to make it a habit of maintaining a checklist for every experiment regardless of how many thousands of times I've done this. All it takes is a moment's lapse in concentration or attention and you end up adding too much of a component or skipping it at all. A checklist helps prevent that.





**67.** To a sterile 1.5 mL microcentrifuge tube add the components of the master mix calculated above. **DO NOT ADD THE Q5 POLYMERASE YET!** Leave the master mix capped and on ice for now and move on to the next step.

**68.** We establish that P1 and W1 will contain the PH-CHSD-WIDE primer pairs, the detection primer pairs will be in P2 and W2, and the cloning pairs (PH-CHSD-SacI-F and PH-CHSD-XbaI-R) will be in P3 and W3. For the P- and W- tubes containing water instead of petunia genomic DNA, we will add the detection primers since these primers have the highest certainty of giving a signal if the gene is present therefore a good indicator of plant genomic DNA contamination in the water we used. Dispense 2.5  $\mu$ L of the corresponding primers into their respective tube. Here is how each tube should look like:

Tube Name	Primer Pair	Volume
P1	PH-CHSD-WIDE-F PH-CHSD-WIDE-R	2.5 $\mu$ L of each
P2	PH-CHSD-DET-F PH-CHSD-DET-R	2.5 $\mu$ L of each
P3	PH-CHSD-SacI-F PH-CHSD-XbaI-R	2.5 $\mu$ L of each
P-	PH-CHSD-DET-F PH-CHSD-DET-R	2.5 $\mu$ L of each
W1	PH-CHSD-WIDE-F PH-CHSD-WIDE-R	2.5 $\mu$ L of each
W2	PH-CHSD-DET-F PH-CHSD-DET-R	2.5 $\mu$ L of each
W3	PH-CHSD-SacI-F PH-CHSD-XbaI-R	2.5 $\mu$ L of each
W-	PH-CHSD-DET-F PH-CHSD-DET-R	2.5 $\mu$ L of each

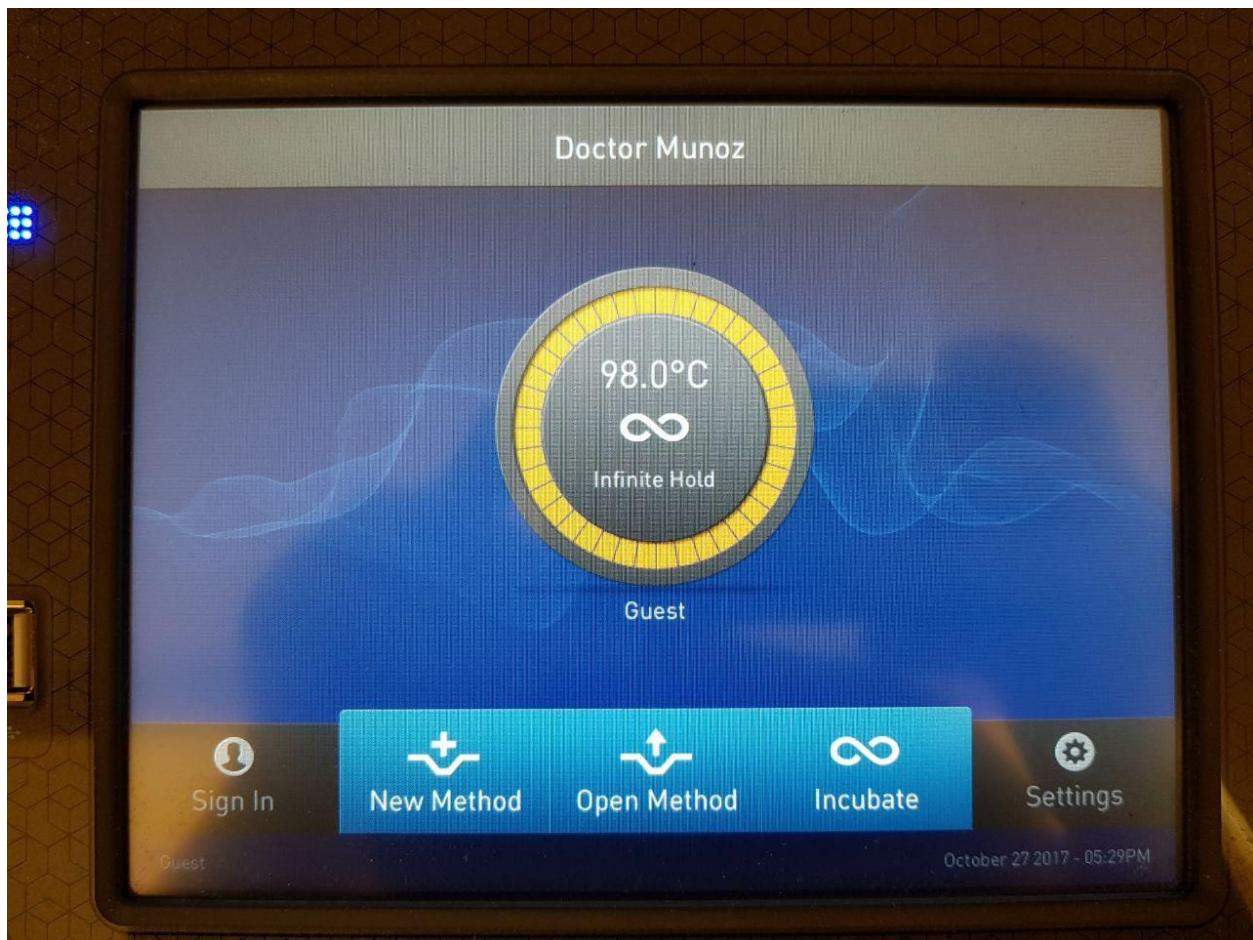
Try to have a system in place that allows you to visually see which tube you just pipetted into. For me, I just place the tube I used one row above the rest of the tubes in my ice block. This helps me recheck if I lose track. Go slowly and carefully.



**69.** Once all of the primers are added to their corresponding tubes, add 5 µL of the Q5 DNA Polymerase to the Master Mix tube, vortex the tube for a few seconds, distribute 43 µL into each PCR tube.

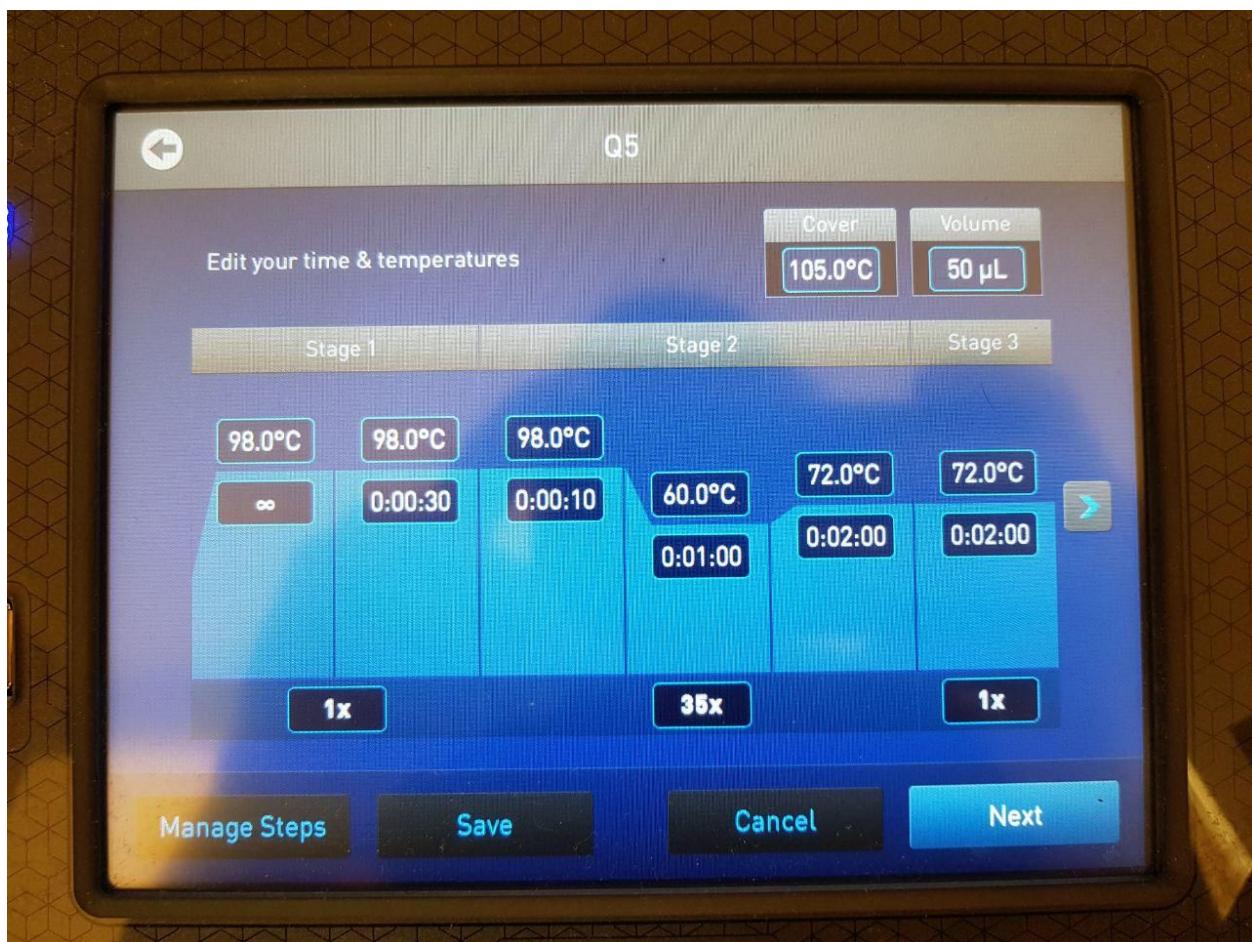
**70.** Cap the tubes and vortex each one to ensure proper mixing. Either spin down the tubes if you have a PCR tube centrifuge, if not just whip the tubes down with your hand to ensure all the contents are on the bottom of the tube. Sometimes, during vortexing, the reaction liquid will end up in the cap. One or two downward whips will suffice. Place the tubes back on ice.

**71.** Preheat the PCR machine to 98 degrees Celsius. Ensure the block reaches the proper temperature BEFORE placing your tubes into the block.





**72.** Program your PCR machine with the following parameters:



The interface of your machine and how to go about programming it varies from device to device so I will use my SimplyAmp's program layout to help you visualize the program itself. Starting from the left most column in Stage 1 we have a 98 degree "soak". This holds forever and I set it up like this so I can immediately go from preheating the PCR machine at 98°C to the program that follows. Most PCR machines can link a "hold forever" command to a cycling program. Again, please refer to your PCR machine's manual for how to do this.

The next stage is called the initial denaturation. This, like it says, is the first denaturation of the DNA present in the sample. It essentially breaks apart the double stranded DNA causing the complementary bases to be exposed. We want the first step to bind properly, so the Initial Denaturation step tends to be longer, in this case 30 seconds at 98°C.



After this step we go to the main stage of PCR, the cycling steps. According to NEB, here is the routine PCR program for utilizing Q5 polymerase:

**Thermocycling Conditions for a Routine PCR:**

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C *50–72°C 72°C	5–10 seconds 10–30 seconds 20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

I tend to use the maximum time for each step when amplifying from genomic DNA so our program does the following:

Denature	98°C	10 seconds
Anneal	60°C	30 seconds
Extend	72°C	<b>60 seconds*</b>

\*Note our gene of interest is 2188 basepairs long so we need the extension step to be 60 seconds long (30 sec per 1000 bp). We will run this cycle 35 times and then do a final extension of 2 minutes for the polymerase to finish extending any parts of the DNA that did not fully extend due to imperfect timing.

**73.** Place your tubes in the preheated PCR block, set the program as above, and run the PCR. Good luck!

**74.** While we wait for the PCR to finish (about 2 hours on my machine, varies depending on speed of heating and cooling but expect at least 2 hours), let's pour an agarose gel. This will help us visualize the resulting PCR reaction as bands on the gel and we will compare the lengths of DNA fragments in those bands against a ladder of known DNA concentrations running alongside the PCR products. More on that in a bit, but for now let's focus on preparing the buffers and gel.



To start, let's gather the needed components: 50x TAE buffer, Agarose, a 250 mL Erlenmeyer flask with rubber stopper, 100 mL graduated cylinder, 1000 mL volumetric flask, 10 mL graduated cylinder, distilled water, weigh boat, GelGreen 10,000x Concentrated DNA Stain, and an analytical balance or scale accurate to 1 mg.

**75.** Weigh out exactly 1.500 g of Agarose powder. Add this powder to a 250 mL Erlenmeyer flask.

**76.** To a 1000 mL volumetric flask add 20 mL of 50x TAE Buffer. Add distilled water to the neck mark and during the pouring fill the 10 mL cylinder used to initially pour TAE and dump out the wash contents into the volumetric flask too. This helps pour out any residual TAE. Mix by inverting. You now have 1 liter of 1x TAE running buffer. Note the concentration or volume needed for a gel electrophoresis tank varies with make and model. The manufacturer always provides some sort of manual explaining that. Read and familiarize yourself with your specific gel box as it may be different from mine.

**77.** Add 150 mL of this 1x TAE Running Buffer to the 250 mL Erlenmeyer flask containing 1.5 g of powdered agarose. Swirl the contents to resuspend the powder. Note this will NOT dissolve unless heated.

**78.** Bring the flask to a boil in the microwave. Take the flask out and give it a swirl. Boil again until the solution becomes crystal clear.

**79.** Once the agarose is completely dissolved and molten, pour 50 mL of molten agarose into a 50 mL centrifuge tube. Add 1  $\mu$ L of GelGreen 10,000x DNA stain. Cap the tube and invert to mix.

**80.** Pour the molten agarose, pre-stained with GelGreen, into the gel casting tray.

**81.** Select the appropriate comb width and count depending on the amount of samples you have and comb selection available. For me, I plan on running two small gels so that the PCR reactions are separated as one gel per isolated plant. Again this is just personal preference so you could easily pour a single gel. For the purposes of publishing a photo of a gel, I prefer wide wells using only 10  $\mu$ L per well. This avoids overloading and gives nice sharp bands.



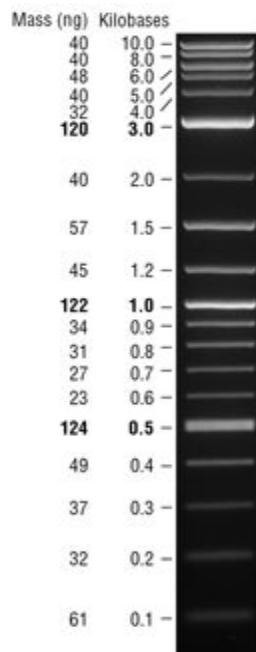
**82.** Ensure the comb is properly seated and there are no bubbles in the gel especially around the teeth of the comb. If bubbles form, pop them with a dry clean pipette tip.

**83.** Allow the gel to set while you wait for the PCR machine. Take a break. Go for a walk. Enjoy your time! :)

**84.** Once the PCR reaction is complete, take the tubes out of the machine and add 10 µL of the 6x Purple Loading Dye to each PCR tube. This will help the PCR sample sink to the bottom of the agarose gel well during electrophoresis. It also helps in visualizing where the sample is as you pipette the otherwise clear liquid into a clear well of a basically clear gel... submerged in buffer. You get the idea.

**85.** Vortex the PCR tubes. Whip them down to move the liquid to the bottom of the tube.

**86.** Thaw the **2-Log DNA Ladder**. This will help us in estimating the size of the DNA present in the PCR tubes after the reaction run. They contain fragments of DNA at known lengths and some ladders also include the actual molecular weight of those bands to compare both size AND brightness (indicating rough concentration). Here is a reference marker from the [NEB.com](http://NEB.com) website regarding this particular ladder:



Note the order of the rungs from bottom to top. We are expecting just above 2 kbp.



