Sarpeshkar Wet Lab Guide 2024

How to Construct, Transform, and Recover a Plasmid from Start to Finish

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Reading a Plasmid Map

The plasmids used in the lab are designed in a software called SnapGene. This software allows us to tailor plasmids to our specific needs based on the sequences of preexisting plasmids, or sequences pulled from the bacterial chromosome itself. These small circular pieces of DNA can be inserted into the cytoplasm of the bacteria where they will replicate and express genes independently of the bacterial genome (1). The preexisting sequences we use are considered our template DNA. With SnapGene, we can design new plasmids with specific antibiotic resistance genes to select for only the bacteria that have taken up our plasmid, and to test genes of interest

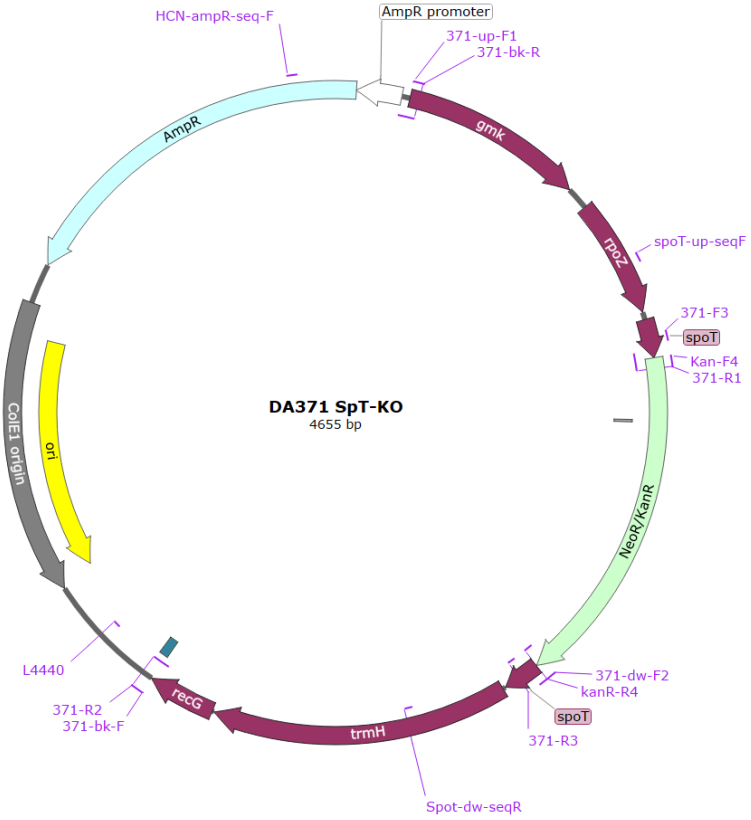


Figure 1. DA371 Plasmid Map.

outside of their original host.

Here is an example of one of our plasmid maps, DA371. The size of each plasmid is listed in base pairs under the name of the plasmid. Each plasmid consists of a backbone, or vector, in which fragments of target DNA are inserted into. While plasmids are not replicated from top to bottom, SnapGene orients the map so that genes with the arrow facing clockwise around the circle are transcribed in the forward direction, and those with the arrow facing counterclockwise around the circle are transcribed in the reverse direction. In the case of DNA replication of the entire plasmid, the leading strand will be the one pointing in the direction of the yellow *ori* arrow, and the lagging strand will be transcribed opposite of the arrow.

For DA371, the backbone is made up of the high copy origin of replication ColE1, the ampicillin antibiotic resistance gene *ampR*, and their corresponding promoter and terminator regions. The inserted region of this plasmid consists of the essential bacterial gene *spoT*, as well as the genes flanking it. The purpose of this plasmid is to knock out *spoT* from *E. coli* strain BW25113 via homologous recombination. The primers are designed to amplify the complementary regions up and downstream of *spoT* so that our PCR fragment can bind and replace the gene with the kanamycin antibiotic resistance gene *kanR*. Theoretically, only the bacteria that have *spoT* knocked out of their genome should be able to grow when plated on LB + Kan plates.

