Undergrad Guide - Spring 2023

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Welcome to the NielsenLab! I hope you are excited for what is to come during your undergrad research journey. While joining the lab might be stressful or scary to some, we have all been in your place at one point, and want to do everything we can to help. So, don't be afraid to ask questions or double check your actions before you proceed.

I created this undergrad guide to help others hopefully not feel as overwhelmed as I did first entering the lab. I knew very little about synthetic biology, and as a college student, did not feel like I had a ton of time to spend studying about things I didn't even know existed. So, I decided for my honors project I would compile a short guide of the basics you would need to know in your first year in the lab, I hope it helps!

Starting off at the lab, you will want to create an account on Benchling, which your mentor will introduce you to, as well as ask Dr. Nielsen to share any important documents with you on Google Drive. Once you have done that, and read this guide, you'll be ready to go!

Contents:

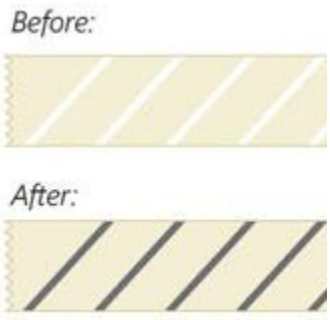
Sterile Technique:

1. Gloves
   Gloves are worn inside the lab as they are easy to sterilize with isopropyl alcohol (IPA) spray and change if they get contaminated. Always wear gloves inside the lab and take them off before going outside the lab. This protects bacteria and other dangers from leaving the lab. Gloves should not touch your phone, clothes, or anything else that could contaminate them. If they do, spray them with IPA, or change them.
2. Isopropyl (IPA)
   IPA is your best friend in the lab, it can be used to clean your bench when it is dirty or when something small spills. IPA is available all over the lab in spray bottles, use it liberally. Before beginning a project, spray your hands down with IPA.
3. Pipetting
   In order to keep our stock containers free of contaminants, always use new pipette tips when pipetting. Make sure they have been autoclaved, you can check if the autoclave tape is brown/black and cream, or just cream colored to check. If your pipette tip touches anything before entering the stock container, or your transfer container, throw it out. It is not worth contaminating the whole stock or your experiment. Even if you are pipetting the same substance, always use a new pipette tip to stop any chance of contamination. When pipetting, be quick but careful. You should only take the caps off of containers for the shortest amount of time possible, as this reduces the chance of contamination, especially if you are working at your bench.
4. Biosafety Cabinet

   The biosafety cabinet is located in the room with the nanodrop and spectrophotometer. The biosafety cabinet is the most sterile field in the lab, and where pouring plates, plating plates, inoculations, transformations, and everything that needs to be kept completely sterile occurs. In order to keep the cabinet sterile, you must follow a series of rules. Before putting anything inside the cabinet, you need to spay the working surface down with IPA and wipe it dry with a paper towel. Then, place your tools inside the cabinet and spray your hands with IPA. If you are using a pipette from your bench, clean it with IPA before using it as well. Anything not needed in the cabinet should be left outside of it. There are tips inside the cabinet as well, that should be used only inside the cabinet. Make sure that you work far inside the cabinet, as that is the most sterile place. If you take something out of the cabinet, or your hands, you need to clean them with IPA again. Also, learn from my mistakes, there are specific microcentrifuge tubes that are sterilized and on your bench in a container. These will be marked with autoclave tape and be

labeled as sterile. Don't ever open these, or anything marked similarly, on your lab bench as they are only for use inside the biosafety cabinet. Some stock solutions, like SOC (which we will talk about later) are also only for use in the biosafety cabinet, but your mentor will tell you about those.

5. Making Plates
   Plates are always made inside the biosafety cabinet. When making and using plates, ensure that your hand or other tools (unless they are the tool making the plate or adding something to it) never pass over the lid or the surface of the plate. The plates should only be open for as long as they need to be, and the lids should always be placed top down inside the cabinet. Plates should always be labeled with the antibiotics they have in them, and the day they were made.
6. Autoclave
   The autoclave is used to sterilize various tools and substances in the lab by using heat/steam and pressure. Things that have been autoclaved will have tape on them that has changed colors (as seen to the right). This means that it is safe for lab use as it cannot have contaminants in it. Glass bottles, media, pipette tips, and many other things are autoclaved.

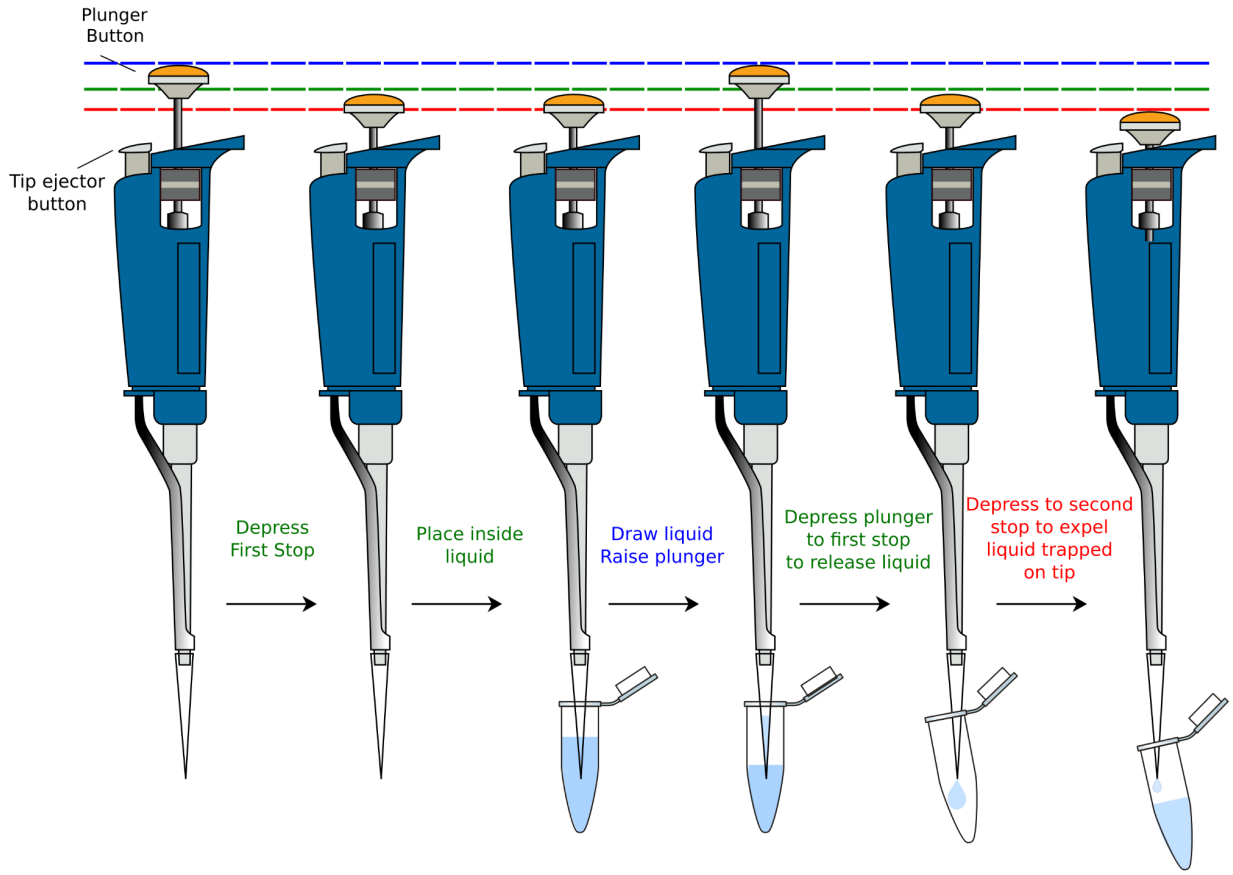


7. Sterile Supplies
   As mentioned above, glass containers are autoclaved to become sterile again. After using them, wash with distilled water three times, and then set them on the rack to dry. Never assume a glass container is clean/sterile unless it has autoclave tape on it. Plastic single-use tools and containers are available all over the lab. Where they are is something that you'll learn over time. If you need to get something out of a jar, like a microcentrifuge tube or PCR tube, always take the lid off and shake them into the lid. Take the number of tubes you need, and then use the lid to put the extras back into the container. This ensures your gloves only touch the tube that you need. When getting tools out of a plastic bag, you can use the outside of the bag to move the items you need up to the top, where you can grab them with your gloved hands.

8. Serologicals
   Serological pipettes come wrapped in paper/plastic, which keeps them sterile. To use a serological, keep it in its wrapper until you are ready to use it. Then open the side that is not fully sealed (like string cheese). Make sure you only open it about an inch or two. With your hand on the plastic outside, push it into the top of the serological machine and then pull the wrapper off. Use it and then dispose of it by slipping it back in the wrapper and putting it in the serological biohazard trash.

Pipetting

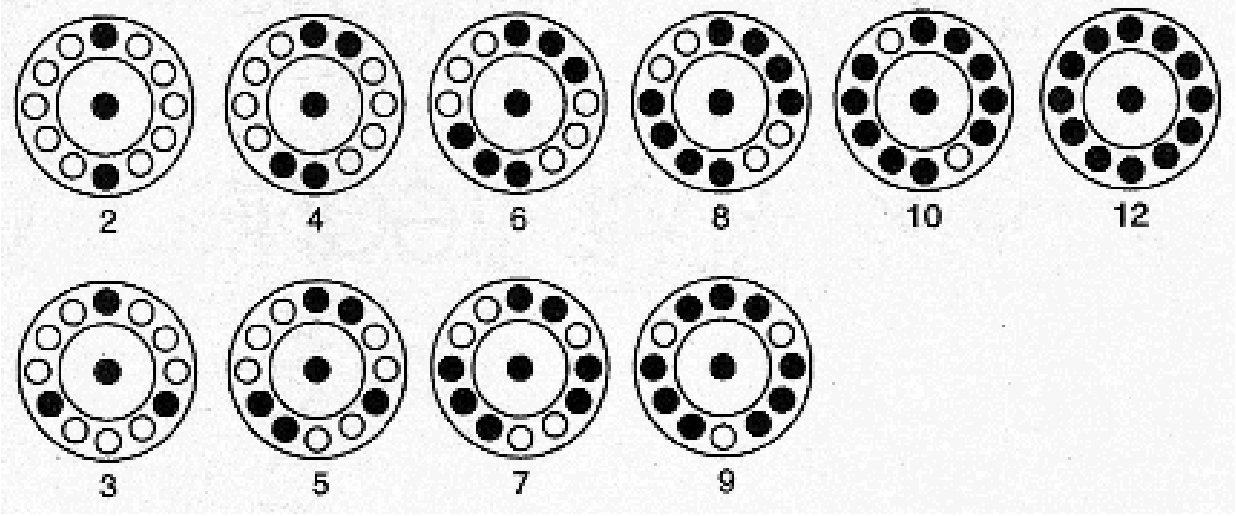
Pipetting is the most used skill in the lab, and while it takes a little bit of time to feel confident in it, it is not too hard to learn. Below is a diagram showing how to use the micropipettes. Besides what the diagram says, there are a few things to remember. These micropipettes are meant to touch a surface when they expel liquid, so either touch the bottom or touch a side of the vial. Also, to mix up a liquid after adding it to your vial (usually if you are dealing with small volumes) you can alternate between the first stop and no pressure on the plunger.