**87.** Take 10 µL of the PCR reaction per well and order the gel from left to right is as follows. Note we use only 5 µL of Ladder to conserve the reagent. If possible, always flank the gel sides with ladders so we can compensate for skewed gels.

#### Ladder

P1 - CHSD-WIDE Size expected: 2176 bp

P2 - CHSD-DET Size expected: 351 bp

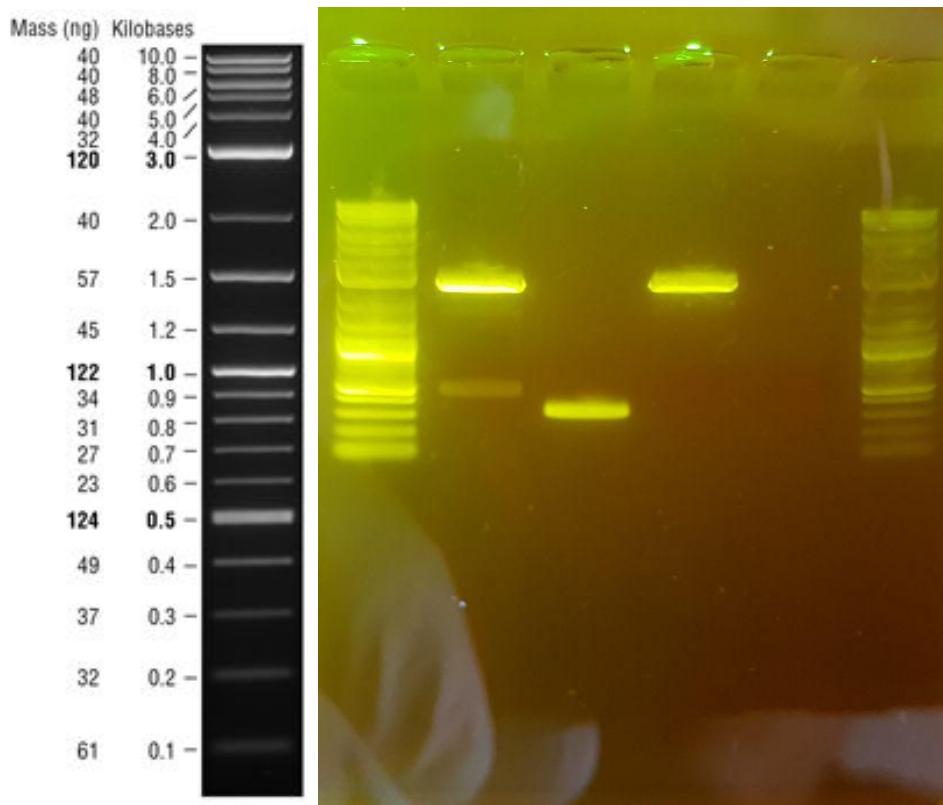
P3 - CHSD-SacI/XbaI Size expected: 2188 bp

P- - Negative control with water Size expected: 0 bp or just primer dimers\*

Note primer dimers are primers that were bound to themselves or each other causing very low (below the lowest rung on the ladder) bands to show up. This indicates no priming of target DNA.

Run the gel. The 2-log DNA Ladder 3 dyes that run at different speeds. You may wish to stop the gel run when the yellow dye reaches about 0.5cm from the bottom. This may take 10-20 minutes depending on the voltage and speed of your gel box.

**88.** Remove the gel from the chamber and place it on the LED transilluminator for analysis.





89. Paying close attention to the bands, with respect to the ladder, we see there is a bright band in the first lane after the left ladder and also a dim band above the 500 base pair mark. The ladder has very distinct bright bands to denote key sizes and help the human eye figure out the spacing without counting too many bands. The dim band is called a secondary amplification, a region of genomic DNA with a similar but not quite identical sequence that amplified along with our primary product. This may happen if the extension time is too long or the annealing temperature is too low. Either way, it actually proves to be an interesting note later. For this primer pair, we expected a fragment about 2100 bp. The bright band lined up just under the 3000 bp ladder rung but due to the compression of the way larger fragments move in 1% agarose, longer fragments do not always move in a predictable manner. Normally one makes a lower concentration gel (i.e. 0.8% or lower) to better resolve larger bands. Also, during microwaving some of the water in the gel evaporates increasing the actual concentration beyond 1.0%. As long as it's around the expected size we can take it for a grain of salt and move on. We do not know how this particular plant's CHSD gene will be in terms of size, since the intron region of genes tend to vary in length from organism to organism. The gene very well could be 3000 bp in this plant. We won't know for certain until we sequence the gene.

The next lane after the ladder in the gel sequence is the detection primer pair. This was a short fragment, INSIDE the gene of interest, that we amplified as a kind of insurance that the gene we are hunting is in fact there in case our WIDE or cloning primers did not bind. The outside of a gene — which is flanked by regulatory elements like promoters, terminators, 5' and 3' UTRs — may vary greatly, so our 25 bp primer binding sites may not always be there. However, the inside of the gene is much more highly conserved, so our detection primers have a much higher chance of matching up with the real life gene sequence in the genome of our plant in question. This seems to be positive as well. Looks like just above the third rung of the ladder from the bottom, which represents 300 base pairs. Our expected size was 351 bp so this is strong evidence the proper sized fragment has been amplified.

After the detection primers is a pair that took me by surprise and amplified when the odds of it working were stacked against it. These primers had an almost identical sequence to the CHSD-WIDE pair but, due to the addition of the adapter sites and landing pads to the primers, there was less base pairs of primer that actually bound to the genomic sequence. Less base pairs binding means less specificity, therefore more secondary amplifications. For one reason or another it actually gave a brighter and cleaner result than the WIDE primer pairs. We got lucky and saved us one whole Q5 PCR run since we were going to use the resulting

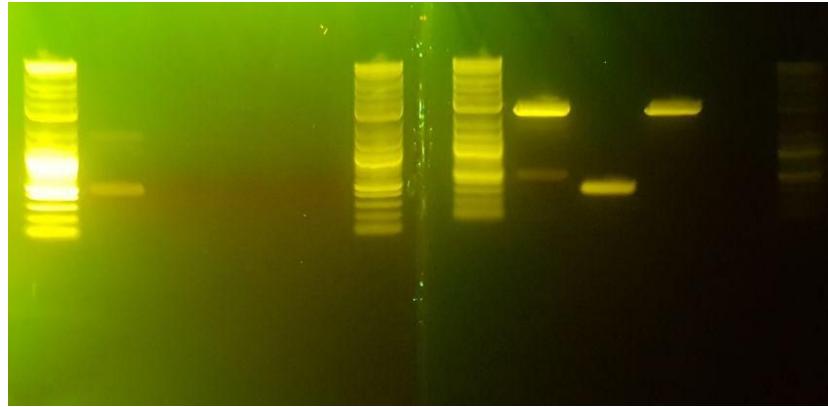


band of PH-CHSD-WIDE-F/R as the template for another PCR reaction, which had a better chance of adding the adapters to the gene, since the only piece of DNA in the PCR tube for the primers to bind to would have been our gene of interest. This process is known as **nested PCR**.

For complex or stubborn sequences, amplifying a larger piece of DNA, that is easier to design primers for, is necessary. Your gene of interest is somewhere within this fragment and we can purify and use the resulting PCR product as template for primers that target our gene of interest within this fragment. A commonly used tool and neat trick if your PCR fails even after a few tweaks.

The last lane on the gel before the rightmost ladder lane is our negative control. This should have ZERO bands anywhere above the primer dimer threshold (25–50 bp). This indicates we do not have any contamination that was amplifiable nor are any of the bands coming from DNA from within the water itself. A good way to avoid fooling ourselves, as Feynman would say.

As a side note, when I ran the white petunia DNA with the same primers I got back totally different results:



The gel on the left is white petunia, the gel on the right is the purple petunia. None of the primary products formed, BUT the secondary amplifications of the CHSD-WIDE DID show up. Curiously, this may mean the secondary amplification bands are common to both purple and white petunias but the exact primer sequences for the actual chalcone synthase sequence appears to be absent. Note this does not mean the whole gene is absent, just that our specific primer pair did not yield results. More PCRs using different primers would be needed for us to definitively say the gene is absent, but for now it does show some evidence that the gene may in fact be either different or absent. So many questions!



**90.** Okay, let's get back to lab work. Now that we know our PCR products gave positive results, let's pour one more gel to purify a concentrated amount of our PCR product from tube P3, the purple petunia gDNA amplified by PH-CHSD-SacI/XbaI. This fragment has the cut sites for the SacI and XbaI enzymes, explained previously during our SnapGene simulation. We will be using these sites to cut and paste this fragment of DNA into our pUC19 plasmid. To the gel, add the following in this order:

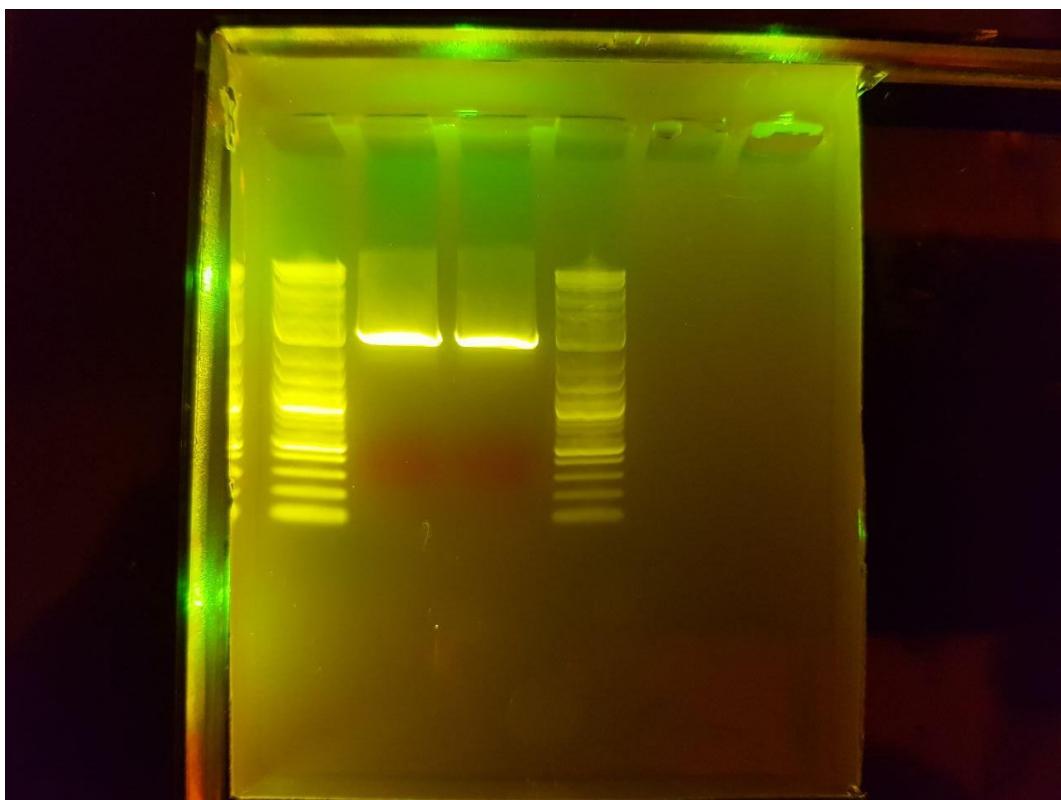
Ladder - 5  $\mu$ L

P3 - 20  $\mu$ L

P3 - 20  $\mu$ L

Ladder - 5  $\mu$ L

Run the gel and it should look like this:

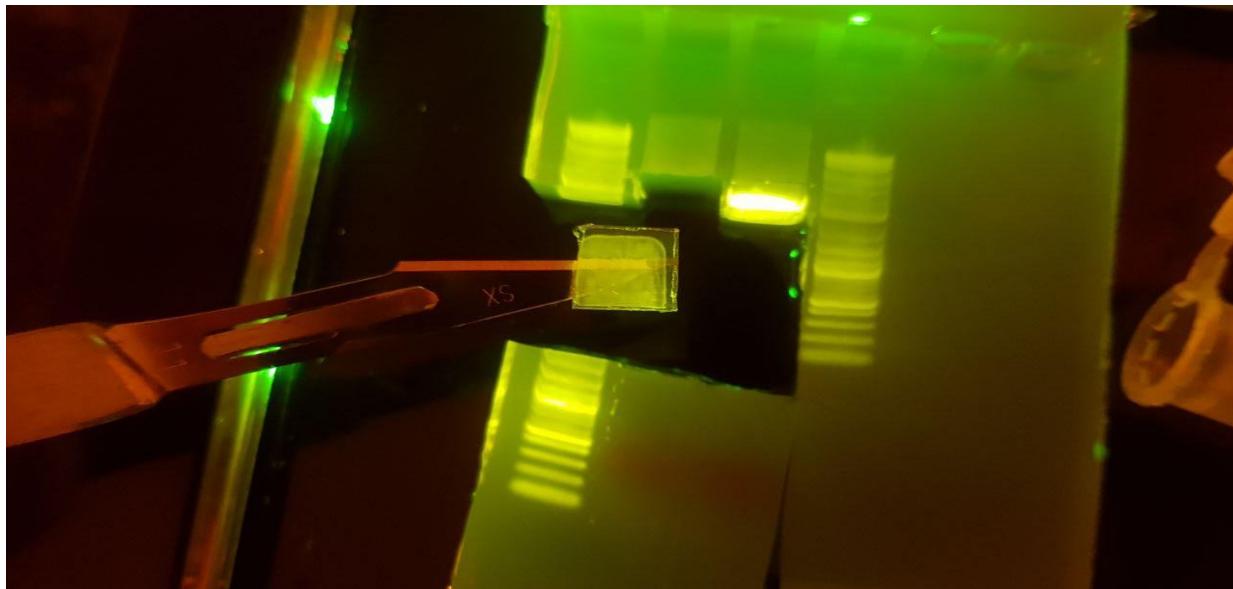


Note the super bright bands, and they line up with the 3000 bp mark, which is higher than expected but it could be an aberration of the gel or some other unknown factor. It could also be that the gene really is 3000 bp or thereabouts. We will know soon.

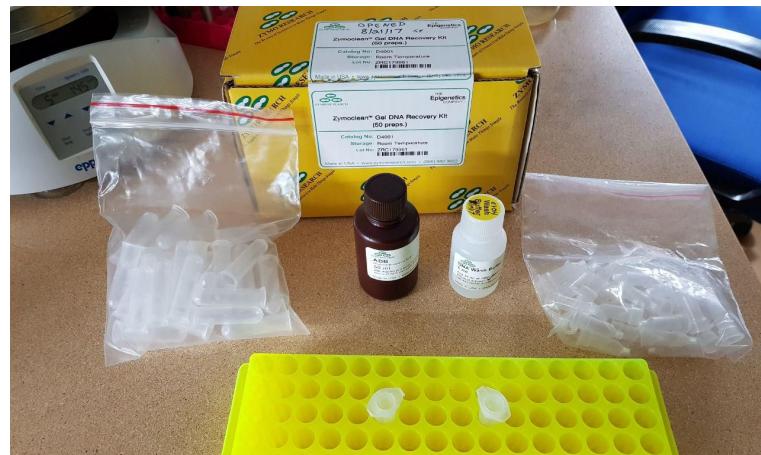


**91.** Using a #11 or #10 scalpel with handle, cut out the bands and place them in a 1.5 mL microcentrifuge tube one band per tube. Weigh and tare the microcentrifuge tube before adding the bands, so we know how much the gel fragment weighs. Be sure to cut as close to the band as possible.

**92.** Weigh the gel fragment and write down the weight. We will need this information for gel purification in the next steps.



**93.** On to DNA gel fragment purification! We are going to use the Zymo Research kit, since it has world-class elution volumes, meaning very little of the DNA you are purifying gets left behind in the column. There are some slightly cheaper alternatives but, since we lose a ton of DNA in the process of purifying, we want as much starting material as possible and the most efficient means of purification.



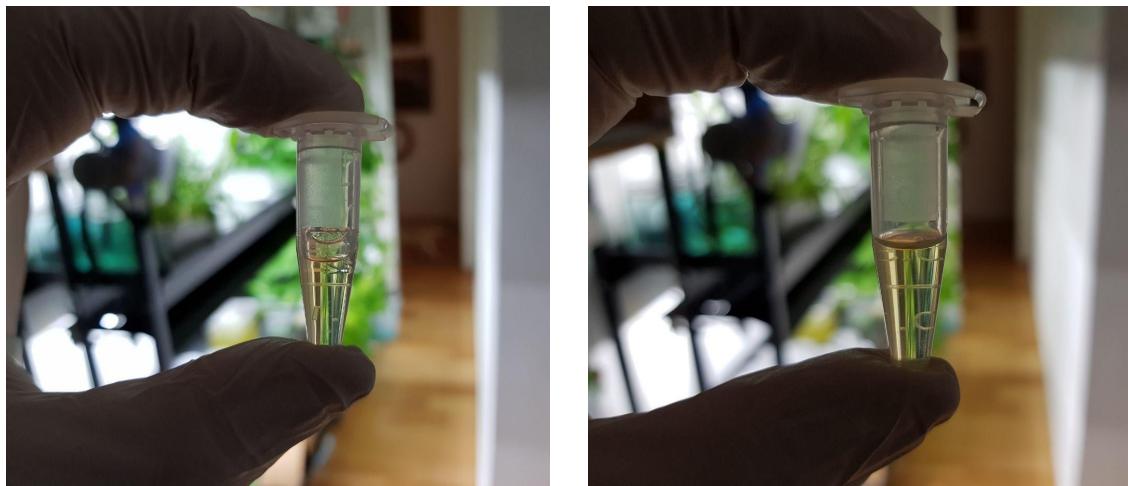
Add the agarose dissolving buffer to each of the gel fragment tubes at a volume equal to the amount of milligrams the fragments weigh **times three**. An example would be: if your gel fragment weighs 103 mg you will add 309  $\mu$ L of Agarose Dissolving Buffer (ADB) to the tube. Place the tubes onto a small tray with a brim (a



PCR tube rack cover will suffice) into the incubator shaker set to 37°C and 250 rpm and let them rattle around for 10 minutes. This will help dissolve the gel fragments in the buffer.

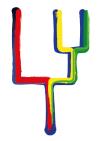


Once fully dissolved it will go from the image on the left (before incubation) to the image on the right (after incubation). Note the lack of any solid gel bits.



Let's move on to binding the DNA to the silica column in the next steps.

**94.** Add the entire contents of each tube to their corresponding silica column. A silica column consists of an upper tube containing a small fiber glass-like plug in the bottom of the tube and a larger open-mouth collection tube to catch the flowthrough during washing. Assemble the two together and add the dissolved gel



fragment liquid to the tube. Cap the tube and incubate for 1 min at room temperature.



**95.** Spin down the tube at max speed in a microcentrifuge for 30 seconds. To increase the final yield by 20%, pour the flow through back into the upper tube one more time and allow to incubate for 1 minute and spin down for 30 seconds. This gives the DNA in the liquid another chance at binding to the silica.





**96.** Discard the yellow flowthrough and proceed to the washing step. Add 200uL of Wash Buffer to each column. Be sure to have added Absolute Ethanol to the buffer bottle and checked off that you did so with date and signature. Note when pipetting volatile (or alcohol containing reagents) to pipette up and down such that the vapor pressure equilibrates to the air in the pipette. This avoids the beginner's trap of a leaky pipette during wash steps.



**97.** Spin down the columns for 30 seconds.

**98.** Repeat steps 96 and 97 once more.

**99.** Label a new microcentrifuge tube with the gene name and any pertinent information. Cut off the caps of the tube, since they will interfere with centrifugation. Keep caps close by. Transfer the UPPER tube containing the DNA bound to silica to this new labeled tube. Discard the bottom collection tube.



**100.** Add 20  $\mu$ L of distilled water EXACTLY to the center of the small white silica plug at the bottom of the tube. Be very careful not to stab into the plug.



**101.** Cap the upper tube and incubate at room temp for one minute to elute the DNA from the silica and into the water we just added. Spin down for 1 minute at maximum speed to elute the DNA. We now have 20  $\mu$ L of purified PCR product and ready for downstream processing.





**102.** We are now going to cut the PCR fragment and the plasmid (pUC19) with *SacI* and *XbaI* for 2 hours to produce sticky ends for later ligation (gluing) to form the final construct pUC19-CHSD. Almost finished!

Thaw on ice the NEB Cutsmart Buffer, your two purified gel fragments, and a miniprep of pUC19 plasmid. Label 4 tubes with either “I” for insert or “V” for vector.

**103.** To the tubes labeled “V” add 16 µL of pUC19 plasmid and 2 µL of Cutsmart Buffer.

**104.** To the tubes labeled “I” add 16 µL of the purified gel fragment, one fragment’s worth of eluted DNA per tube.

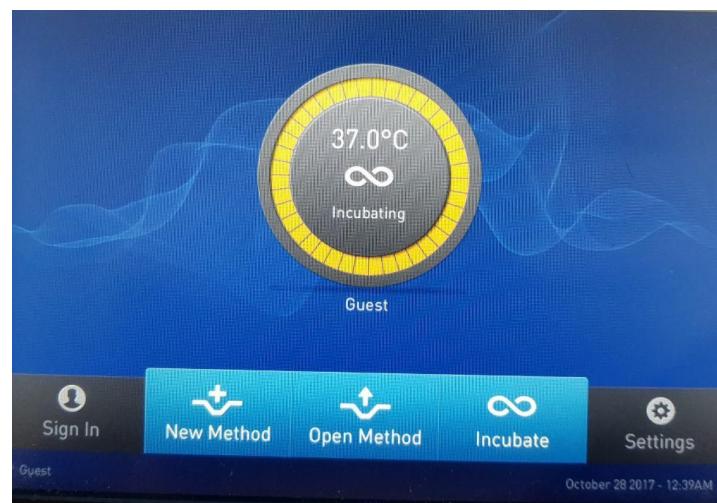
**105.** Take the *SacI* and *XbaI* restriction enzymes out of the freezer and either keep them in an Ice Block Rack (**HIGHLY RECOMMENDED**) or on ice.

**106.** Add 1 µL of *SacI* to all the tubes.

**107.** Add 1 µL of *XbaI* to all the tubes.

**108.** Vortex the tubes for 3 seconds, whip down if needed.

**109.** Incubate at 37°C for 2 hours to ensure complete digestion (cutting) of the PCR fragment AND pUC19 plasmid.

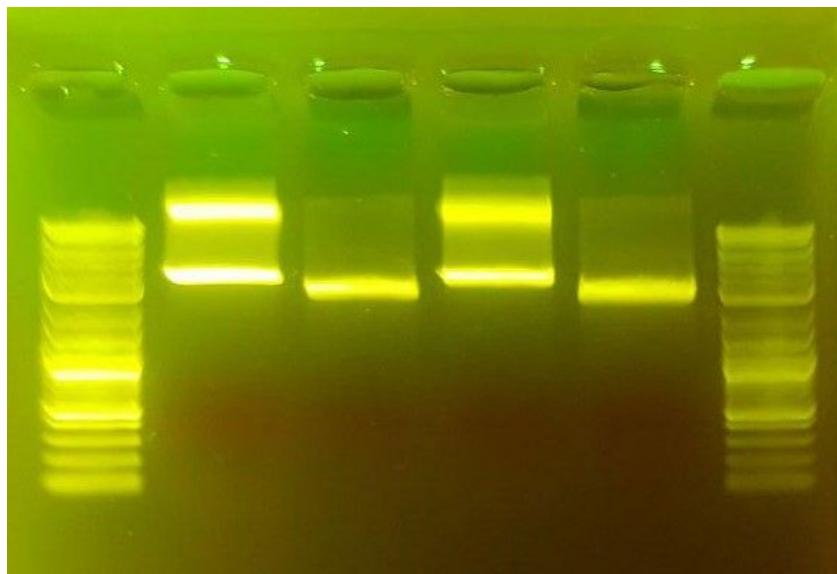




**110.** Take the tubes out, add 4  $\mu$ L of 6x Purple Loading Dye to each tube. The final volume has to be divisible cleanly by 6 so the nearest value to the 20  $\mu$ L restriction digest is 24 so by adding 4  $\mu$ L the total becomes 24  $\mu$ L and the final concentration of Purple Loading Dye goes from 6x to 1x.

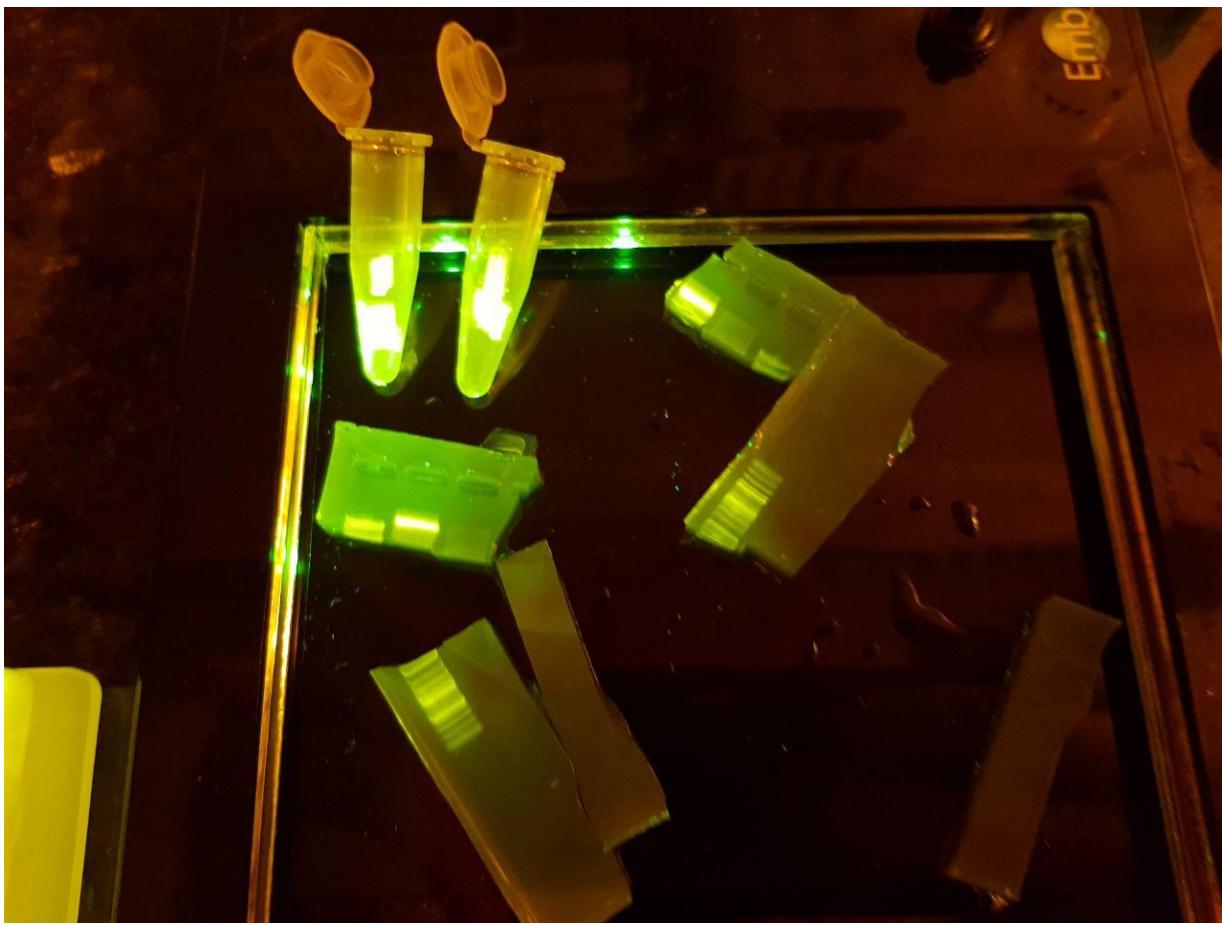


**111.** Pour a gel and allow it to set. Run the samples as follows: Ladder, V, I, V, I, Ladder.





**112.** Cut out the bottom bands of each lane. Note the pUC19 plasmid has two bright bands. This is due to the incomplete digestion and supercoiled nature of natural plasmid. By only taking the bottom most band, we only take plasmids that have been completely opened up via *Sac*I and *Xba*I. If you take the upper band, many of the resulting colonies during the ligation and subsequent transformation will result in empty pUC19 plasmids. We wish to avoid this entirely so be sure to ONLY cut the bottom bands. Double up two “V” fragments in a single new 1.5 mL microcentrifuge tube. Do the same for the “I” fragments. Make sure you weigh the 1.5 mL tubes before adding the bands, so we know the weight of the fragments for later purification.

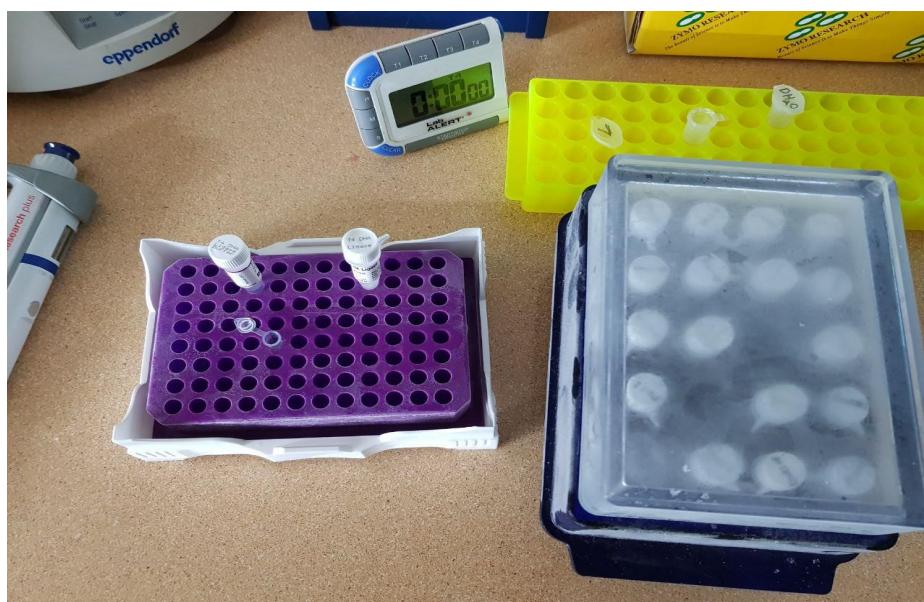




**113.** Gel purify the fragments using the Zymo kit as explained earlier. For a quick cheat sheet, please refer to your gel purification manual. Elute in 20 µL of distilled water into a new microcentrifuge tube.



**114.** Now we are going to set up a ligation reaction which will glue the PCR fragment to the pUC19 vector using an enzyme called T4 DNA Ligase. This enzyme requires ATP, the molecular currency of cells, and thus its buffer is **VERY** sensitive temperature. Be sure to thaw this on ice and let it thaw naturally. You could also leave this buffer in the fridge during the restriction digest gel run, which should give you ample time to slowly and safely thaw this buffer. If in a rush, melt in ice water.