Serological pipettes are also used in the lab, they are much easier to use and just consist of a button that pulls liquid up, and one that expels it. Serologicals deal with larger volumes of liquid.

Centrifuge

In the lab we use large and mini centrifuges. It is extremely important that you balance your samples, as they can be dangerous machines if not balanced. If you need a blank to balance the centrifuge (like if you only have one sample, or an odd number that you can't balance) there are blanks available by each centrifuge. Look for one with a similar volume, and if one doesn't exist, feel free to add or subtract water as needed. I have forgotten many times to put the inner lid on the mini centrifuge, so don't be like me, and remember the lid before starting it. A good rule of thumb also is that you should stay by the centrifuge until it reaches the speed you set it to, and then you can walk away. This ensures that you can stop the machine if there is going to be a problem. And, as always, if you are not sure about what you are doing, or how to balance your samples correctly, ask for help.



Making Media, Pouring Plates, Freezer Stock

Media:

There are many different types of media for different organisms. A researcher will change their media for a variety of reasons, to promote growth, to restrict growth, to make it selective (for a specific plasmid or added gene), or to test a specific pathway (if the organism is using the given "ingredients" correctly), just as a few examples. Some suggestions for making media include:

1. Stir liquid stock before adding
2. Always use DI water
3. Always add on autoclave tape and label what you are making
4. For A+ media, add KH_2PO_4 second to last because it often precipitates out of solution, and and $NiSO_4$ last after changing the volume to its final amount
5. When using solids that need to be dissolved, add water to the final container about halfway (or less if you are adding a lot of volume of solids), then add additional water (or solvent) to bring it up to your final volume. This ensures you don't go over the final volume. Use the large graduated cylinders by the sink to add water. Pour in your solution first, then bring it up to the final volume. This is much more precise than any of the large containers the solution could be in.
6. If you need to shake something to mix it, ensure the lid is on very, very tight, as pressure will often build up

Plates:

Plates serve a similar function as media, they are meant to grow organisms, but usually they are more focused on testing a transformation or something specific in an organism. This means that plates usually have antibiotics in them, which are meant to kill off non-transformed organisms (if you are transforming an organism). Making plates starts off very similar to making media, except they end up solid (which means they usually have agar) so therefore they have a few extra steps. I would follow all of my above suggestions, plus here are a few more for just plates:

1. When making plates, autoclave the agar and water mixture separately from the rest of your solution, this will prevent free radicals, which are harmful to growing cells. Before making the plates, mix the two solutions together
2. Label the plates with the antibiotic that will be used
3. Plates are made in the biosafety cabinet. Use a 50 mL serological pipette to be able to get enough liquid to fill at least two plates before having to refill. Make sure bubbles don't get in the plates, and if they do, suck them back up with the serological pipette. You can use the pipette or shake the plates gently to make sure the solution covers the whole bottom of the plate evenly
4. Move quickly as the solution will solidify. After finishing a plate, immediately cover it so its chances of contamination are lower

5. Finally, place solidified plates inside the bag the petri dishes came from. Label the bag with the date they were made, the antibiotic used, and the concentration of the antibiotic. Place these back in the fridge.

Freezer Stock:
Freezer stocks are made when you want to freeze a sample so you could use it in the future. These are small volumes of cells, often transformed. Freezer stocks should be made after each transformation as a sort of progress check. These need to be made before the cell culture becomes unsterile (aka before it is open outside the biosafety cabinet). Some suggestions to follow include:

1. Add glycerol to your freezer stock, this stops ice crystals, which could damage the cells. It is best to add the glycerol first, as it is easier to mix it with the cells that way
2. Remember to label the plasmid, antibiotics, and date it was made. If you want, you can add in what is in the plasmid, but this is not necessary
3. Make two of each, this ensures that there is a backup copy if anything goes wrong (don't need to do this, but it can be helpful)
4. Shake up the sample cells before pipetting cells into the freezer stock tube. It is also good to use a filter tip to pipette the cells into the stock. Filter tips protect both the pipette, as well as the sample. They stop any cells from accidentally going up into the pipette, or any contaminants from inside the pipette from getting into the sample. This is especially helpful with cyanobacteria, which likes to clump together and can "jump" up into the pipette.

This is one type of filter tips

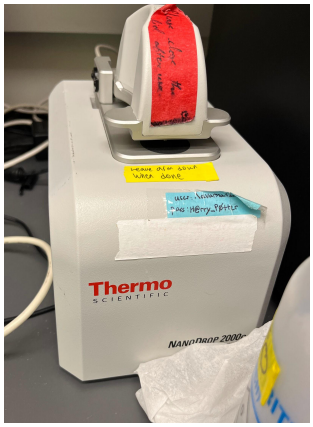
Vacuum

The vacuum is one of the most time saving tools in the lab. It is very simple to use too, and can be used on many procedures as long as the bottom of the DNA column can fit on the vacuum manifold. The only directions you would need is that you place the column onto the vacuum, and make sure you don't touch the actual column, as you don't want to contaminate it. Make sure when you pick a vacuum to use, it is not cracked. To turn on the suction, rotate the valve 180 degrees. The valves are fragile, and only turn one way on, and the opposite way off, so be careful. This makes procedures so much faster!!

This is an example of a column with a vacuum manifold attachment.

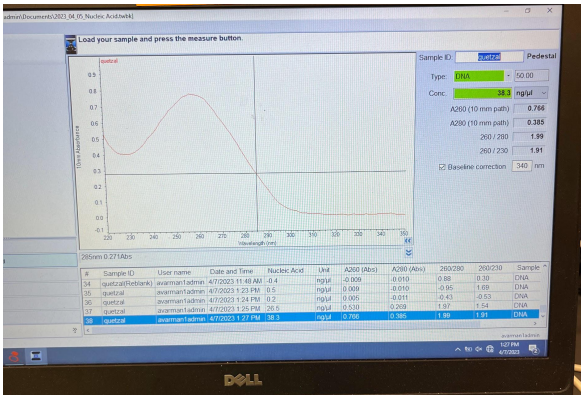
Nanodrop

The nanodrop is a spectrophotometer for extremely small volumes. This is extremely useful as the volumes we are working with are usually very small, and you don't want to lose your entire sample trying to determine the concentration of DNA in it. The nanodrop can be used to determine DNA, RNA or protein concentrations in the UV/visible light range.



When using the nanodrop for measuring nucleic acids, start by ensuring that the computer shows that you are measuring nucleic acids. Then, take a part of a KIM wipe and dampen it with the DI water there. Wipe off the stage (where the sample is placed) and then take a new part of a wipe and dry off where you wiped. Then, take MilliQ water or whatever solutions you dissolved the sample in (which will be leftover from when you prepared your sample) and pipette 2 uL of it onto the stage. Press the blank button, and let it scan. When this is done, clean off the nanodrop again, this time with a dry wipe, then a damp one, and then a dry one again. This will always be the cleaning procedure. Following that, take 2 more microliters of MilliQ water (or whatever you used to blank the machine), and place it on the stage. Then press measure and proceed to the next step only if the reading is less than 0.5 ng/uL.

Following blanking, clean and run each of your samples. Follow the cleaning procedure from above, and use 2 uL of each sample to gather your data. Before adding your sample you should mix it up, either by pipetting up and down, or by tapping the microcentrifuge tube. You will record three values for each sample, the DNA concentration (in ng/uL), the 260/280 ratio, as well as the 260/230 ratio. Pure DNA will have a 260/280 of around 1.8, and a lower value means there is more protein or other contaminants present in the solution. Pure DNA will have a 260/230 of about 2.2. To increase these values, and get more pure DNA, one can do a PCR Cleanup. When not using the nanodrop, always leave the lid closed.



Spectrophotometer - Biomate

The spectrophotometer is the larger version of nanodrop, and can be used when doing large scale experiments, or for checking cell density when trying a longer term experiment, rather than editing a plasmid into a select few number of cells. When using the Biomate to check cell density, it is important one knows what absorbance their sample should be read at. For example, samples of *E. coli* should be tested at 600 nm, while cyanobacteria are sampled at 730 nm.

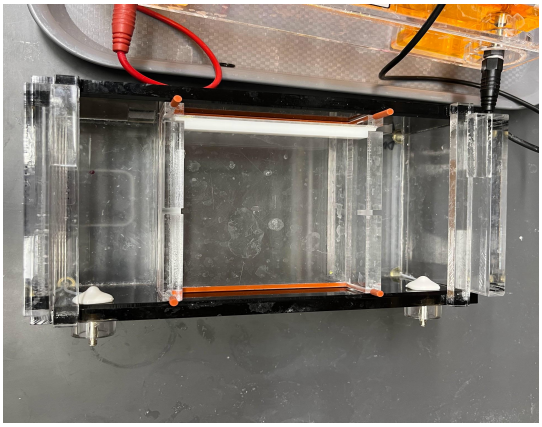
When using the Biomate for cell density, begin by setting the type to cell growth, the wavelength, and the number of samples. Make sure you use clean cuvettes, and that your samples don't have any bubbles in them, as this will mess up the readings. If there are bubbles, remove them with a micropipette. To load the Biomate, make sure your blank goes in the slot labeled with a "B", and line up the arrow on the top of the cuvette with the side with the hole inside the spectrophotometer. This is where light will go through the sample, and ensures the distance for each sample is the same. Continue placing your samples, making sure the arrow corresponds with the hole for each sample. If your samples have been sitting for a while, mix them before loading .When all the samples are loaded close the lid, press run, and enter. When you are not using the machine, make sure the lid is closed.

Troubleshooting:

- If you are using cuvettes that have been used before, run them through the spectrophotometer first with just water, or the same solution as your blank. If any give off a number greater than zero, you can use that as error, and subtract it from your final readings to correct for the "dirty" cuvettes.
- If your values are outside the linear range (0.07 to 0.6) dilute your sample less or more to get your readings inside the range. Then redo them, and do the math (according to the dilutions) to get the actual values. This must be done because measurements outside of the linear range are inaccurate

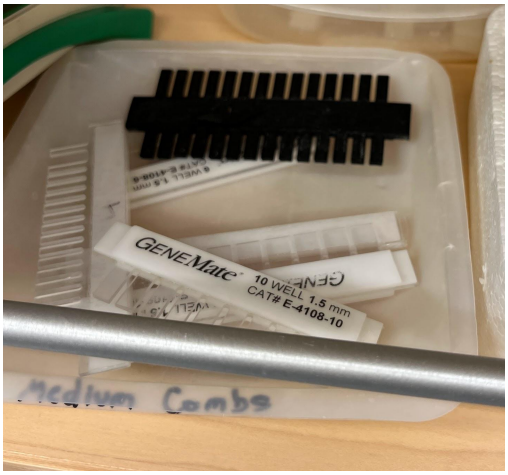
Gel Electrophoresis

Gel electrophoresis is a process used to separate DNA fragments based on their size, and is often done after a PCR replication to determine if the correct fragment was replicated. Gel electrophoresis works because DNA has a negative charge, so when the electric current is applied, the DNA moves toward the positive electrode. Gels of different concentrations are used for DNA fragments of different sizes, the bigger the size the smaller the concentration you want to use, because it will make the pores in the gel larger, and make it easier for separation to occur, and the fragment be seen.



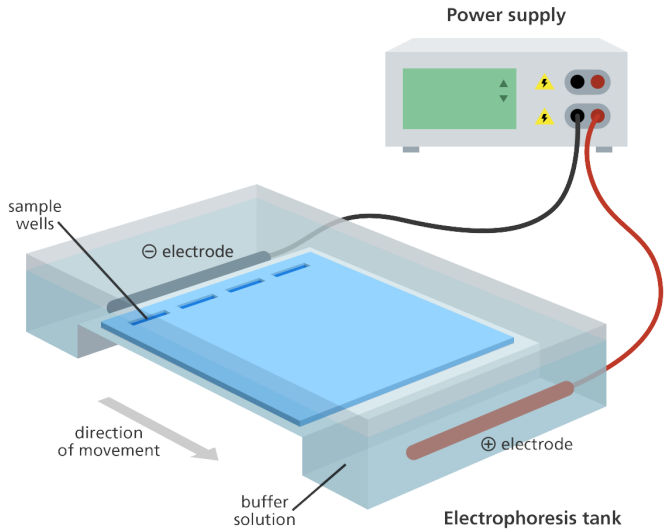
To make a gel, take one of the empty gel trays and place it into a broken electrophoretic chamber (fancy name for a gel box), with the rubber on the sides (as seen to the right). This will create a liquid proof seal to pour and solidify the gel.

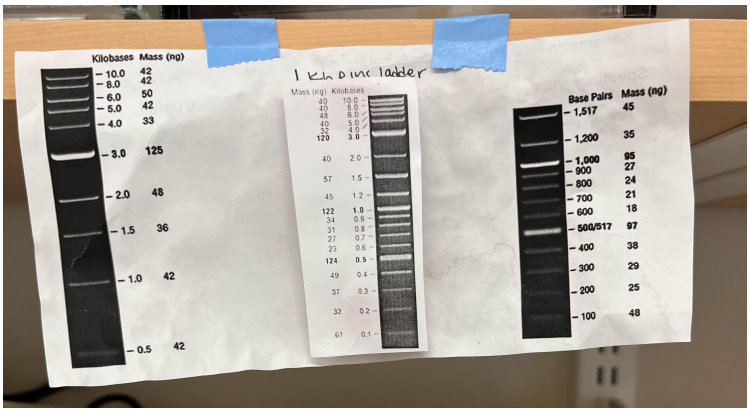
Continue by deciding which percentage of gel you want, and measuring the agarose accordingly. Gel percentage will depend on the length of your DNA fragment. For a 1% gel, 0.6 g of agarose is used, for a 0.5%, 0.3 g of agarose is used, and for a 2%, 1.2 g of agarose is used. For each of those listed, they are added to 60 mL of TAE buffer. These recipes can be used for the little or big gel boxes, unless you want to gel extract from a large gel. Then, the wells will need to be deeper, so more solutions will need to be used. Then, the agarose must be dissolved by microwaving the solution for usually around a minute. Just make sure there aren't any chunks or granules left in the solution. Then, add 4 µL of safe DNA stain, which is located on one of the shelfs in the gel electrophoresis room. Next, slowly pour your solution into the gel tray, making sure there aren't any bubbles, and continue by taking one of the well combs and placing it into your gel. Pick a comb based on how many samples you have, and what our purpose for running the gel is. This will make more sense later, but if you are running to extract some of the DNA, pick a comb with wider wells, as it will make cutting the specific band easier. Finally, take your gel to the fridge to solidify. Make sure you set it on a level surface.



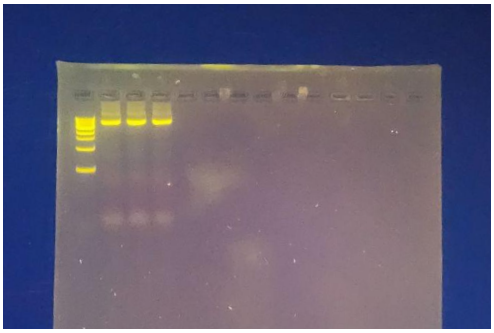
When it is time to run your gel, take it out of the fridge and remove the gel tray. Place the gel into one of the working electrophoretic chambers (gel boxes) with the side with the comb closest

to the side of the black electrode, as this is the negative end. Check the level of TAE buffer available. If the buffer does not cover the gel, add more (up to the fill line). Continue by removing the comb, make sure you pull gently and straight up, as you don't want to tear the gel. Once you check that all your wells are intact, get your samples. Gel loading dye will need to be added to the samples. This ensures that the samples are visible. Gel loading dye also has glycerol in it, which ensures that the samples have enough weight to go to the bottom of the wells, and not just dissolve into the solution. This dye is located in the fridge, next to the ladders, which you will also need. The ladder you use is specific to the fragment you are running. Fragments that are expected to be less than 1000 base pairs should be ran with a 1 kb+ ladder, while fragments larger than 1000 base pairs should be ran with a 1 kb ladder. The picture to the right shows how the 1 kb+ ladder is much more detailed within 1000 base pairs, which is why it should be used for smaller fragments. The ladder is meant to show you approximately how long your fragments are, as it is made up of a series of fragments that are a specific length. Once a ladder is chosen, load 6 µL of it into one of the wells. Then, mix the dye with your sample, and load your sample. If you are using samples from a PCR, you can use 4 µL of sample, and 2 µL of dye. Otherwise, the dye is 6X, so dilute accordingly. Be very careful not to puncture the gel with the pipette tip. This requires some practice.