**115.** To a single PCR tube on ice, or in an ice block rack, add 17.5  $\mu$ L of both Vector and Insert.

**116.** Add 4  $\mu$ L of T4 DNA Ligase Buffer.

**117.** Add 1  $\mu$ L of T4 DNA Ligase Enzyme.

**118.** Cap, vortex, whip down to ensure it's at the bottom.



**119.** Incubate in a PCR machine at 16°C overnight (16+ hours).





**120.** At this point the ligation reaction is complete and we need to prepare the new DNA construct, namely pUC19-CHSD, for transformation into *E. coli* cells... the exact cells we made not too long ago.

First step is to purify the ligation reaction and get rid of all the salts and buffer used in the ligation. Any additional ions to the cells directly inhibit electroporation by making the cells more conductive. To do this, we will use a similar kind of purification kit to the gel fragment kit known as PCR or liquid purification.

Add 2x the original ligation reaction's worth of volume in NTI buffer to the ligation tube. This will help remove all the enzymes, buffer, and ions as described above.





**121.** Split the NTI-buffered ligation reaction across two liquid purification columns for easy centrifuge balancing as well as insurance in case you make a mistake purifying one of the reactions. We've come this far, it would be a shame to lose everything over human error and not have a backup plan.



**122.** Allow the reaction to incubate at room temperature in the column for 1 minute, then spin down for 30 seconds.

**123.** Transfer the flow through into the column once more to increase yield. This step of course is optional.

**124.** Spin down once more. Discard the flowthrough. Add 700  $\mu$ L of Wash Buffer to the column and spin for 30 seconds. Discard the flowthrough and repeat this step once more.

**125.** Spin the tube down empty to remove any trace wash buffer.



**126.** Transfer the upper column tube to a new 1.5 mL microcentrifuge tube. Like before, cut off the caps to ensure it won't interfere with the centrifugation.

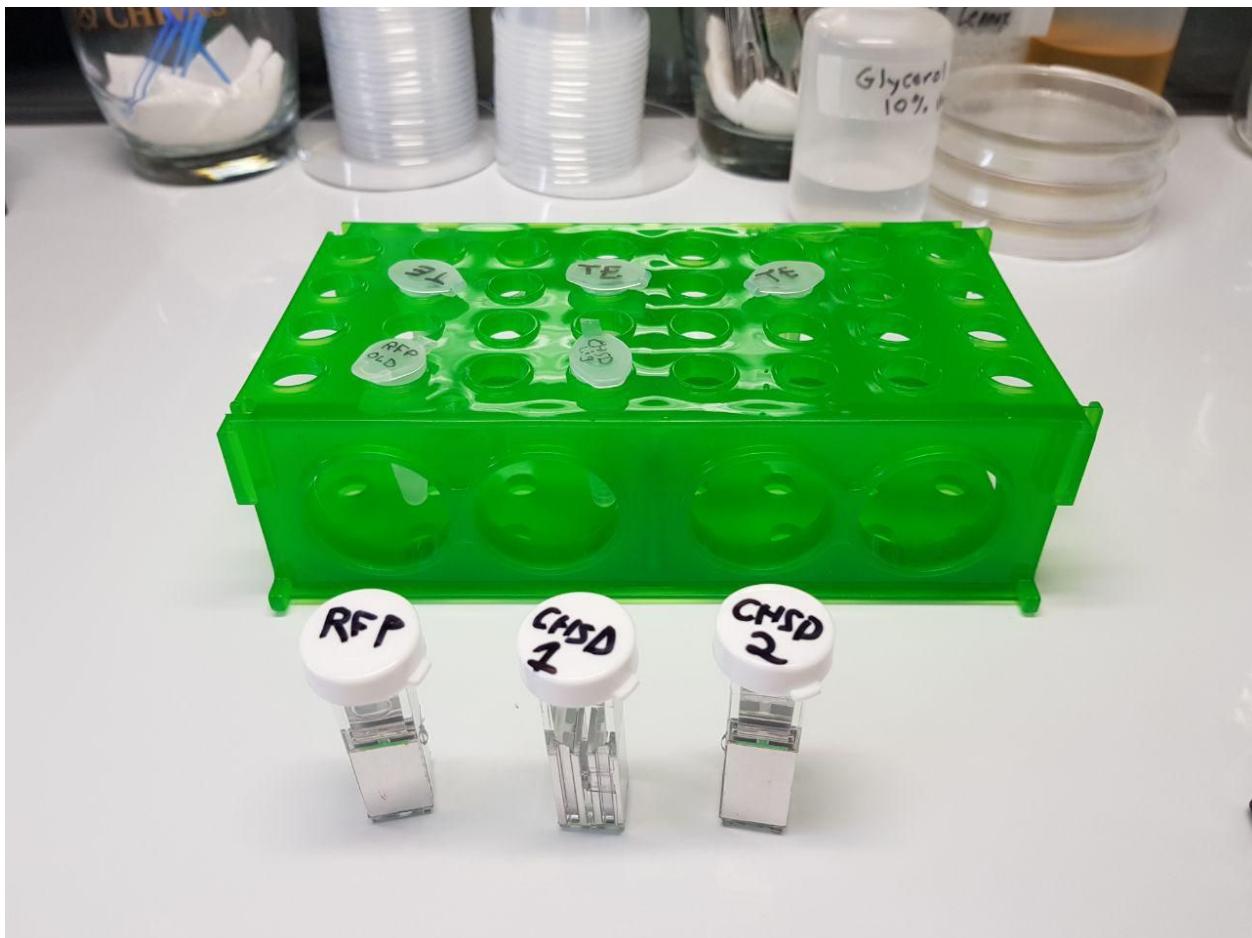
**127.** Add 20 µL of distilled water to the column, allow the reaction to incubate for 1 minute, and spin down the columns.



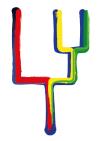
**128.** We have now done all we could in terms of amplifying, cutting, and pasting our gene of interest into the pUC19 vector. The next step is to transform these plasmids into *E. coli*, where they will be repaired, copied, and maintained ad infinitum. The plasmid will hijack *E. coli* DNA replication machinery and make tons of copies of pUC19-CHSD and, in doing so, will also express the ampicillin resistance gene encoded in the plasmid. This will give *E. coli* resistance to ampicillin, so we can select only cells carrying our plasmid and ultimately our gene of interest. The next steps will explain how we get this newly constructed plasmid into *E. coli* via the method known as electroporation.



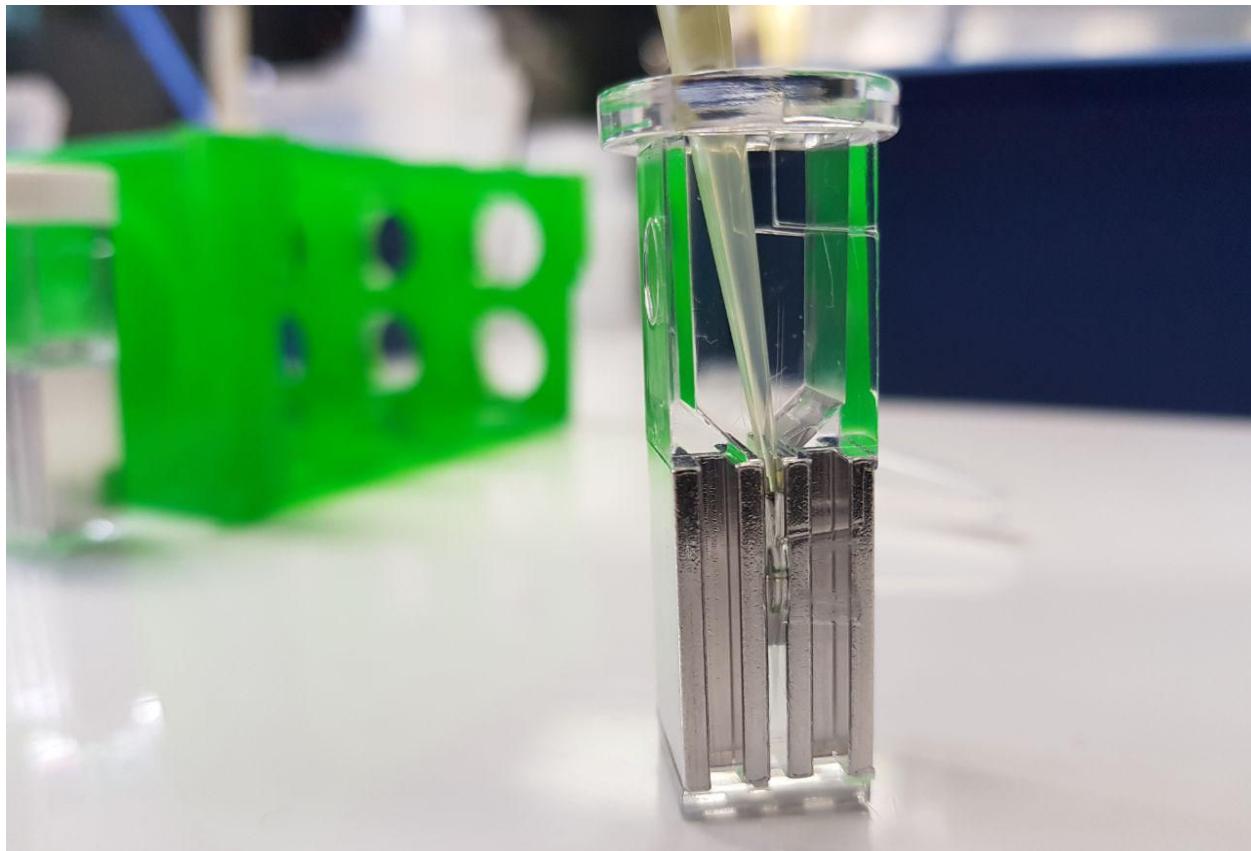
Prepare 3 electroporation cuvettes with a 1 mm plate gap. This is the distance between the two aluminum plates and plays a crucial role in the efficiency of the transformation. Label each tube with the following names: CHSD 1, CHSD 2, and RFP. The RFP stands for Red Fluorescent Protein, this is a plasmid I use often as a positive control since it is reliably transformed into *E. coli* AND it expresses a red protein that, as the name suggests, acts as a secondary indicator that the cells took up the plasmid.



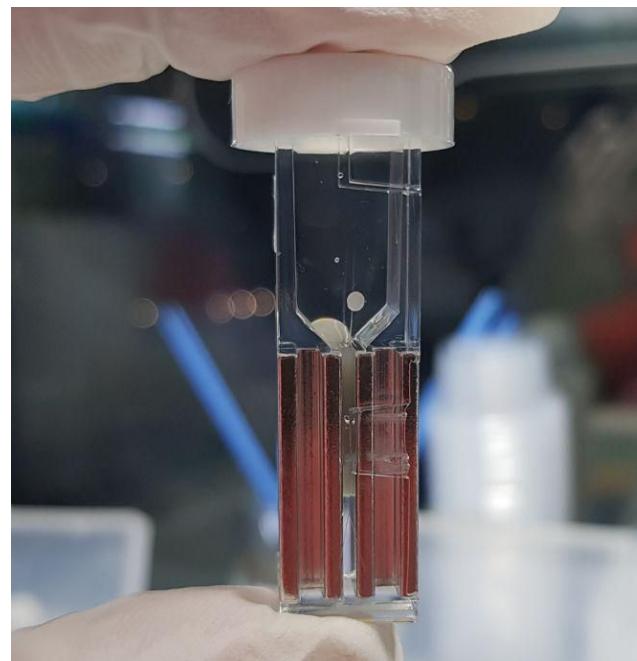
**129.** Thaw a tube containing the RFP plasmid (or any other plasmid you've transformed before as a control). Bring all of the ligation tubes and positive controls into the laminar flow hood. Along with the DNA tubes, take 4 tubes of electrocompetent *E. coli* out of the freezer. They will thaw as you work.



**130.** To the CHSD 1 tube, add 10  $\mu$ L of the ligation reaction to the electroporation cuvette in one corner of the plate opening against the wall.



**131.** Add 100  $\mu$ L of electrocompetent *E. coli* (one competent cell tube's worth of cells) to the cuvette. Gently pipette up and down to mix the plasmid with the cells.





**132.** Turn on the electroporator, set the voltage to **1800 V**, and load the cuvette into the device. In the laminar flow hood, prepare a 1000  $\mu\text{L}$  pipette set to 1000  $\mu\text{L}$  and a bottle of SOC medium for immediate recovery. As soon as the electroporation is over, you need to quickly add 1000  $\mu\text{L}$  of SOC media to recover the fragile cells. Delaying this step will drastically influence the efficiency of your transformation.



**133.** Immediately add 1000  $\mu\text{L}$  of SOC media to the shocked cells and gently pipette up and down 3 times to resuspend the cells.





**134.** Cap the cuvette and place the cuvette in the incubator shaker in a brimmed tray. Set the shaker to 250 rpm and 37°C and allow the cuvette to rattle around for 30 minutes.

**135.** Finish shocking the other CHSD 2 ligation into the cells in the same manner.

**136.** Shock the RFP plasmid but only use 2 µL of the plasmid, since it is already very concentrated. Because DNA is naturally negatively charged, adding too much DNA will cause the solution to become conductive: an electrical arc will form across the plates of the cuvette and through the solution, drastically reducing transformation efficiency or, simply put, it will kill your cells.

**137.** Allow all the cuvettes to shake for at least 30 minutes at 37°C, 250 rpm.

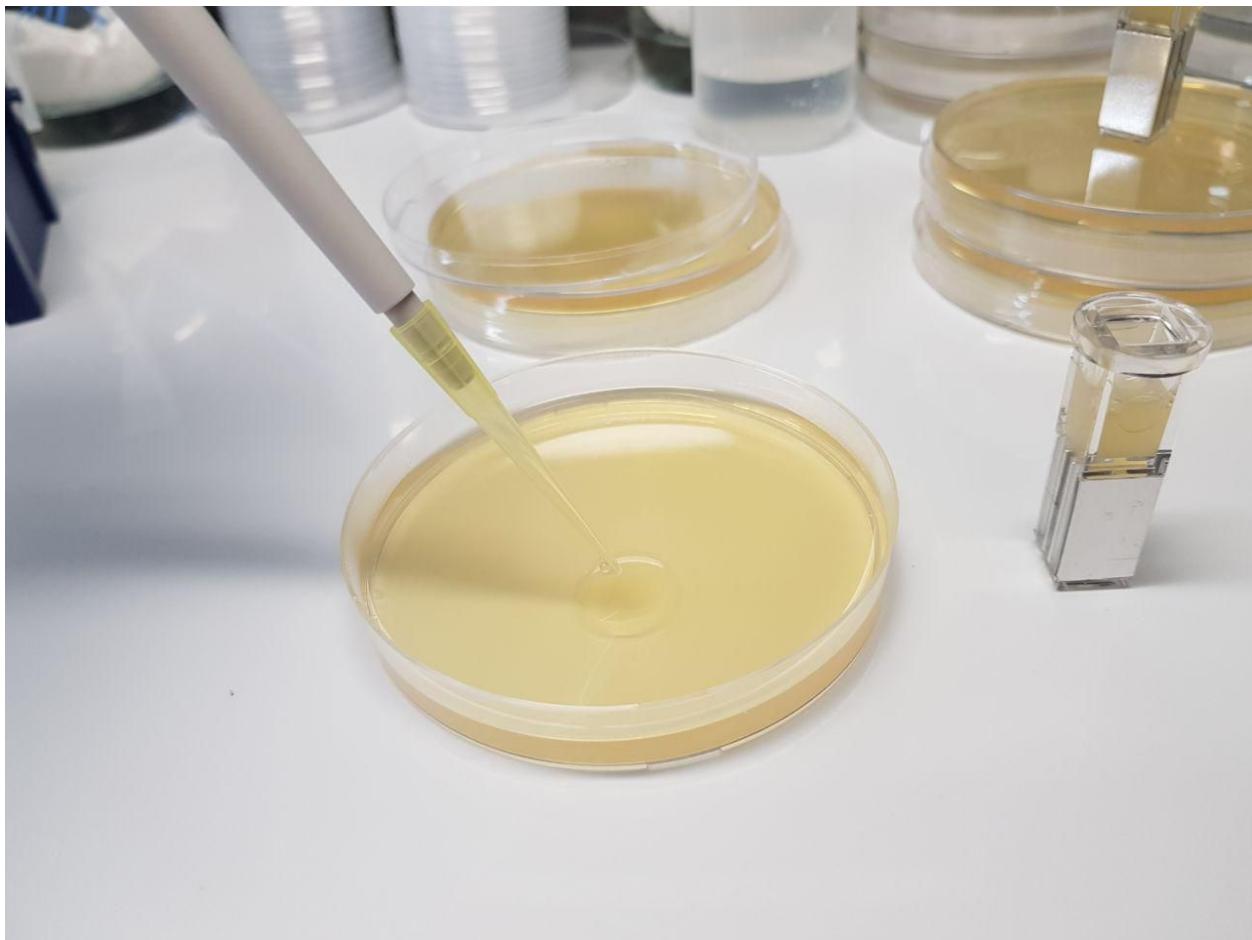
**138.** In the meantime, melt and pour LB-Amp plates by microwaving a 250 mL LB media bottle full of 150 mL of LB-Agar. Allow the media to cool to the sensation of a hot cup of coffee you are still willing to touch and then add 150 µL of 100 µg/L ampicillin solution. To make this solution add 1 g of ampicillin powder to 10 mL of sterile distilled water.