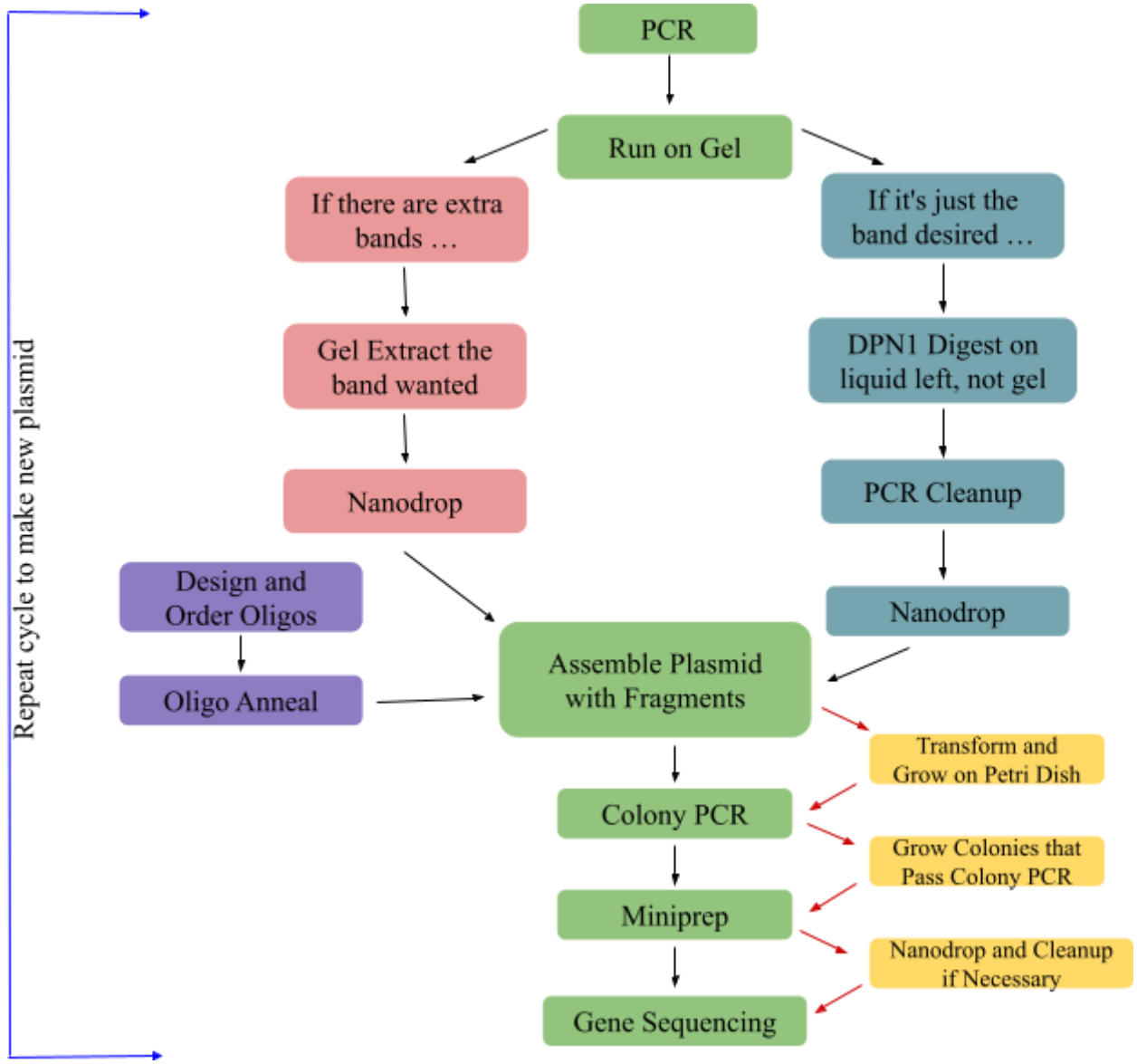


To run your gel, turn on the power pack, and set your voltage and time. The amperage does not need to change. For a small gel, usually the voltage will be 110, and it will run for an hour, and for a large gel the voltage will be 140. These can change however, so ask your mentor if you are not sure. Plug the black cord into the black hole on your power pack, and the red cord into the red hole. Then slide the lid onto the gel, pressing the cords into the electrode until the lid is fully on. Make sure the black cord is on the side where you loaded the samples, and then press run on the power pack.



When your gel is complete, there are many different things you can do with it, depending on why you were running it. First, shut off the power pack. Then take the gel tray out, and put your fingers blocking either side of the open ends so it doesn't fall out. To check the separation, you can take the gel to the UV light machine. Slide the gel out of the tray and onto the screen by tapping it. Place the viewing box over the screen and then turn the UV light on. Never turn the light on before the box is covering it, as UV is bad for your own DNA. You also want to work quickly as the UV can damage the DNA in the gel if left on for too long. You should get results like those seen above. The ladder in that picture is on the left, as you can see the multiple bands clearly. Use your fingers or a spatule to get the gel off of the screen, and back into the tray. When you are done with the gel, place it in the biohazard trash and put the tray back.

Cloning Flow Chart

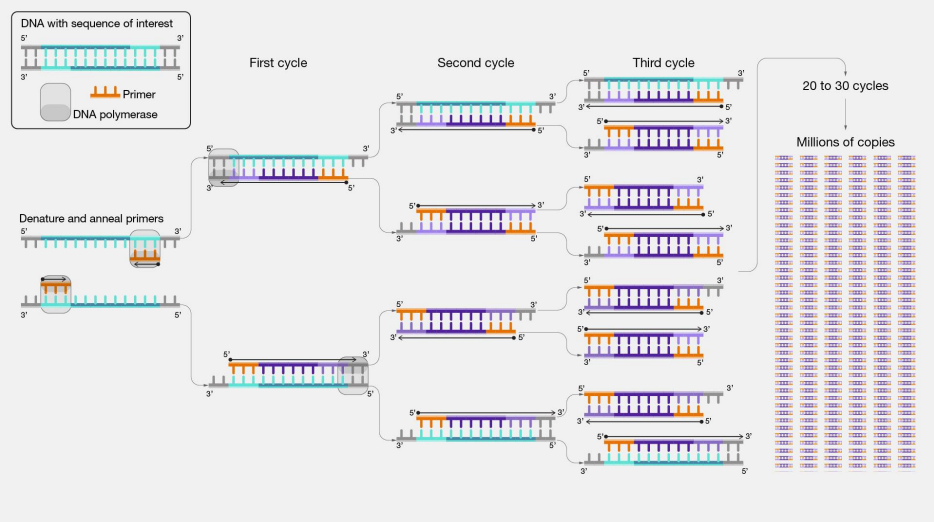


Cloning and Editing Plasmids is extremely important to synthetic biology, and therefore your work in this lab as an undergrad. My mentor, Cody Kamoku, drew a version of this diagram one day on the whiteboard when I, and some other undergrads, were confused on how everything we were doing was connected. My plan is to go through each section, and explain what it is, why we do it, and how it works, rather than how to do them, as you will be given a procedure for most of these actions.

For More Information:

What is a Plasmid? - Plasmids 101

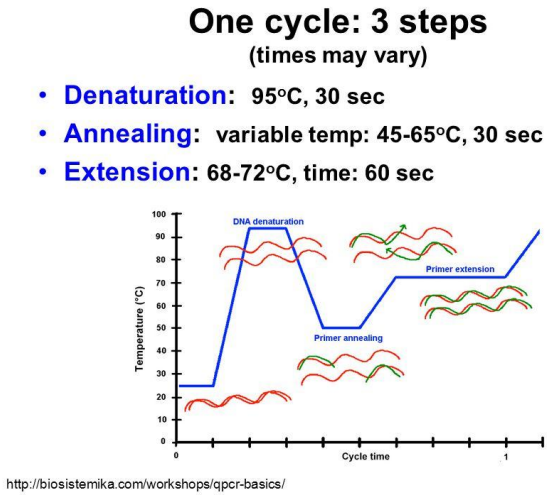
PCR



What it is: PCR stands for Polymerase Chain Reaction, a process meant to make a bunch of copies of a specific DNA fragment outside of an organism. PCR's work by making use of deoxynucleoside triphosphates (dNTPs), primers, polymerase, and a template strand of DNA to create copies of a specific fragment of DNA from the template.

Why we do it: PCR is done to make copies of DNA, usually of a specific gene or fragment that one wants to add to a plasmid. PCR products can then be run through a gel (gel electrophoresis), cloned into a plasmid, or even sent to another lab for sequencing. PCRs can also be used for diagnostic tests, to check if transformed cells have the correct added DNA fragments.

How it works: PCR works because of the primers that are chosen. Primers are single stranded DNA fragments that will bind to the beginning and end of the specific fragment of DNA you want to make copies of. Benchling, which your mentor will likely show you, has sequences of all of the plasmids/primers/DNA fragments the entire lab has. This tool is used to determine good primers that will lead to the PCR copying the correct fragment of DNA. The PCR needs primers in order for the polymerase to bind to the DNA and begin DNA synthesis. In this lab, we have both taq polymerase, and Q5 polymerase. Taq polymerase is much cheaper, and more likely to make errors, whereas Q5 is extremely expensive, but results in almost perfect or perfect results. Taq polymerase is used for diagnostic tests such as colony PCRs, and Q5 is used for assembling plasmids. PCRs take place through many cycles of heating and cooling, which is done in the thermocycler. As you will see in the given protocol, the mixture is heated up to 95 degrees Celcius first which will denature the template strand. Then 30 cycles of heat changes occur, following the pattern of heating to denature (around 95 degrees), then cooling to anneal primers (around 45-65 degrees), and then heating a little more to allow the primers to extend and DNA to be synthesized. The exact



http://biosistemika.com/workshops/qpcr-basics/

temperature for annealing will depend on the primer used, and multiple temperatures may even be set on the thermocycler in order to find the most effective one for those specific primers (*Polymerase chain reaction (PCR) (article)*).

Hints:

1) When using Q5, preheat your thermocycler as it is very temperature sensitive. Leave your mixed up solutions on cold beads until the thermocycler reaches a temperature of 85 degrees, then you can put them in
2) Always add enzymes in last, and keep the enzymes in the freezer until the last possible second
3) When using anything frozen, mix by tapping or pipetting before use
4) Annealing temperature and extension times will change, so always make sure you have the right ones
5) Q5 has an extension time of 25 seconds/1000 base pairs and Taq has an extension time of 60 seconds/1000 base pairs

For More Information:

Polymerase Chain Reaction (PCR) Protocol

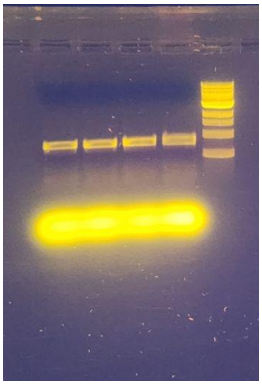
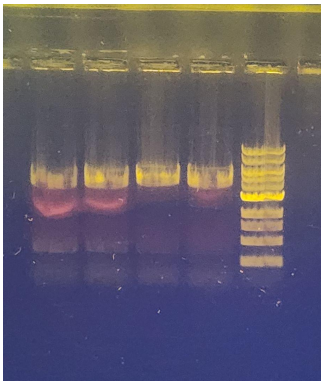
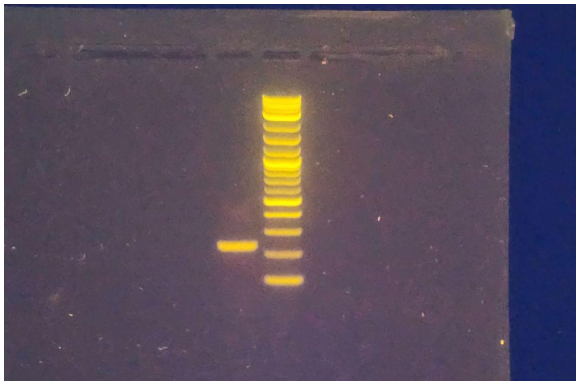
DNA Structure and Replication: Crash Course Biology #10

Gel Electrophoresis

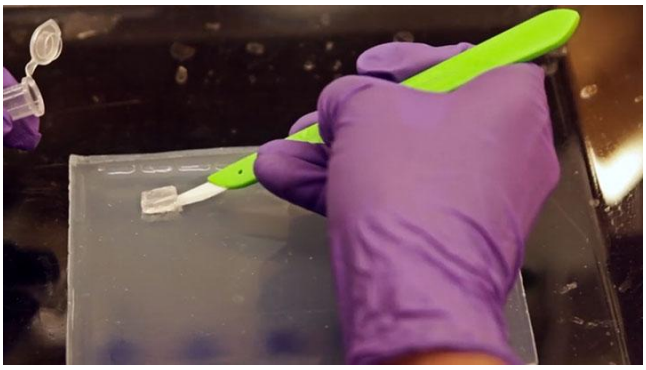
Okay, so your PCR is done, what is next? Typically gel electrophoresis follows PCR.

Hint: When Taq is used, it typically has dye in it, so dye does not need to be added to the sample when it is run on a gel, but for Q5, loading dye should be added as normal.

While I discussed what a gel is, why we do it, and how to run a gel earlier in the guide, let's talk about reading the results, which is the most important part for the cloning flow chart.

Extra Bands	Single Band
 	
Multiple bands are much more common than single bands. This means that the PCR resulted in multiple fragments being copied. To determine which fragment is likely the one wanted, you can compare the fragments to the ladder to determine their approximate length. If one of the bands is close to the length you meant to achieve with the PCR, you can perform a gel extraction, which will be discussed later. This gel can be thrown away if you have more PCR product, but you will have to run another gel with a larger volume of liquid so you can extract the wanted fragments. If you don't have liquid left, you should keep the gel and extract the wanted bands.	A single band is much more rare (unfortunately) than multiple bands. A single band means that only one fragment of DNA is present in the sample. To ensure that the fragment produced is the right one, you can compare its length to the ladder. If the base pairs are approximately the same as your expected base pair count, you can assume your PCR worked. If a single band is achieved, you can take all the PCR product (all the liquid left from the PCR) and do a DPN1 digest, which will be discussed later. Since you have all the liquid left from the PCR, and you know it is all made up of one DNA fragment, you can throw away your gel.

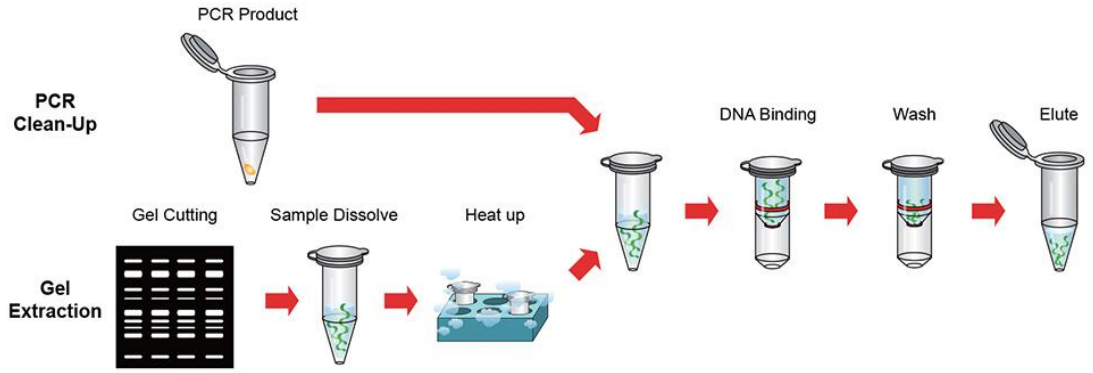
Gel Extraction



What it is: Gel extraction is the process that takes place after running a PCR and getting multiple fragments, instead of just the one you targeted in the PCR. Gel extraction allows one to select the band/fragment they want from the gel, and then transform it back into liquid form so the DNA strands could eventually be added to plasmids.

Why we do it: Gel extraction is done when PCRs don't work exactly how we want them to. We used gel extraction to select for the fragment of DNA we wanted to get from the PCR. While this is not an ideal situation, as liquid from gel extraction is typically more dilute than liquid straight from a PCR, it still can produce the same results, and if needed, purification via PCR Cleanup can be done.

How it works: Gel extraction begins by running the rest of the PCR product, through another gel. It works best if you use a wider well comb, and put all of the product, or most of it, in the one well. Once your gel has been run, you find the band you want by using the ladder, and then cut out the band from the agarose. You want to run all the liquid in one well so that you get the least amount of agarose and other things that could dilute your final solution in your gel extraction. You also want to only get your desired fragment in the final product, so skip wells or leave space between samples if you use multiple. After getting the piece of the gel, you will follow the steps on the given procedure to create a solution with just the desired DNA fragment present.

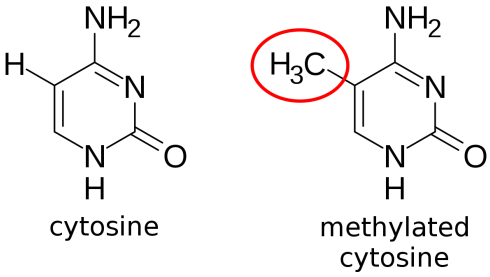


This picture shows how Gel Extraction and PCR clean-up, which will be discussed next, have similar steps, with the gel extraction just having a few extra steps to dissolve the agarose (*PCR clean-up & gel extraction kit*.).

Hint: This is kind of more of a universal hint, but (at least with my mentor), we always eluted DNA with warm water, and I would usually forget to warm up the water until the end, so start any protocol where you have to elute DNA by heating up your water! We use MilliQ water, which is super filtered water, for DNA.

DPN1 Digest and PCR Cleanup

DPN1 Digest:



What it is: DPN1 digest is a process to delete the template DNA that is left in a PCR product after a PCR is run. The template DNA is the original DNA that the primers first attached to, and replicated a fragment of.
Why we do it: DPN1 digest is done so that the PCR product only has the specific fragment of DNA the primers targeted left in it. While this is only done on PCR product that has shown in gel extraction (by only having one band) that it is pure, and only made up of that fragment, we still need to purify the PCR product and remove the template DNA. This needs to be done before assembly to ensure that the assembled plasmid will not have the template DNA in it, and only the fragment of DNA that we want. We also will have a higher success rate if we use pure PCR product.
How it works: DPN1 works by targeting and destroying methylated DNA. Only the template DNA is methylated, not any of the copies made during the PCR reaction, so only the template DNA is destroyed. DNA is methylated when a methyl group is added onto cytosines and adenines in the DNA sequence. This occurs when cells grow. DPN1 works by cutting out the methylated DNA, which will only be the template DNA, so only the replicas from the PCR are left.

PCR Cleanup:
What it is: PCR cleanup allows PCR products, as well as other DNA elutions, from mini/midipreps or gel extractions to be purified. This will remove impurities such as remaining primers, loose nucleotides, remaining enzymes, agarose (in the case of a gel extraction), and other contaminants in the DNA solution. It will also increase the concentration of the DNA solution, which is why cleanup is sometimes done after the nanodrop step for gel extraction or mini/midiprep.
Why we do it: We do this to create a product that is more concentrated and pure for use in plasmid assemblies. Having a clean, concentrated product will increase our chances of having a successful assembly.
How it works: PCR Cleanup works similarly to gel extraction, just with less steps. In simple terms, the PCR product (original DNA solution) is mixed with a binding buffer and centrifugation (or vacuuming) and a filter system separates the DNA and the contaminants, which flow through into a waste container, and are thrown away. Then an elution liquid (usually water) is applied to the filter, which causes the DNA to flow through the filter and into a microcentrifuge tube, once the contaminants have been removed.
Hint: This is one of the protocols where you will need to elute the DNA with water, remember to heat it up at the beginning if that is the elution solution your mentor uses.

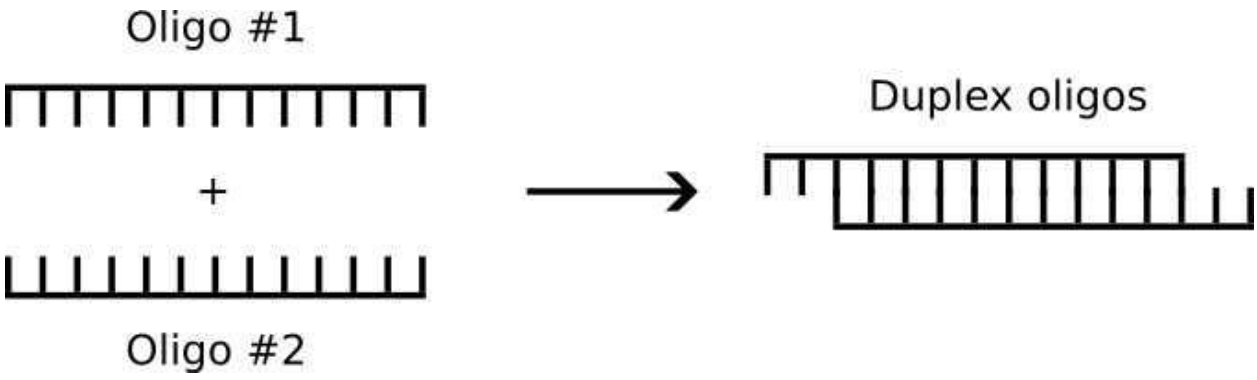
Nanodrop

What it is: As explained earlier, the nanodrop is a tool to find the concentration of DNA solutions. It is a spectrophotometer, so it uses light of various wavelengths to determine the concentration of various substances in the solution, including nucleic acids and proteins.
Why we do it: We do this to ensure that the concentration of DNA solution, and its purity, is of a high enough level before it is used in a plasmid assembly.
Note: While the cloning flow chart does not say Cleanup after the Gel extraction step, if the concentration or purity of the DNA solution is too low after the gel extraction is “nanodropped”, it can be purified with the Cleanup procedure.