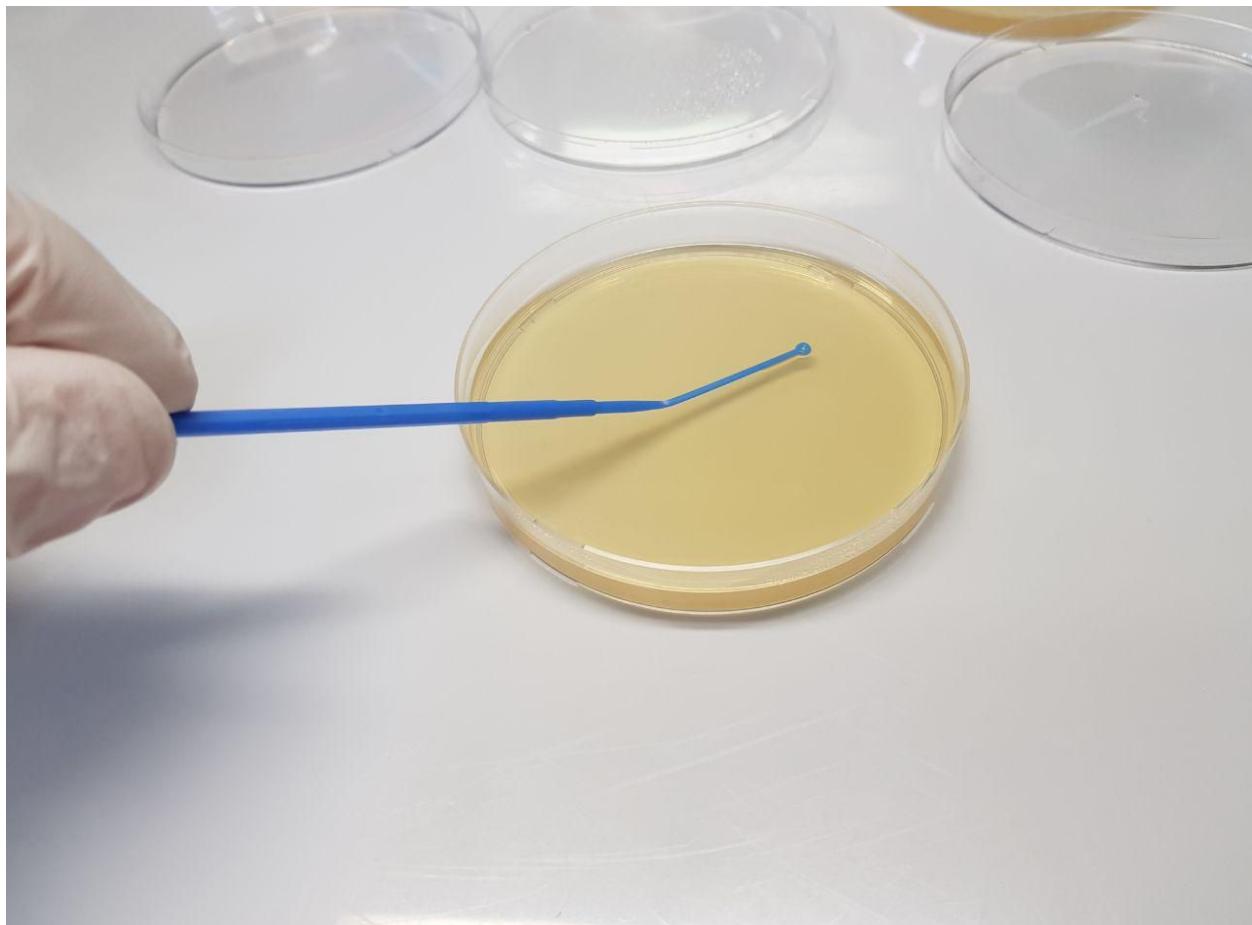
**139.** Once the 30 minute timer for the transformation recovery elapses, and the Petri dish media has solidified, bring the cuvettes into the laminar flow hood.

Add 100  $\mu$ L of one of the electroporated cuvettes onto the surface of an LB-Amp Petri dish.

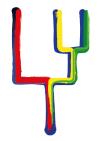




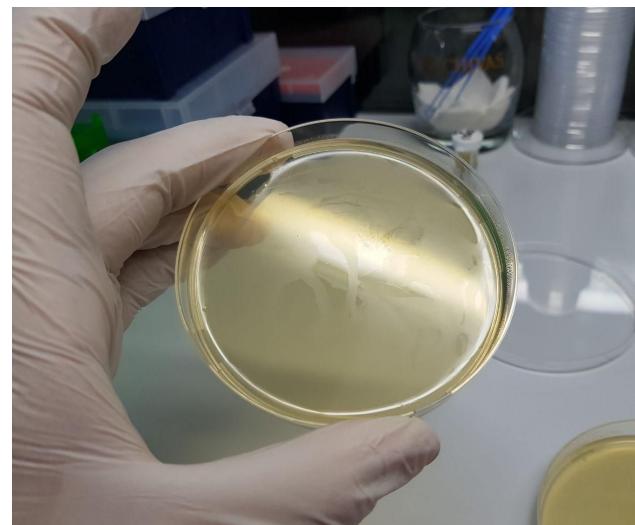
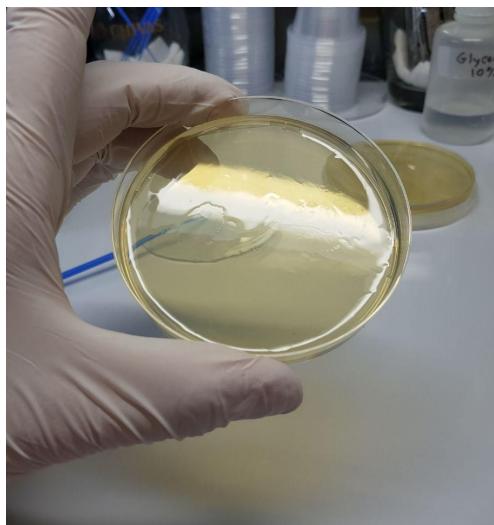
**140.** Bend the thin end of a sterile disposable inoculation loop by pushing it against the sterile inner lid of the Petri dish you just inoculated, and spread the bacteria across the media surface like you would a wide mop or rake. Gentle spread the cells across the entire surface evenly, by rotating the dish 45 degrees after every pass. Use the reflection of your light source to detect any missed spots.



**141.** Allow the plate to dry by setting it aside and continuing to repeat steps 139 and 140 for each subsequent electroporation cuvette. Streak 2 Petri dishes for each of the CHSD electroporation cuvettes to hedge our bets in case of low efficiency. After you have successfully streaked out a sample of each cuvette, take 100  $\mu$ L of cells from the 4th unused competent cell tube and streak it onto one of the LB-Amp dishes. This will be our negative control to ensure the cells we started with were not already resistant to ampicillin. This plate should not produce any colonies when incubated with the rest.

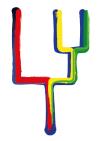


**142.** Allow all the plates to fully dry. This will take about 10 minutes and you will know they are dry when the once shiny surface of the dish takes on a matte sheen. The image below on the left is freshly streaked and on the right is adequately dry.

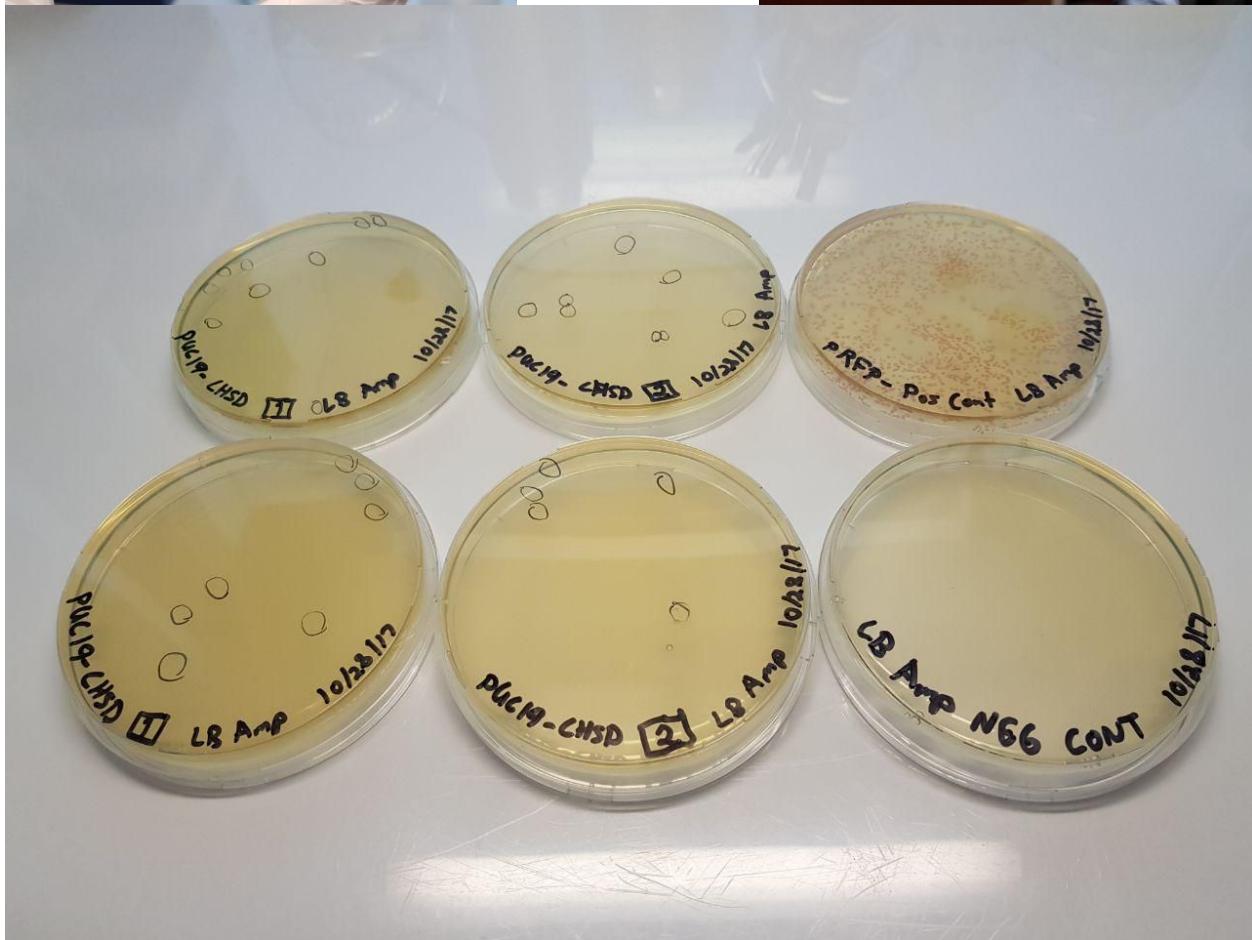
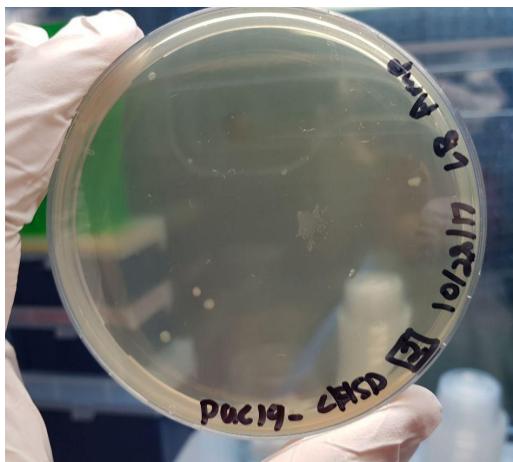


**143.** Ensure all of the plates have been properly labeled with their corresponding plasmid name, media contents, *E. coli* cell line, and date. **DO NOT MIX UP LABELS!** Incubate the plates upside down overnight in a regular incubator set to 37°C.





**144.** The following day (12-18 hours), inspect the Petri dishes for colonies and circle any colonies visible in the CHSD 1 and CHSD 2 with a Sharpie marker. Below are photos of one of the CHSD plates, the positive control RFP, and the collection of plates from this experiment. Note the surprisingly low efficiency of transformation despite the cells being decently competent. Congrats on the colonies!! You did it!!