Oligo Order and Annealing

What it is: Oligos are short single stranded sequences of DNA (or RNA) that can be custom made and ordered for synthetic biology applications.
Why we do it: We don't use oligos a lot, but we use them when we need a specific, very short fragment of DNA. This could be for a plasmid assembly for example. Oligos are designed and ordered when we either don't have that sequence in any of the plasmids we have (which are all cataloged) or doing a PCR for it would be too difficult as the fragment is so short.
How it works: Oligos are ordered from IDT and shipped to the lab in a very concentrated form. First, the oligos must be diluted. They will come with a paper that says how much MilliQ water one should add to reach a standardized dilution of 100 µM. Because the oligos are different lengths, the amount of water that is needed will be different. To anneal the single stranded oligos to make double stranded DNA fragments that can be used in plasmid assembly, one must mix the oligos, 10X T4 ligase buffer, T4 PNK, and MilliQ water. This solution is annealed in the thermocycler. PNK is the abbreviation for poly-nucleotide kinase, which phosphorylates things. This is needed by the ligase, which does the horizontal linkages between the DNA fragments. After being annealed, the mixture should be diluted by a factor of ten with MilliQ water, and this solution will be useable for assembly.
Hints:

1) Use the thermocycler at the end of the NielsenLab bench for oligo annealing (iCycler)
2) Parafilm around the lid of the PCR tubes any time you use the iCycler, this will stop the lids from popping open



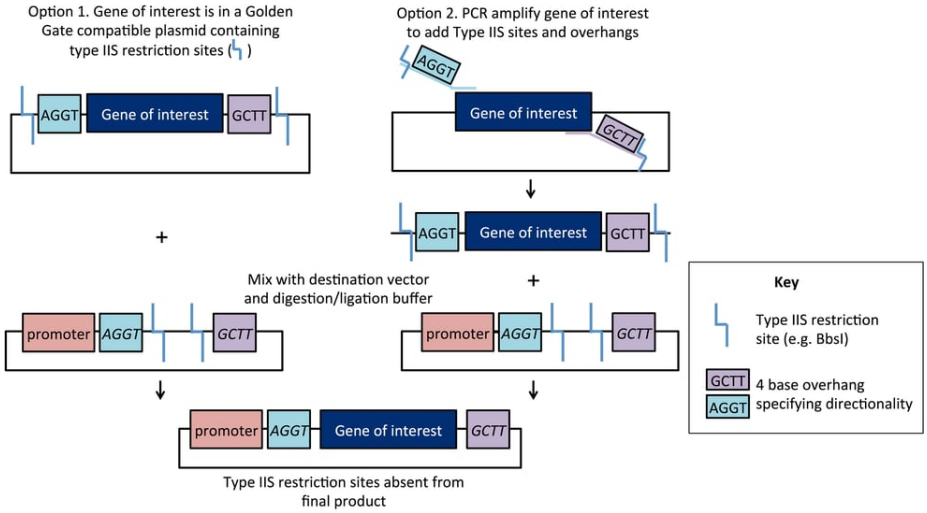
Plasmid Assembly

What it is: Assembly is the process of taking all the fragments of DNA you isolated in the PCR or made with Oligo Anneal and combining them into a plasmid that can be transformed into cells at the next step. There are many ways to assemble plasmids, but I am just going to cover Golden Gate Assembly and Gibson Assembly.

Why we do it: We assemble plasmids so we can make cells do specific things. Plasmid assembly allows us to create a plasmid, which acts as the delivery van for foreign DNA to bacteria. Plasmids are the key to genetic engineering. The DNA fragments we made in all the steps above are useless until they can be placed into a cell, and plasmid assembly is the key to that process. These fragments can contain antibiotic resistance, operators, systems to promote protein synthesis, and much more.

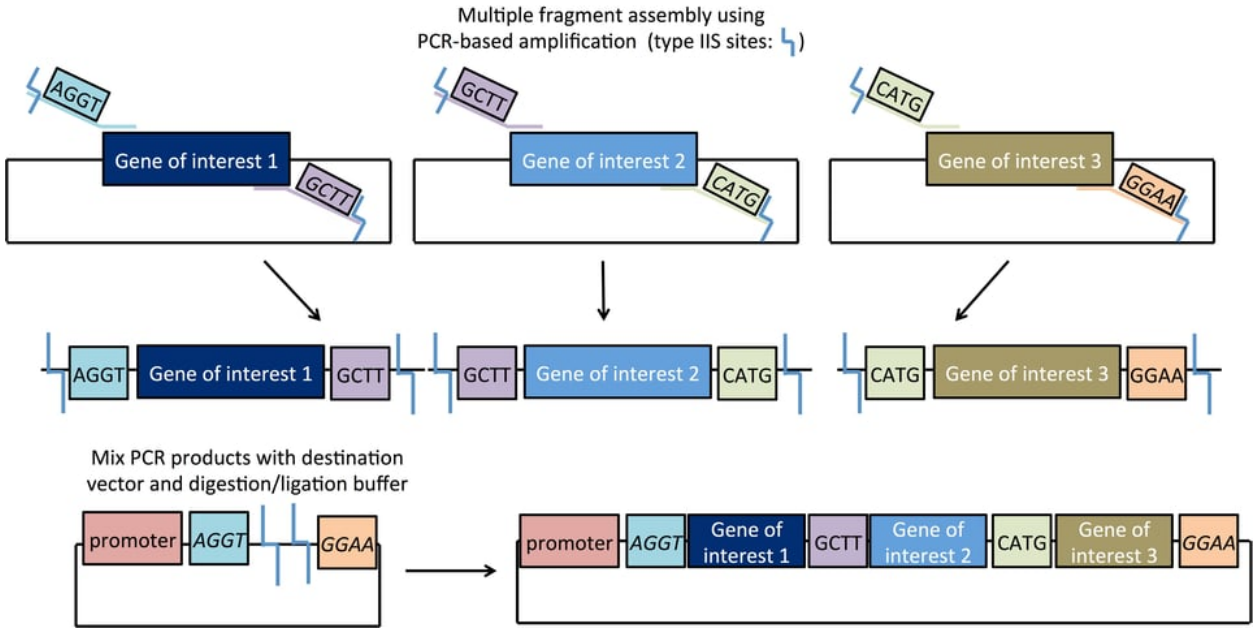
How it works: Assembly using involves taking a compatible backbone and compatible DNA fragments you want to add, and then using the thermocycler to insert the fragments into the backbone. The final product will be a curricular plasmid that is ready to be transformed into cells.

Golden Gate Assembly:



- Golden Gate Assembly is unique because it uses Type IIS restriction enzymes, which cut the gene outside of its recognition sequence. This means the overhangs left that will help with assembly are not part of the recognition sequence. The overhangs can be customized, so one can make them part of the DNA fragment sequence. When the plasmid is assembled then, there are not any recognition sites left, and the assembly is said to be scarless. This can be seen above, as none of the restriction sites are in the final product.
- In other words, the overhangs can be designed for these restriction enzymes, so the enzymes cut out only the restriction site and leave the overhangs that are the same as the ones on the backbone the fragment is being inserted into. It is important that these overhang sequences are located in the right space in the backbone so the DNA fragment is inserted where you want it to be

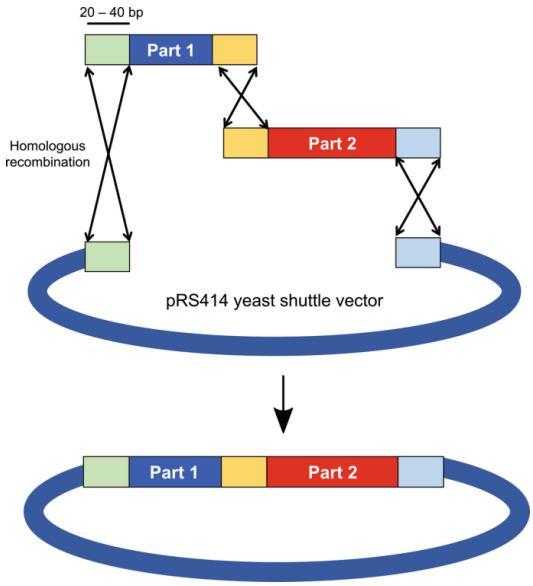
- Custom overhangs can be present in the original DNA fragment, which is seen in the left side of the image, or they can be added via a PCR reaction, which is seen on the right side of the image above
- Golden Gate Assembly works by mixing a plasmid backbone, the DNA fragment(s), MilliQ water, T4 DNA ligase, T4 DNA ligase buffer, and BsaI (which is the Type IIS restriction enzyme). This solution is made and then the solution is ran through the thermocycler, with the times and temperatures dependent on what is being added. The BsaI works to digest the restriction sites on the fragment(s) and template, and the ligase works to connect the digested fragment(s) and template.
- The product of Golden Gate assembly is irreversible because it no longer has the restriction sites, so once it is assembled, the BsaI cannot break it down. This makes Golden Gate assembly very efficient
- Golden Gate assembly is also popular because multiple fragments of DNA (up to 10) can be assembled in one reaction. As long as they each have their own unique four base pair overhangs, they can be assembled. The overhangs specify the order of the fragments (which is seen in the image below), and since the restriction sites are gone after the BsaI does its job, the assembly of all of them is favored. This process gets less efficient as more fragments are added (Gearing).



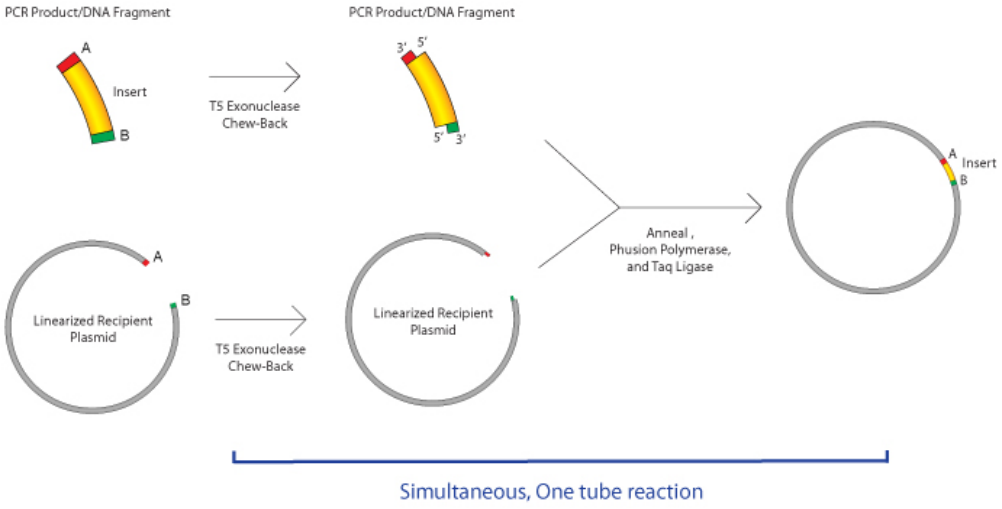
For More Information:

A Detailed Look at Golden Gate Assembly

Gibson Assembly:



- Gibson assembly is known for allowing easy assembly of multiple DNA fragments into a plasmid. DNA fragments need to have regions of homology on their ends in order for Gibson assembly to work. Homology regions are basically just regions of overlap that can overlap and therefore match. These can be created by a PCR, where the DNA fragments for homology can be added to the ends of the wanted fragment.
- These segments then are transformed into the plasmid backbone by mixing them with the backbone and three enzymes. The enzymes include an exonuclease, which creates long overhangs which allows the single stranded regions with homology to anneal, and then a polymerase which builds/binds the DNA, and a DNA ligase, which connects the fragments.
- These enzymes all work at the same temperature, which makes the process really efficient and easy to do. Gibson assembly can combine up to six fragments at once, with the regions of homology determining the order. The result is scar free and doesn't need specific restriction sites (Pyhtila).

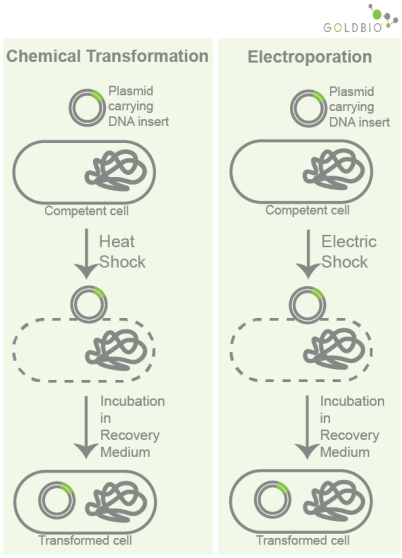


For More Information:

A Detailed Look at Gibson Assembly

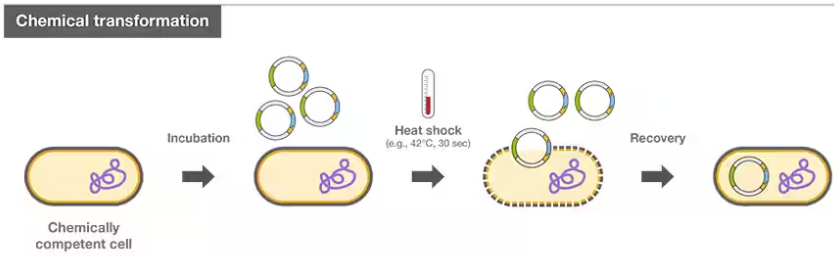
Transform Cells and Grow on Plate

What it is: Transforming cells is the act of inserting the plasmid we just created into the cells we want to grow. These cells are then grown on a plate that is selective (typically with antibiotics).



Why we do it: We have to transform the cells so that they have the plasmid we created. The plasmid is useless until it is in a cell, which can then express the DNA we insert. We grow these cells on selective plates to see which cells took up the plasmid, and if they can survive with the DNA we added. We transform and grow cells so we can do a Colony PCR to check if the plasmid was assembled correctly, and if it was taken up by the cells.

How it Works: Cell transformation works by taking a cell that is competent for the plasmid we want to add, and adding the plasmid to the cell via a series of steps. By doing a series of temperature shocks and adding buffers to help cell growth after transformation, plasmids can be added into cells. The 30 second heat shock is meant to make the membranes of the cells more permeable for the plasmids, and the SOC buffer, or super optimal broth with catabolite repression medium, is meant to help the transformed cells (specifically *E. coli* cells when using SOC) recover from the temperature shocks and addition of a plasmid. This medium, as well as others used for cell recovery after transformation, is a specialized mix of salts, magnesium, and glucose, which promotes cell growth (Kroemer).



The plate's selectivity by antibiotics works by plating the transformed cells on plates with antibiotics that the transformed plasmid has antibiotic resistance for. This ensures, barring unusual circumstances (which are prone to happen in the lab), that any colony that lives on the plate has the plasmid, as it has antibiotic resistance. The only way the cells should be able to grow is if they have antibiotic resistance. This means that one must check what antibiotic resistance they put in their plasmid, and plate the cells on the appropriate (matching) plate.

Steps for Transformation:

1) Add 1 µL (usually) of plasmid in the biosafety cabinet - once inserted, tap 4 or 5 times to mix
    a) Put the plasmids back in the freezer and cells in fridge for 30 minutes

2) Put sample on the heat block for exactly 30 seconds, then put them back on cold beads
3) Add 500 µL of SOC buffer in the biosafety cabinet
    a) Put the tubes (tightly shut) in an erlenmeyer flask and in the shaking incubator for 30 minutes (temperature is dependent on the plasmids added to the cells)
    b) Get the number of plates you need, checking that they have the correct antibiotic, and place them in the 37 degree incubator for 30 minutes, gel side down
4) Plate the transformed cells on the appropriate plates, in the biosafety cabinet - make sure to label the date, antibiotic, your initials, the cell type and plasmid, as well as the volume of cells added
    a) Use glass beads to spread around
    b) Place in the incubator, gel side up, overnight

Hint: Use the light reflection in the biosafety cabinet to check if you are getting the transformed cells all over the plate

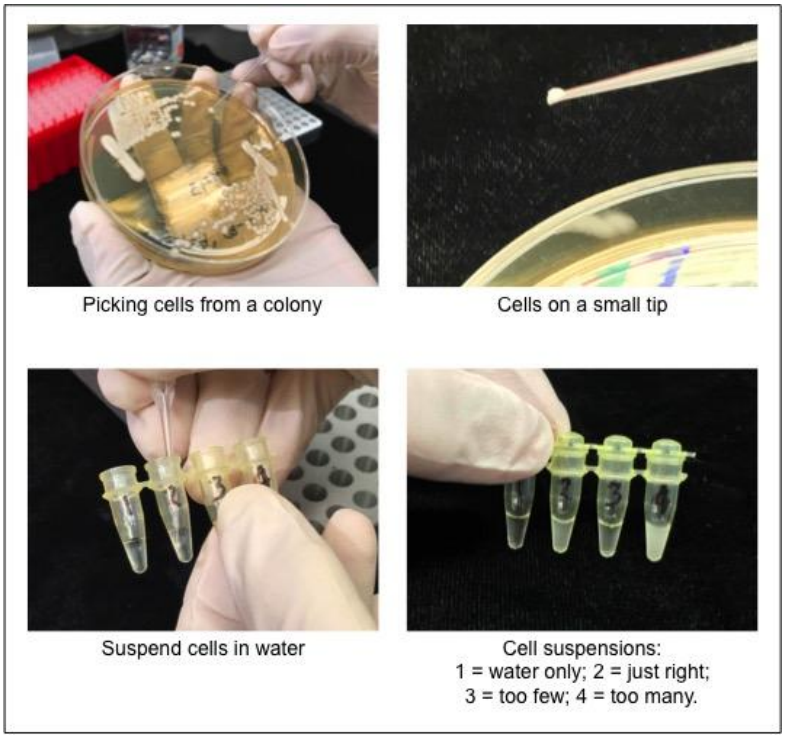
For More Information:

The Mechanism of Transformation with Competent Cells

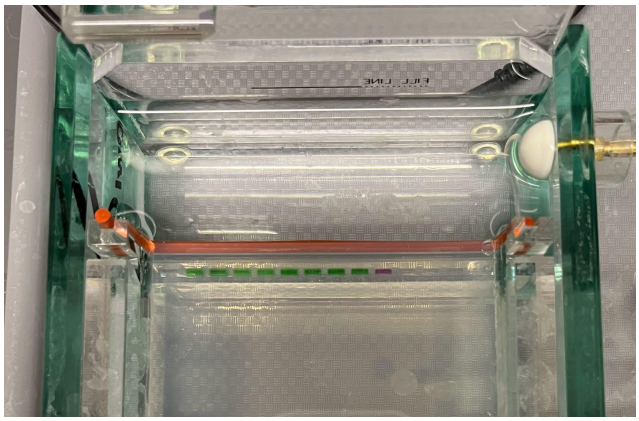
Heat-Shock Transformation Protocol (for Bacteria)

Colony PCR

What it is: A colony PCR is another PCR test, this time using a lower fidelity of polymerase (Taq), that ensures the plasmid wanted is in the cell.
Why we do it: We do a Colony PCR to check that the transformed cells from the last step that grew on the antibiotic plates actually have the full plasmid inside of them, and aren't growing on the antibiotic plates just because they mutated or somehow survived the antibiotics. We do this because we want to create a lot of cells that have been transformed, to use in the future. By testing the cells for the plasmid with a PCR, we use a very small volume of the cell mixture and can grow cells from whichever colonies produce good results on the PCR test.
How it works: A Colony PCR works the same as the general PCR test I explained earlier, as it has the same ingredients, including a forward and reverse primer. Generally in a Colony PCR test taq polymerase is used, as it is low fidelity, but this is just a diagnostic test. The forward and reverse primers will amplify the plasmid that was transformed into the cells.



The Colony PCR starts slightly differently from a regular PCR, as the "template" is actual colonies mixed with MilliQ water, not a sample from the freezer. Begin by putting the selective plates into the biohazard cabinet with labeled PCR tubes and MilliQ water. You will need PCR tubes for the colonies to be mixed with water, as well as PCR tubes for the actual PCR to run in. Singular colonies will be gently scraped off the plate, and mixed with water until the solution is translucent, not cloudy. This value will likely be eyeballed, and depends on the sizes of colonies on your plate. Two important reminders are that it is better to have less concentrated cell solution than over concentrated, as the PCR will not work with too many cells, and it is important you only take one colony at a time. Once the cell/water solution is made, take 1 µL of the solution and place it in the PCR tubes for the Colony PCR. The labels on these tubes, and the ones with the cell/water mixture should be the same. Repeat these steps for as many samples as you want to have (this will be up to your mentor).



The Colony PCR works past this step the same as a regular PCR. The PCR amplifies the plasmid, and the samples are then run through gel electrophoresis. When loading samples made with taq polymerase, loading dye is not needed, as the ‘gotaq’ solution has green dye in it already. You can see this in the picture to the right. The purple sample is the ladder. After the gel runs, analyze the samples and grow the cell/water mixtures that showed proof of the plasmid in the Colony PCR. This will result in a large cell culture with the plasmid inside.