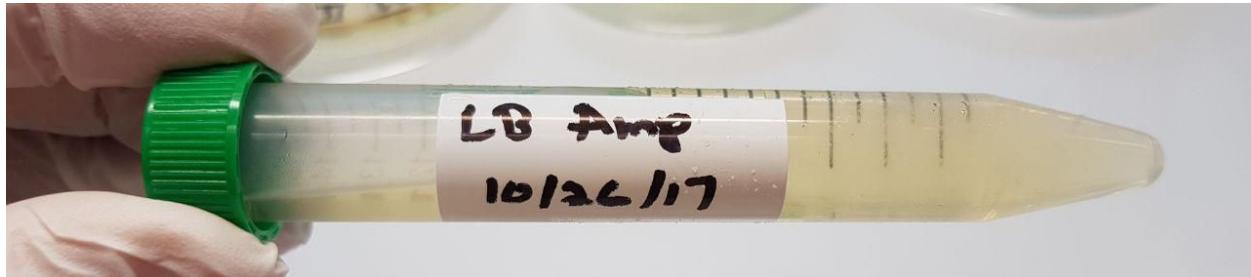




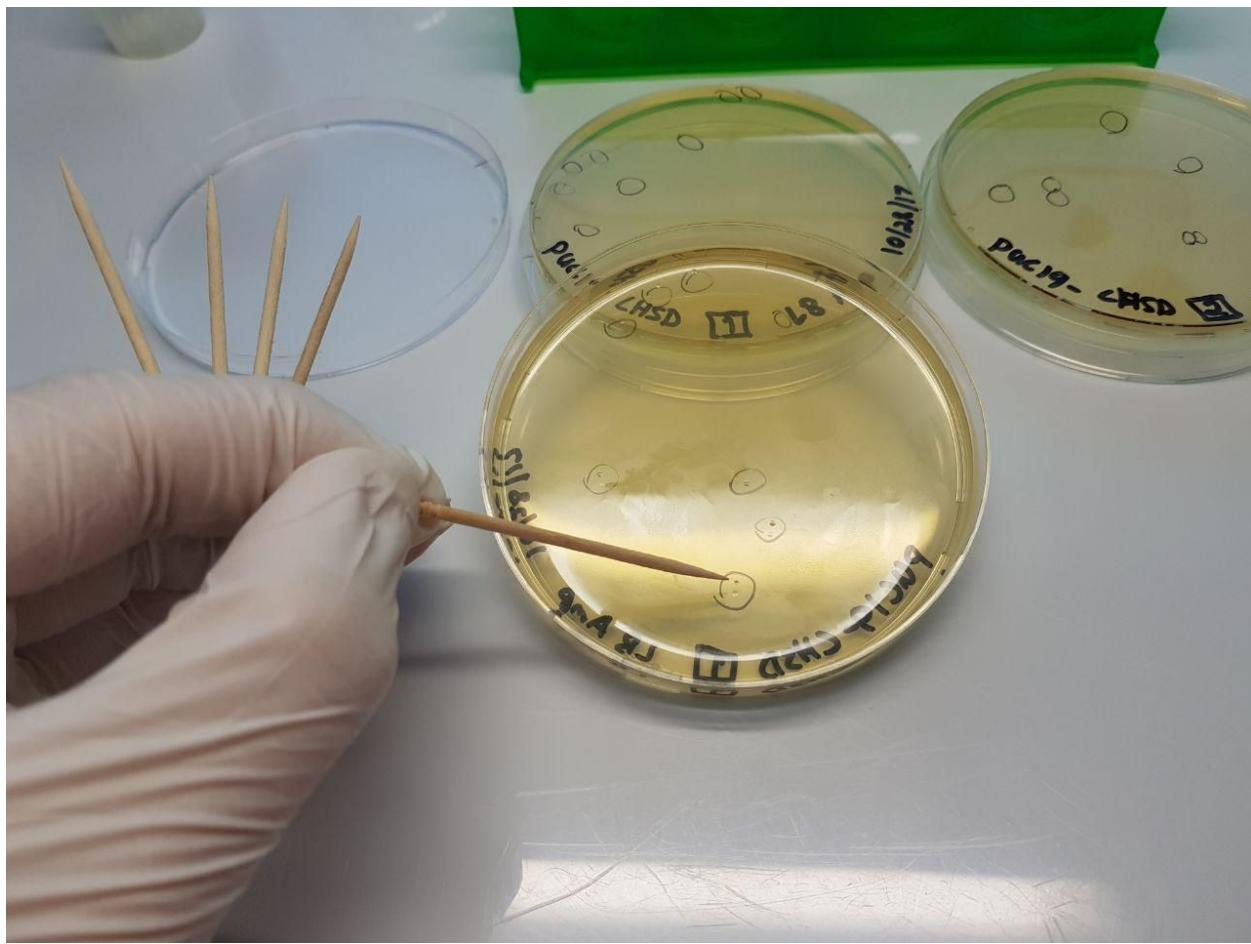
**145.** Next step will be to verify if the colonies we have are indeed carrying our gene of interest. This process is known as colony PCR and is rather straightforward.

Place 10 sterile 1.5 mL microcentrifuge tubes into a tube rack under laminar flow.

**146.** Fill each of the tubes with 200  $\mu$ L of liquid LB amp.

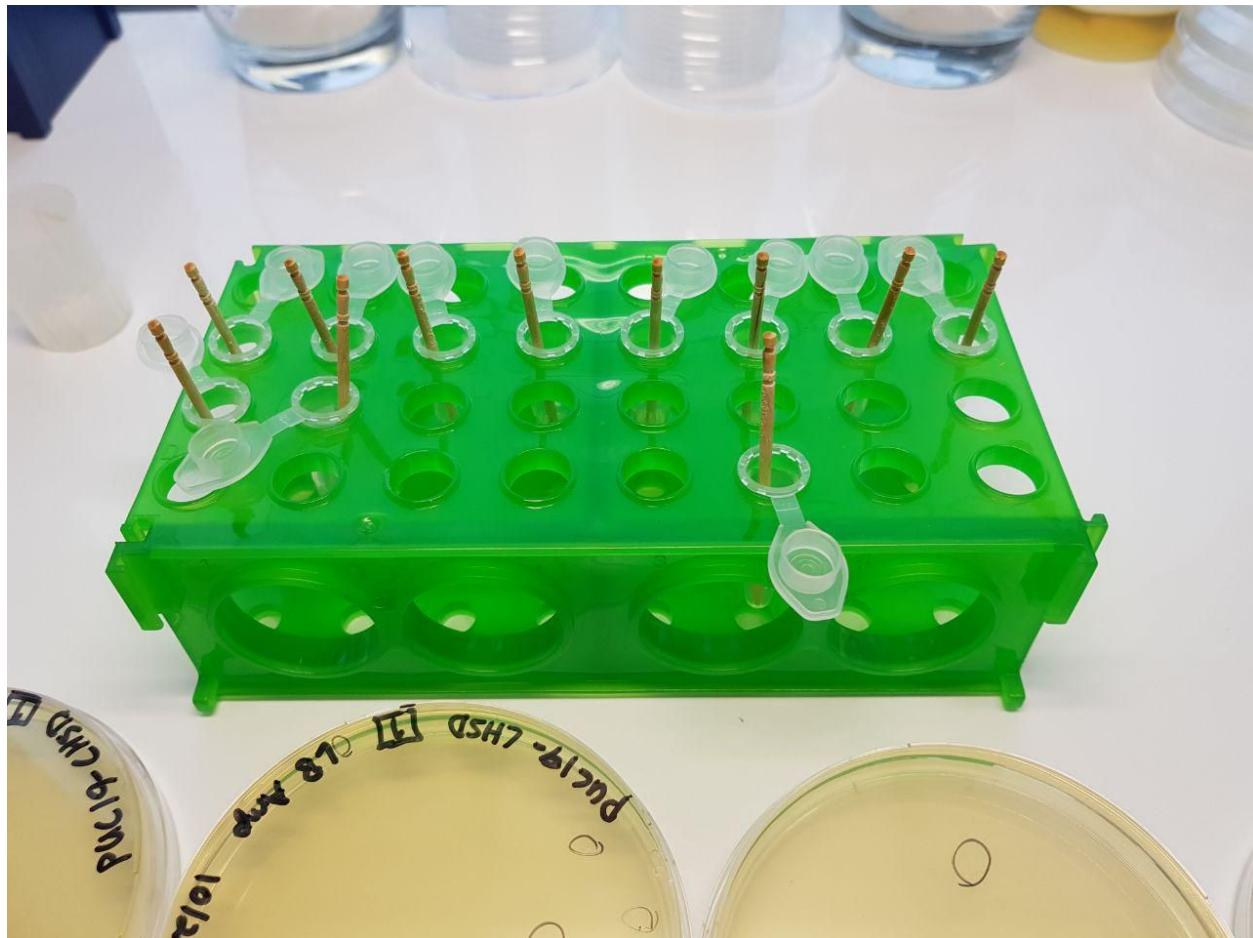


**147.** Using sterile toothpicks, pick up the colonies by gently poking into the colony and just a millimeter or so into the agar.



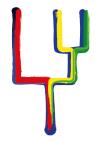


**148.** Place each toothpick in its own tube of LB-Amp, swirl around the bottom of the tube with the toothpick and repeat the process for 9 of the 10 tubes.

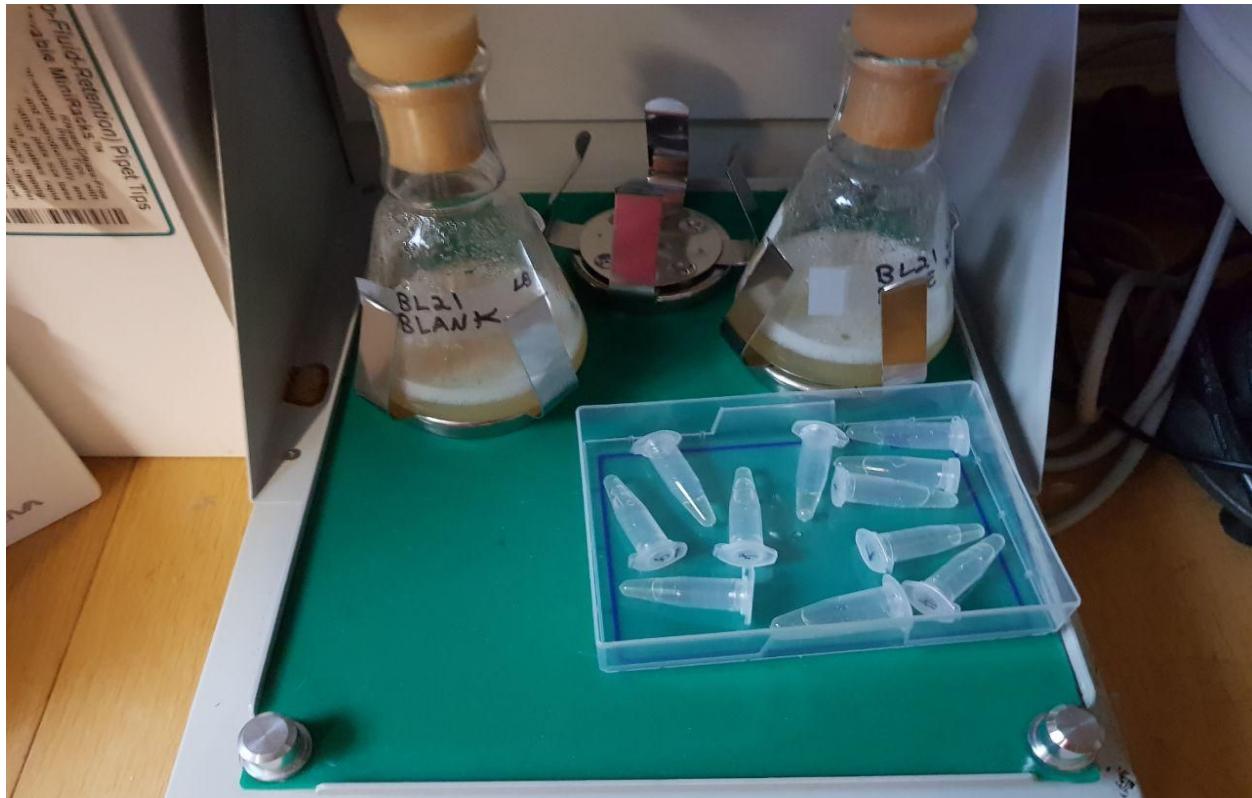


**149.** For the final tube, just dip a sterile toothpick into the LB-Amp. This will serve as a negative control for the colony PCR we will conduct in a few hours time.

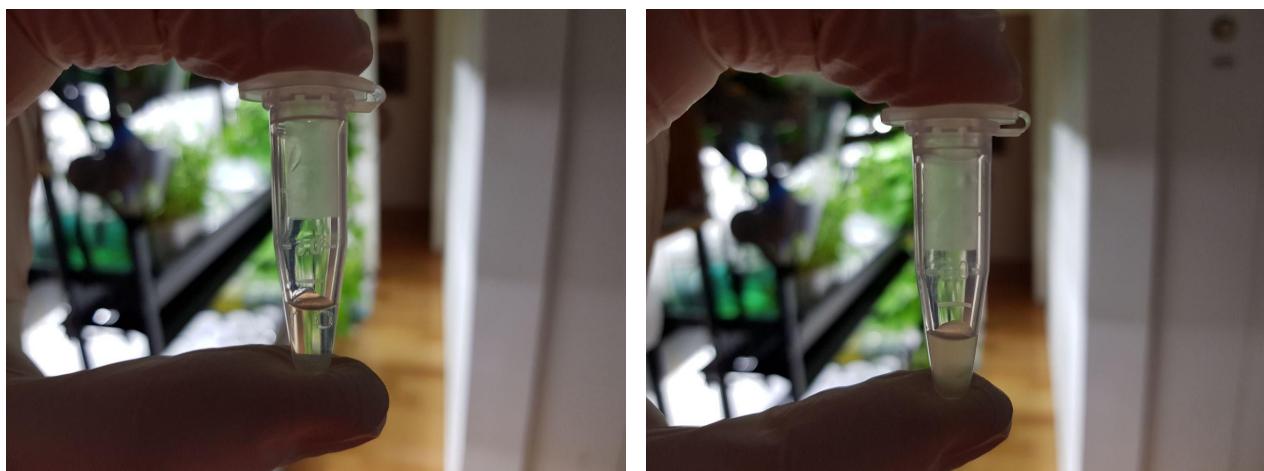
**150.** Once all the tubes have toothpicks, remove the toothpick from the tube that will serve as our negative control, cap it, and label it with a "C" for control. Remove all the other toothpicks and label each tube an incrementing number from 1 to however many colony tubes you have, in this case 9. Be sure to underline the 6 and 9 numeral so that they do not get mixed up.



**151.** Place the tubes in a brimmed tray or lid in the incubator shaker set to 37°C and 250 rpm, and allow them to rattle around for 4 to 6 hours depending on your cell line's growth rate (determined empirically).

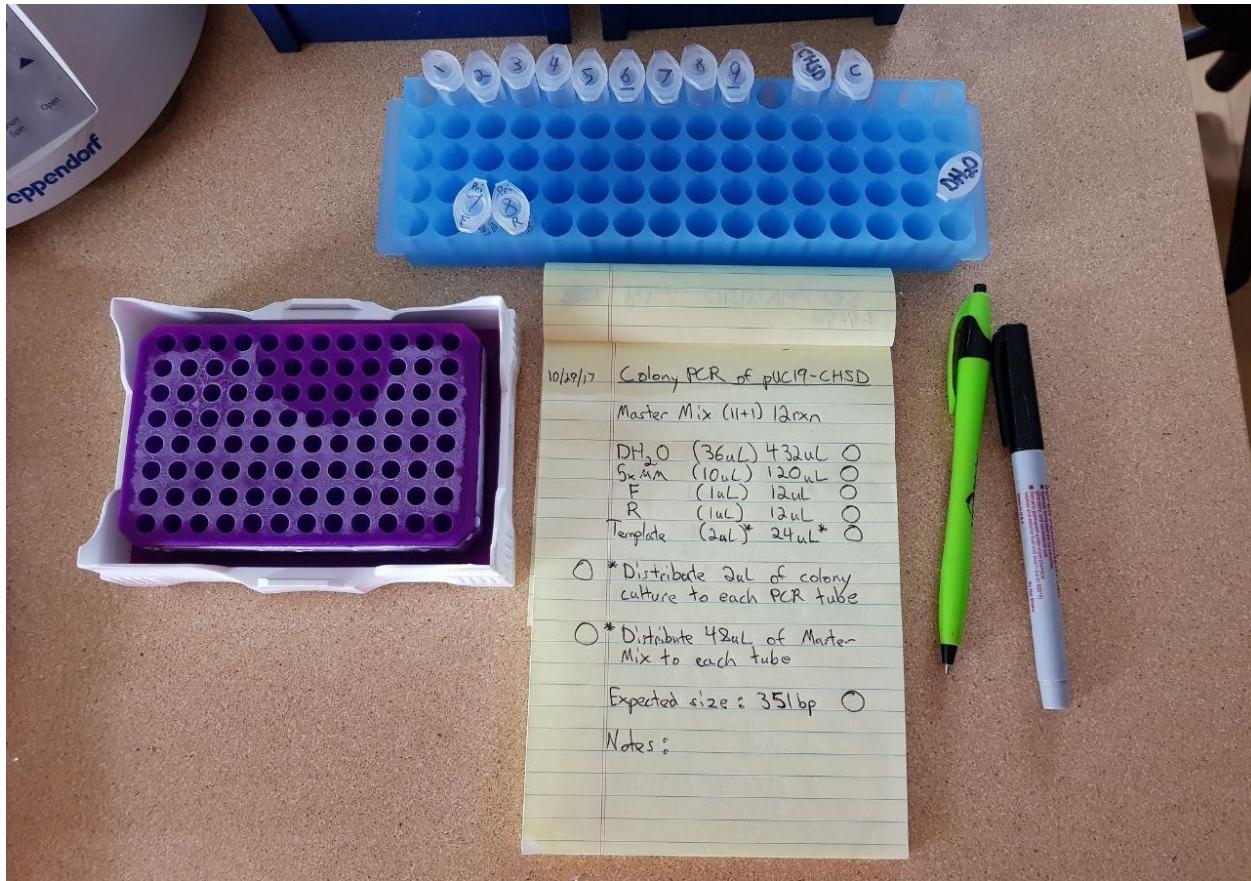


**152.** After the colonies have grown in the shaking liquid media, take the out and inspect the tubes against the control. There should be a distinct difference in turbidity. Control on the left, Colony 8 on the right.