Hint: If you need primers that are frozen and in their stock tubes (which will be stored in the -23 degree freezer), these primers will need to be thawed and diluted.

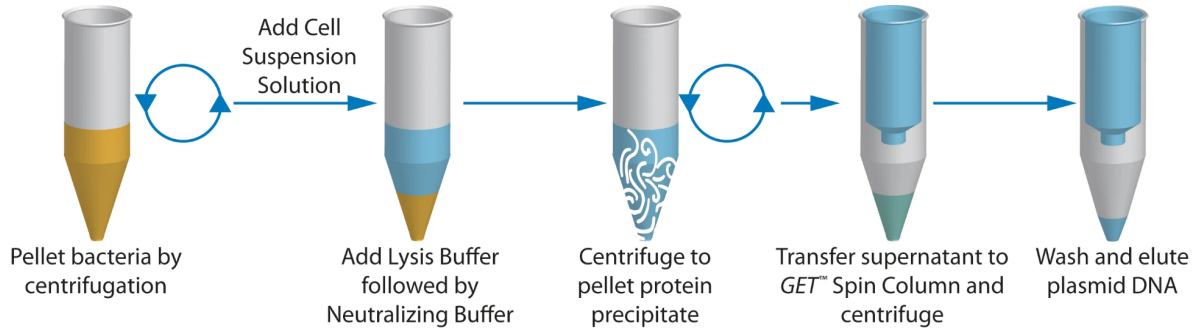
For More Information:

colony PCR

MiniPrep or MidiPrep

What it is: Miniprep and Midiprep are the same thing, just with different names that correspond to the volume of cell culture one is using. These processes are used to purify DNA from bacterial cell cultures. It is another way to purify DNA, with similar results to gel extraction and PCR cleanup procedures.
Why we do it: Mini/Midiprep is done so the DNA from the successful bacterial cells can be sequenced, or used for other purposes, such as taking fragments of them in PCR, transforming the plasmid into more cells, etc.
How it works: Mini/Midiprep includes a series of several steps that lyse the cells, isolate the DNA, and then elute the DNA into a (hopefully) highly concentrated solution.

Mini/Midiprep begins by centrifuging the cell culture so the bacteria is in a pellet at the bottom of a tube. The supernatant is then discarded, and the cells are resuspended with the addition of the first solution. This solution will also include RNase A, which is meant to degrade cellular RNA during the cell lysis, as RNA is not wanted. Then, the cells are lysed from the cell culture. This is done by making the solution alkaline (basic). Next, the cells are returned to a normal pH. The solution should now be chunky, with the cell components such as proteins, cell membranes, and genomic DNA all precipitated. The smaller plasmid DNA should stay in solution. This solution is then centrifuged, which turns the unnecessary cell components into a pellet. They might also float on top, so when you pour the supernatant (which now has the plasmid DNA in it) into a tube for filtration, rotate the tube you're pouring so neither the floating cell components, or the pellet, get inside the collection tube. Then, depending on Mini or Midiprep, your steps for filtration will be different, but the ideas are the same. The DNA is pushed through a filter (the column) that catches the plasmid DNA and lets everything else go through. The DNA is washed several times, to try to purify it as much as possible. Finally, the DNA is eluted, which frees it from the column and allows one to capture it in the final collection tube.



(*Plasmid isolation: Overcoming the challenges for isolating plasmid DNA*.)
This image is not a perfect example of the procedure, but the basic ideas are illustrated, which I think helps with understanding the process.

After Mini/Midiprep is done, the product (DNA solution) will be nanodropped to see its concentration.

Hints:

1) This is one of the procedures that can use hot water to elute the DNA, so make sure you heat up MilliQ water at the beginning if you need to
2) The vacuum can be used on Midipreps, and sometimes Minipreps, I highly suggest you use it when you can, it will save lots of time
3) Some of the solutions in this kit, and a few others, require that ethanol is added to them before they are used. Make sure this has been added before you use any solution
4) Make sure to fully resuspend the pellet on the first step, the vortex can be used to help do this
5) When adding the water to elute the DNA, make sure you add it directly to the column, not to the sides

For More Information:

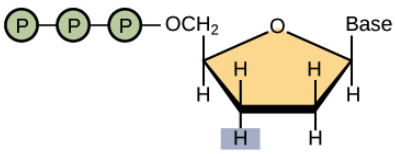
Plasmid Purification In Just 20 Minutes - Protocol - we don't follow this exactly, but it shows the general protocol

Gene Sequencing

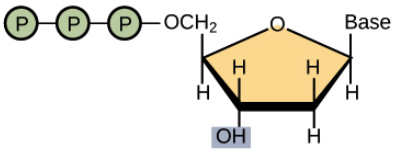
What it is: Gene sequencing is when you send off your DNA, either as fragments or in plasmid form, to a company who can use either Sanger sequencing or Nanopore sequencing to determine the exact base pair sequence.

Why we do it: We do this in order to check our work, especially after a plasmid transformation worked. This can check for mutations, especially if one wants to use the plasmid to do other PCRs and take segments from. It is important to know what the actual base pair pattern is because a lot of the DNA made in the lab is used for everyone's projects. This can also help to explain weird behavior that could be due to a mutation.

How it works: We do not sequence DNA at the lab, instead we send it to Plasmidsaurus, and they sequence it for us. There are two common ways to sequence DNA however: Sanger and Nanopore.

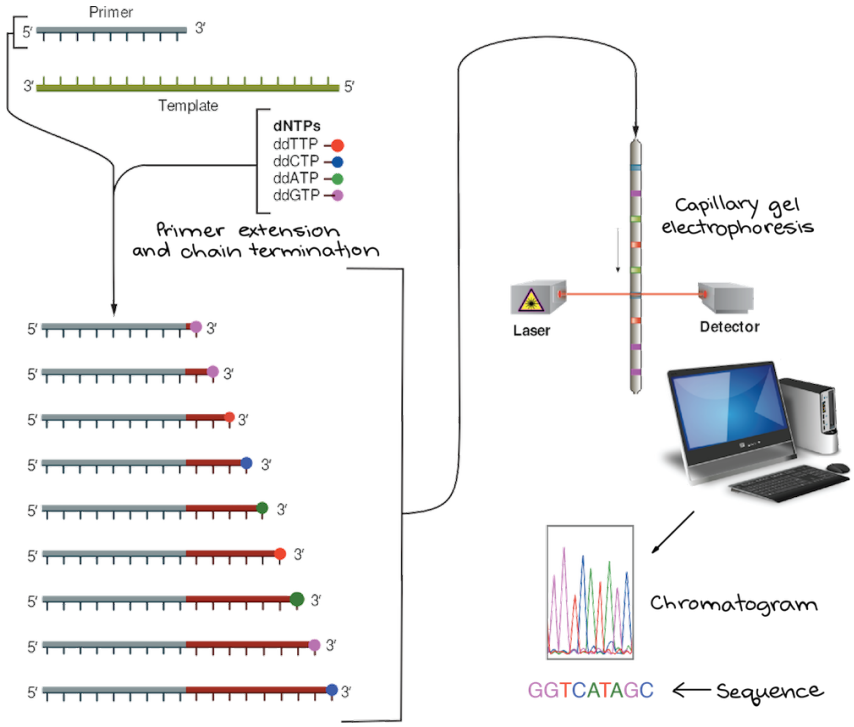


Dideoxynucleotide (ddNTP)



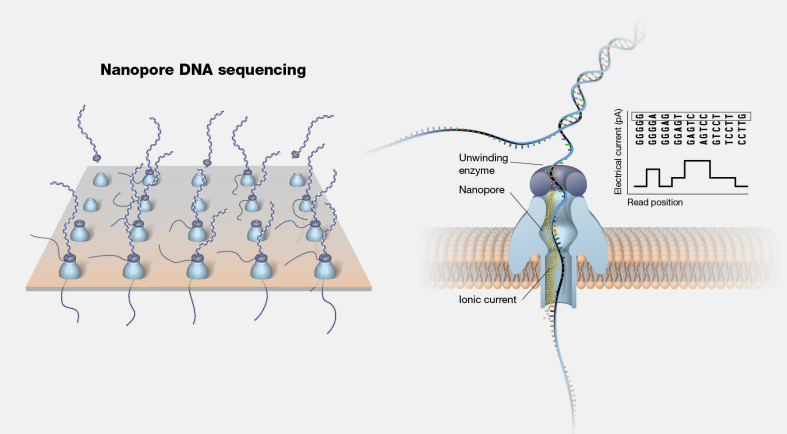
Deoxynucleotide (dNTP)

Sanger Sequencing is used when a short segment of DNA needs to be sequenced. It begins with the DNA that needs to be sequenced being copied to make many segments of various sizes. The sequence is determined with the addition of fluorescent nucleotides, known as "chain terminators". Sanger sequencing works for fragments that are less than 900 base pairs. The Sanger sequencing reaction involves many similar ingredients as in a PCR, including a DNA polymerase, a primer, dNTPs, as well as template DNA (the DNA you want sequenced). The final ingredient is ddNTPs, which stands for Dideoxy, or chain-terminating, NTPs, which are all labeled with a unique dye. The ddNTPs are different from the dNTPs because they lack a hydroxyl group, which means when they are added to the DNA being replicated (which is a very similar process to PCR), the next nucleotide cannot be added, and the strand ends. This will result in many strands, all with different lengths. This is repeated a lot of times, with



the idea that there will be a ddNTP in the DNA copies in each place from the original whole template strand. Next, capillary gel electrophoresis is used to separate the DNA fragments by length. When each fragment crosses the end of the tube used for capillary electrophoresis, a laser illuminates the nucleotide sequence, allowing the dye attached from the ddNTPs to be read and the proper sequence to be found (*DNA sequencing (article) | biotechnology)*.

Nanopore Sequencing is used when a whole plasmid needs to be sequenced. Nanopore sequencing involves reading the DNA sequence of single stranded DNA as they get threaded through tiny pores (aka nanopores) in a membrane. The DNA makes a signal as it goes through the pore, which corresponds to what base it is (*Nanopore DNA sequencing*). Nanopore sequencing is fairly cheap.



Repeat!

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