**153.** We will now prepare a Taq polymerase (routine) PCR reaction using an already prepared polymerase, DNTP, and buffer master mix commercial available from [GeneAndCell.com](http://GeneAndCell.com). The reaction volume will be for 12 reactions (9 colonies, 1 positive control, one negative control and the devil's cut).



**154.** Prepare 11 PCR tubes and label them as 1 through 9 then “+” and “-”.

**155.** To a sterile 1.5 mL microcentrifuge tube labeled “MM”, add 432  $\mu$ L of sterile distilled water.

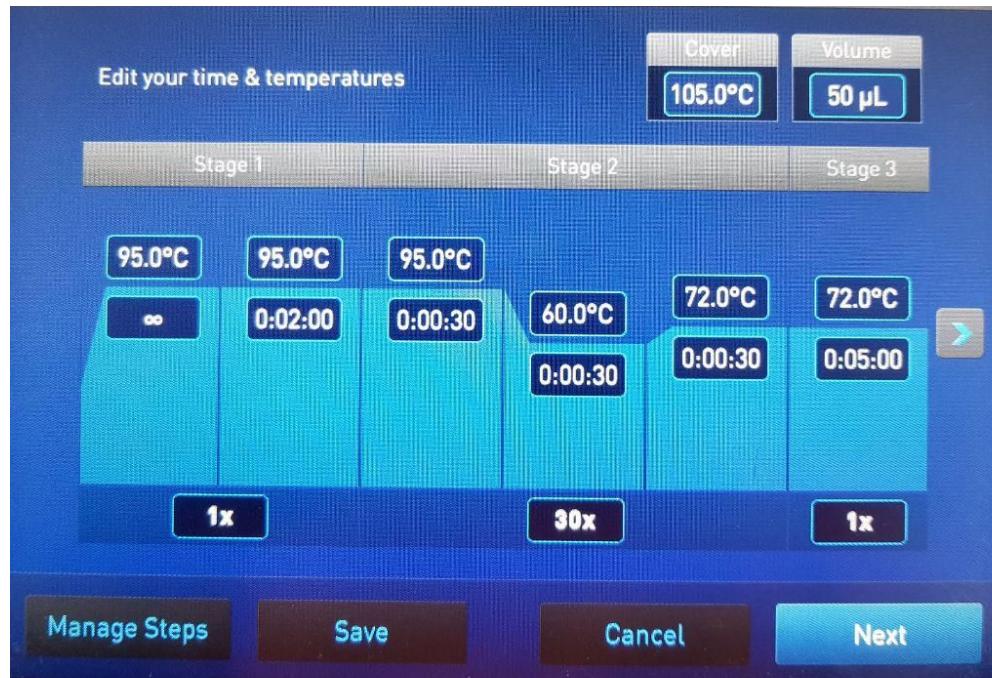
**156.** Add 12  $\mu$ L of each primer, in this case PH-CHSD-DET-F and PH-CHSD-DET-R.

**157.** To each PCR tube, add 2  $\mu$ L of the colony culture from the tubes you shook for 4-6 hours. To the “+” tube, add 2  $\mu$ L of one of the ligation reaction tubes left over from purification. You only used 10  $\mu$ L of each tube so there should be another 10  $\mu$ L leftover. To the “-” tube, add 2  $\mu$ L of sterile distilled water as a negative control.



**158.** Add 120  $\mu$ L of the 5x Taq Polymerase Master Mix to the tube labeled “MM”. Cap, vortex, keep on ice.

**159.** Apply the following program to your PCR machine. This is a routine PCR program that will work with most Taq polymerases available commercially.

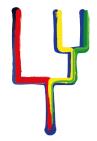


For your convenience, here is a table of the thermocycler program I use:

Initial Denaturation	95°C	2 minutes
Denaturation	<b>95°C</b>	<b>30 seconds</b>
Annealing	60°C	<b>30 seconds</b>
Extension	72°C	<b>30 seconds</b>
Final Extension	72°C	5 minutes

Note\* Bolded cycling stage is set for 30 cycles

**160.** Preheat the PCR machine to 95°C and hold at that temperature.



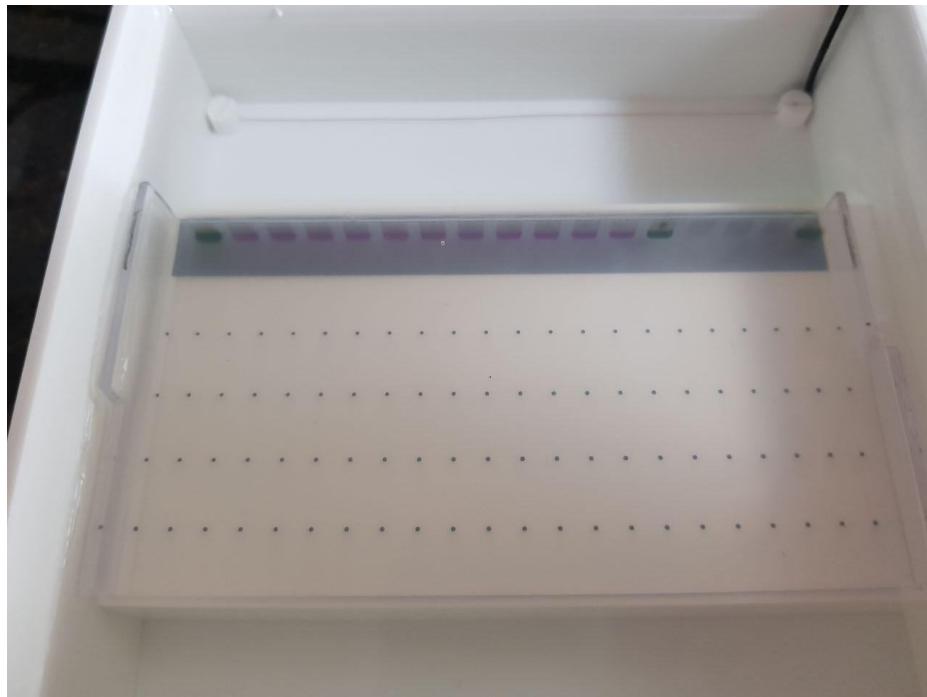
**161.** Dispense 48 µL of the master mix into each PCR tube. Ensure you use a different tip for each tube to ensure no cross contamination. Start with the control tubes. Vortex the tubes, whip them down, place them in the PCR machine, and change the holding program of 95°C to the protocol written above and run the PCR.



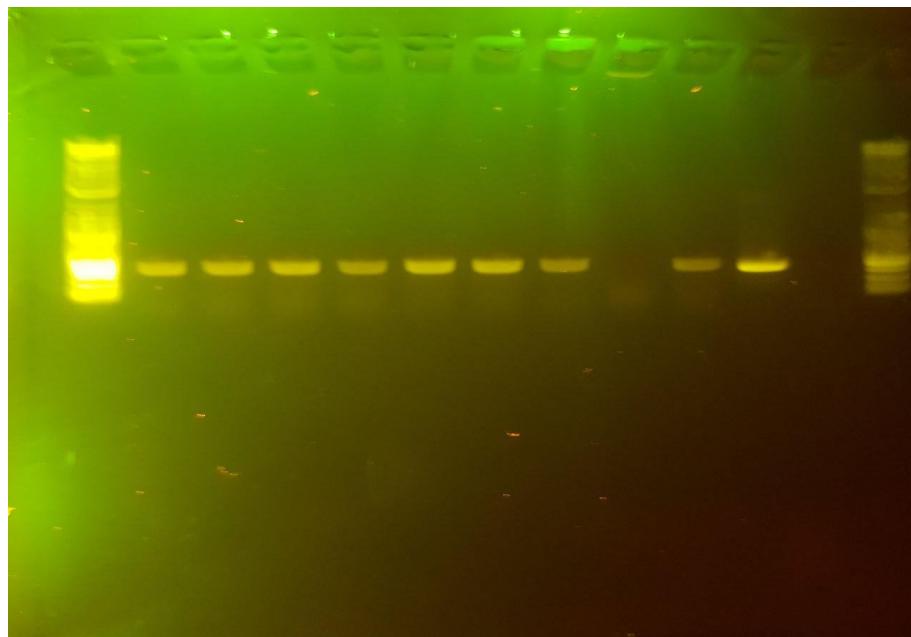
**162.** While you wait for the PCR machine (about one hour and change), pour a wide gel that can fit at least 13 wells. This may be a narrow toothed comb if need be. This PCR gel will tell us which colony is positive for our transgene. Four of the resulting positive colonies, if any, will be selected for further processing. Hang in there, we're almost done!



**163.** Once the PCR is complete, take the samples out, thaw the DNA ladder from the freezer, add 10 µL 6x Purple Loading Dye to each PCR tube, load the gel and run it the gel as follows: Ladder, Colonies 1-9, Positive Control, Negative Control, Ladder.



**164.** Place the gel on the visualizer and analyze results.





**165.** According to the colony PCR gel, we're expecting a band around 350 bp if positive and no band in the lane if negative. Seems that all of the colonies except colony number 8 are positive. Notably, colony number 8 grew up fastest and sooner than the other colonies during the shaking phase of the colony culture steps. Will save this tube for later analysis since it most likely is contamination.

Pick 4 numbers between 1 and 9 at random, ideally using a random number generator from [random.org](https://www.random.org/). Exclude colony number 8 for obvious reasons. Take those colony culture tubes to the next step.

**166.** Prepare 50 mL of liquid LB Amp and distribute it among four 125 mL Erlenmeyer flasks with sterile foam stoppers, 12.5 mL of media into each.

**167.** Label the flasks with the corresponding colony tube number it is about to receive as well the name of the plasmid, namely pUC19-CHSD.





**168.** Dispense 100 µL of the colony tubes into their corresponding flask. Set the flasks to shake overnight at 37°C and 250 rpm.



**169.** The following morning, we will begin the process of miniprepping the samples according to our Bioneer Accuprep kit. Label the resulting tubes with the colony number, plasmid name, and date. These are our completed and miniprepped plasmids, newly constructed by hand with love.

**170.** Before we send out these samples for sequencing, let's first do one last test on our end to ensure we don't waste our money sequencing empty plasmids due to false positives. It never hurts to add certainty to things you pay for out of pocket. To do this we will do a restriction digest on the plasmids we isolated using the same exact enzyme we used during the initial cloning process, namely SacI and XbaI. We will cut these plasmids and compare the resulting gel with respect to the original pUC19 plasmids from the same tube we took from when we started this project.



**171.** Label 5 PCR tubes with the following: #, #, #, and #, where “#” corresponds to the colony number of the plasmid tube. Label the last tube with a “C”. This is where the original pUC19 will go.

**172.** To each PCR tube, add 16  $\mu$ L of the corresponding plasmid. Be sure not to mix up the tubes and their labels.

**173.** Thaw and distribute 2  $\mu$ L of Cutsmart buffer to each tube.

**174.** Add 1  $\mu$ L of SacI to each tube.

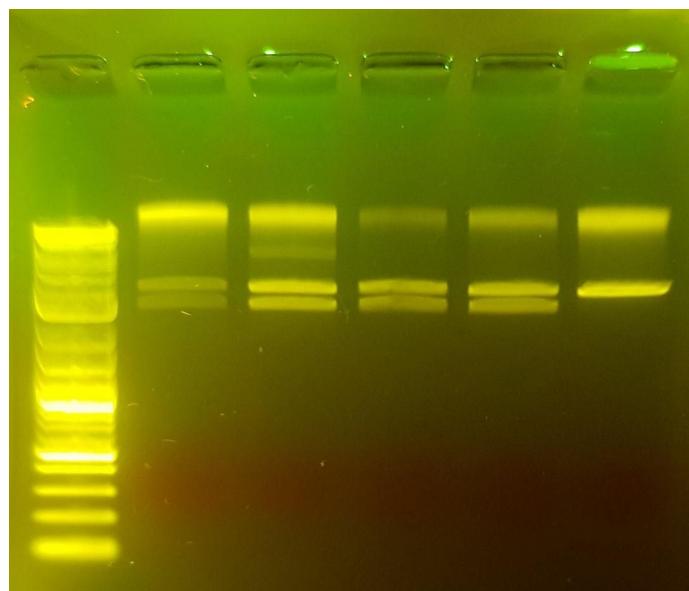
**175.** Add 1  $\mu$ L of XbaI to each tube.

**176.** Vortex the tubes, whip them downwards to force the liquid to the bottom of the tube, and place them in a preheated PCR machine set to 37°C. Incubate for 2 hours.

**177.** Pour a gel enough for 5 samples and a ladder.

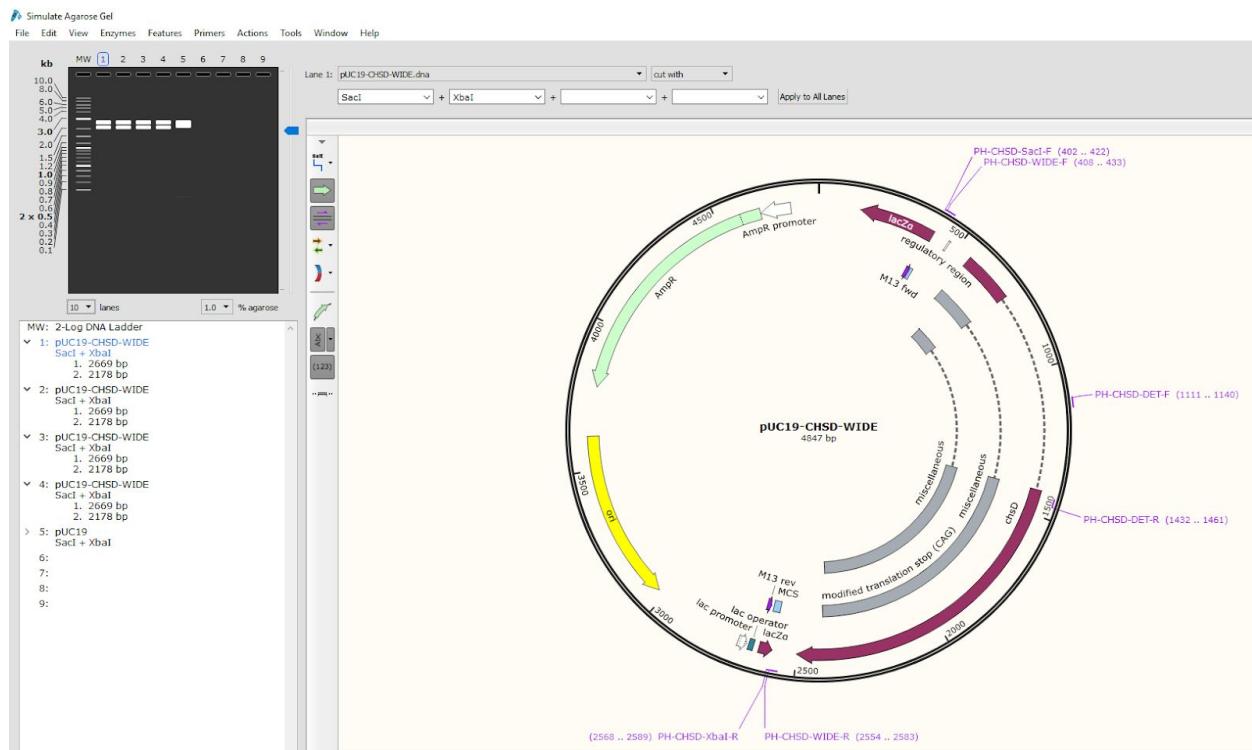
**178.** Once the enzyme digest is complete, add 4  $\mu$ L of 6x Purple Loading Dye to the samples and run the gel.

**179.** This is the resulting gel whose order is:  
Ladder, Colony, Colony, Colony, Colony, pUC19 Control.

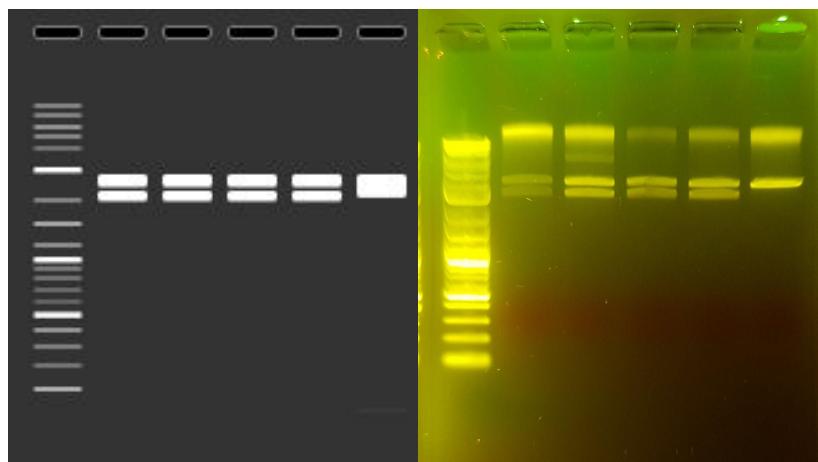




**180.** All of the colony plasmids yield two bands in the 3000 bp region while the pUC19 plasmid shows just one. Our insert is 2188 bp and the plasmid backbone is 1836 bp, so it seems that our four colonies are positive for an insert of that size. To be sure, here is a simulation of the expected gel in SnapGene. You can generate your own by going to Tools > Simulate Agarose Gel and use the final plasmid construct for the colony lanes and the original pUC19 plasmid for the control lane. Cut all of them via SacI and XbaI and here is the resulting simulation. I used the agarose slider to mimic the time I let my gel run.



And here is the actual gel alongside the simulation in black:





**181.** The last step is to prepare the samples for sequencing. We will be using GeneWiz for the Sanger Sequencing services. The cost is normally \$3-\$6 per read (a read is 700-1200 bp in one direction) and, due to their geographic location with respect to my lab, I save on shipping costs. Do shop around and ask for discounts. Paying with a purchase order instead of a credit card can also gain discounted pricing. Develop rapport with your local representative and never settle for the sticker price.

Lay out 10 PCR tubes. Label 5 with the letter "F" for forward and the other 5 with the letter "R" for reverse.

**182.** Next, label every pair of F and R with one of the colony tube numbers you are going to sequence. For example (F9, R9, F4, R4, etc.) Label the last pair FC and RC for negative control.

**183.** To each pair, add 12.5 µL of the corresponding colony plasmid to each of the tubes. For the control tubes FC and RC add 12.5 µL of pUC19.

**184.** To each tube labeled F, add 2.5 µL of primer M13-F.

**185.** To each tube labeled R, add 2.5 µL of primer M13-R.





**186.** Cap the tubes, vortex them, and lay them out on a strip of scotch tape to secure them for shipping.



**187.** Now let's set up a sequencing order with GeneWiz. They have an amazingly simple automated system and very good customer service. Here is my final screen after selecting Sanger Sequencing and saying my tubes are Pre-Mixed (plasmid AND primer)

GENEWIZ®

Live Chat Contact English Wish List Cart Sebastian Cocloba Order / Quote

Home / Sanger Sequencing Download/Upload New Excel Form Auto Save this form in: 0:25

IMPORTANT: When using the "Upload Excel" feature, you must use the new Excel form (version 2). Download it here.

DNA Type\* Plasmid Service Type\* Premix Order Name Patron Monthly Methods 2

Service Priority\* Standard Same Day Order Comments

Save for Prep Special ID

# of Samples\* 10 Apply Upload Excel

Promotion Code

Coupon Code

SAMPLE SUBMISSION GUIDELINES 1 To 10 samples of total 10 samples Preview

Tube View Plate 1

Filled Valid Invalid

Ask Gene

Samples at GENEWIZ

Fill All Columns Clear All Samples Export Samples to Excel

Spreadsheet Functionality Key

Fill all sequentially Fill all values Add primer(s) details Select value from list

Ctrl-C / Ctrl-V for Copy and Paste Double click to edit Drag down to fill like in Excel

Sample			Primer			Notes		
Plate	Tube	Sample #	DNA Name	Length (bp)	My Primer	GENEWIZ Primer Selection Tool	Difficult Template	
1	A:1	1	EXAMPLE	ex:4000-6000	exM13F	ex:T7	ex:hairpin	
1	SC1	1	9F	2001-4000	▼	▼	▼	
1	SC2	2	3F	2001-4000	▼	▼	▼	
1	SC3	3	4F	2001-4000	▼	▼	▼	
1	SC4	4	7F	2001-4000	▼	▼	▼	
1	SC5	5	CF	2001-4000	▼	▼	▼	
1	SC6	6	9R	2001-4000	▼	▼	▼	
1	SC7	7	3R	2001-4000	▼	▼	▼	
1	SC8	8	4R	2001-4000	▼	▼	▼	
1	SC9	9	7R	<501	▼	▼	▼	
1	SC10	10	CR	<501	▼	▼	▼	

Cancel Save As Draft Save and Review



**188.** For primer, enter in M13-F for tube names starting with F and M13-R for tube names starting with R. For length, we will enter “2000-4000” for all of the colony tubes and “< 500” for FC and FR, the pUC19 control tubes. Once you are happy with the order form, click save and review, pay for the order, and they will ask you to supply the invoice form with your order. Print that page out and bring it with you to the post office. I had to mail it overnight to try to meet deadlines but you can mail it ground as well. The samples do hold up fairly well.



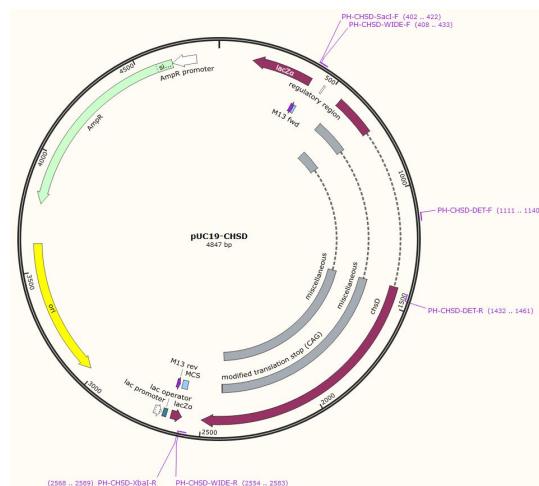
**189.** Now all we have to do is sit back and wait for the results to be emailed to us. In about 24-72 hours after they say they received the order, they'll email you the results along with a link to access it. If there is a delay they'll offer you some free sequencing in return and sometimes they let you redo certain samples if need be.



**190.** Results are in! The email takes you to your personal account page where they show you if things worked. The two most important numbers are the QS and CRL scores. These judge the quality of the reads. QS needs to be higher than 40 and CRL has to be higher than 500. The CRL score stands for contiguous read length, meaning the overall length of each run in terms of basepairs sequenced. The quality score states what is the probability that the bases called were in error. One read did not work at all, the rest are decent but could be better. Concentration of the plasmid due to a rushed miniprep and not letting them grow long enough to get a decent miniprep yield may have been the culprit. Lesson learned, if you are going to setup a culture to shake for miniprep tomorrow, don't process it in the morning if you started it at 3 AM. :)

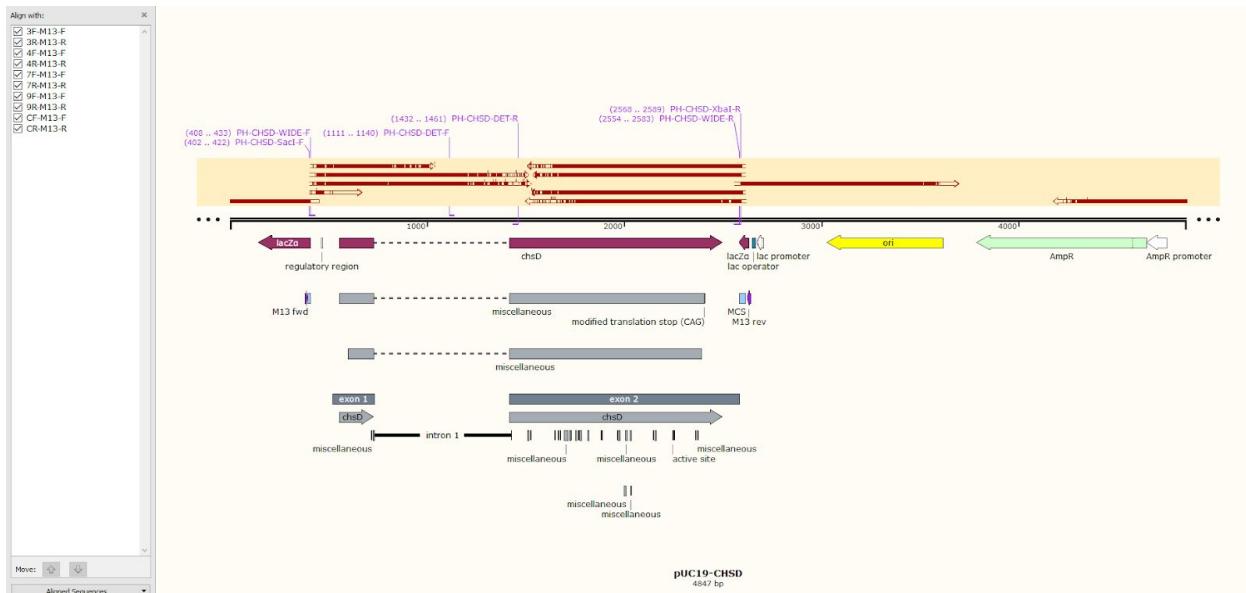
	Reaction	Tube	#	Sample	QS ?	CRL ?	Failure Cause
	SC01	SC1	1	9F-M13-F	15	49	No Priming
	SC02	SC2	2	3F-M13-F	30	526	Early Termination
	SC03	SC3	3	4F-M13-F	44	966	
	SC04	SC4	4	7F-M13-F	45	1001	
	SC05	SC5	5	CF-M13-F	48	1005	
	SC06	SC6	6	9R-M13-R	39	909	Poor Quality
	SC07	SC7	7	3R-M13-R	48	974	
	SC08	SC8	8	4R-M13-R	49	1041	
	SC09	SC9	9	7R-M13-R	50	1044	
	SC10	SC10	10	CR-M13-R	48	1011	

**191.** We can now download the sequences and align them to our simulated plasmid map pUC19-CHSD and see how it turned out. Click on the top left box in the photographed region above to select all of the samples, then go to the top right region of the GeneWiz website and press download selected sequence files. You will get a zipped folder containing the reads. Unzip this folder to a desktop location and open snapgene, namely the pUC19-CHSD DNA file.





**192.** Once on the map, go to Tools > Align Multiple Sequences and they will ask you what sequences to select. Go to the folder you just unzipped and select all of the sequence files. Once they load up, your screen should look something like this:



The red arrows above the main black DNA line are the sequence runs. You can click on each of them and see how long they are. Note that the beginnings and ends of the sequence runs are choppy, denoted by white lines. These are where the signal either started or began to fade. As a general rule of thumb, the first 40 basepairs from a Sanger run is kind of garbage and disregarded. We'll take a look at the **chromatograms**, the actual fluorescence signals read by the Sanger Sequencing machine. Sanger is essentially PCR but instead of using regular DNTPs (free floating As, Gs, Cs, and Ts), they use ones that are fluorescent and each letter glows a different color. A laser inside the machine reads the letters as they come down an extremely thin tube filled with a substrate that allows DNA to flow as a linear fragment. For every letter read there is a peak and the height of the peak relative to background gives the quality score.

For now, let's first look at the actual sequence itself. Switch the viewing tab to Sequence at the bottom left of SnapGene. Now we are in the actual sequence viewer. If you press on the small triangular arrows next to the words "Original Sequence", you can pan the plasmid for the first place where the aligned sequences match up with the plasmid.



Note in this case that the signal starts right after the “M13 fwd” feature of this plasmid. The highlighted letter “N” stands for “aNy basepair” meaning that we cannot determine which letter that part represents, since the signal is just starting and is noisy. As the fragment begins to move down the capillary, the signal becomes more clear and uniform so we can visualize the discrete base pair signals.



Red letters indicate a mismatch. If multiple reads have the same mismatch in the same region, chances are that your sequenced data is the correct version and you may want to order another run to confirm. If the returned data are giving the same result, edit the original sequence and make a note. Now let's look at the chromatogram of 7F-M13-F, my longest read. Back onto the GeneWiz website, select all of the reads and download the Selected Trace Files. Unzip that archive into a new folder and double click the read you are interested in seeing.





Note the very large peaks at the beginning, these correlate to the start of the signal and are the ones we disregard. Now, sometimes the robot makes mistakes and you can use your own vision to see if any suspected peak is actually miscalled. I would not do this if you do not have experience in spotting peak shoulders. Just repeat the sequencing and see what comes of it.

**193.** We now have confirmed that we did indeed clone the Chalcone Synthase D gene from the petunia genome into a bacterial plasmid. This entire process can now be applied, with slight modification, to isolating ANY gene from ANY organism and cloning it into bacteria. There are more advanced methods like cDNA Library Preparation, Bacterial Artificial Chromosomes, etc that take this to the next level. You could have easily just sequenced the first Q5 PCR reaction as is and got back similar results but the added benefit of cloning the gene is you are now able to manipulate it. Add elements, mutate it, transfer the modified gene back into the plant it came from, add it to your library of parts, etc. Once you have the gene in plasmid form, there are countless things you can do with it and countless hours you can spend discovering new genes in known organisms or known genes in new organisms. This is just scratching the surface to the wonderful world of biotechnology and we strongly encourage you to dive deeper and pursue your own curiosities using the tools and techniques illustrated here. Tools for conducting **Small Thoughtful Science**.

I hope you enjoyed this lesson and thank you so very much for supporting our cause! If you have any data that you'd like to share that's been generated during your use of these materials, please send it our way so we can publish it in our annual data booklet.



## References, Resources, and Further Reading

- Serghini, M. A., Ritzenhaler, C., & Pinck, L. (1989). A rapid and efficient “miniprep” for isolation of plasmid DNA. *Nucleic Acids Research*, 17(9), 3604.
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- The Dolan DNA Learning Center — <http://labcenter.dnalc.org/dnalc.html>
- AddGene’s Plasmids 101 Blog — <http://info.addgene.org/plasmids-101-topic-page>
- Primer3Plus — <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>
- GenBank — <https://www.ncbi.nlm.nih.gov/genbank/>
- Uniprot Chalcone Synthase — <http://www.uniprot.org/uniprot/P22925>

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