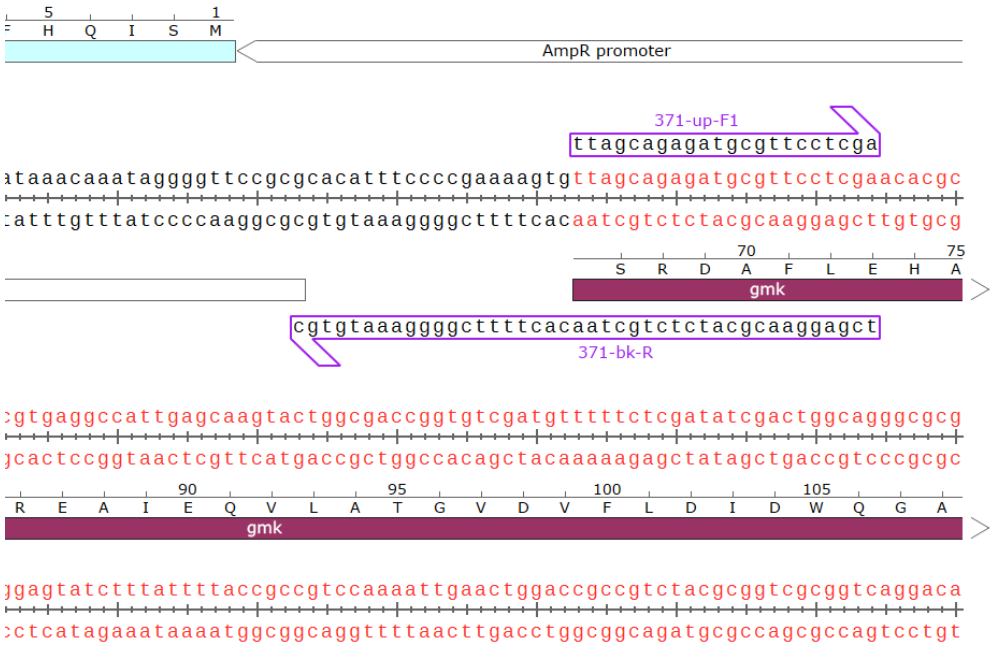


Figure 2. DA371 primers joining the backbone to the insert.

The purple markers on the map are the names and directions of our primers used for PCR or for sending samples out for Sanger sequencing. They are short, single stranded sequences of DNA typically between 15 – 60 bp in length. The primers are oriented from 5' to 3', with the half arrow signaling the 3' end and the direction the polymerase will transcribe towards.

Selecting Primers

When running a PCR reaction, you will need to select a forward and a reverse primer that will bind to only one site on the target DNA and has an annealing temperature within 2-3 degrees of the other primer. This is to ensure that only one site on the DNA will be amplified, and to ensure that both primers can bind fully. It is not always reliable to use the annealing temperature (Tm) provided on the primer stock itself. If the primer is longer than 20-25 bps or is GC rich, the annealing temperature will appear much higher than is necessary. Typically, we want to choose a primer with an annealing temperature between 57°C to 60°C as this is approximately the range the Q5 polymerase works best at.

Check the predicted annealing temperature of the primer by highlighting 18-22 bps on the 3' end of your primer. For 371-up-F1, the predicted annealing temperature is 60°C.

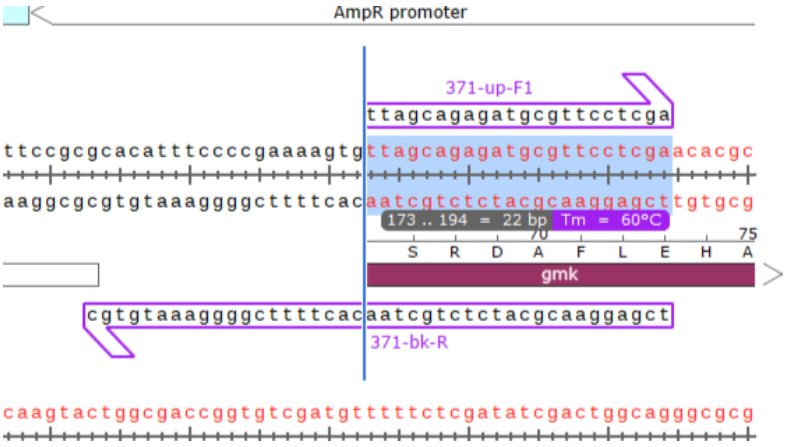


Figure 3. Predicted annealing temperature for DA371 primer 371-up-F1.

To join the backbone to the insert(s), at least one of the primers we choose must have a 20 bp overlap on the 5' end complementary to the insert. This can be seen in Figure 2 in which the 371-bk-R primer has a 20 bp overlap with the black backbone DNA on the 3' annealing end, and a 20 bp overlap with the red insert DNA on the 5' end. After PCR amplification, the linear fragments will be subject to exonucleases that will chew back at the exposed 5' ends, leaving behind the 3' single stranded complementary overhangs that will join the two fragments together during Gibson assembly.

Running Q5 PCR Amplification

We use the highly accurate Q5 polymerase in the lab to construct all our plasmids. Not only is Q5 highly accurate, but the mixture does not contain any added dyes that may interfere with purifying our PCR product.

BEFORE YOU START! Get a bucket of ice and the metal PCR tube holder. The Q5 polymerase is heat sensitive and needs to remain on ice the entire time it is out of the -20°C.

Unless otherwise specified, each reaction mixture should contain:

0.4 µL Forward Primer
0.4 µL Reverse Primer
0.5 µL Template DNA (Approx. 25 ng DNA, adjust volumes as needed)
18. 5 µL ddH_20 (Adjust as needed)
20.0 µL 2x Q5 High Fidelity Master Mix (mm)

_____

40.0 µL Total Volume

Note: Add water first, then primers, and Q5 last as it needs to stay on ice. After mixing your reaction, follow the instructions below that have been bolded.

On the thermocycler, go to home, saved files, select Q5 on the right most dropdown list, then select edit. Depending on your specific primers, you will need to adjust the annealing temperature and the elongation time. Unless specified, nothing else needs to be changed.

There are 7 columns listed after you select edit. The first column is the initial denaturation stage. During this, the hydrogen bonds between the double stranded DNA are broken, allowing for the primers and the Q5 DNA polymerase to bind to the single strands of DNA in the next stage. Column 2 is another denaturation stage, however a shorter one that occurs at the start of each subsequent cycle.

Column 3 corresponds to the annealing stage. During this, the temperature is reduced, and the primers bind to their complementary sites on the DNA. Select the temperature that is already listed, and it will pop up a screen for you to adjust it.

Column 4 corresponds to the elongation stage. During this stage, the Q5 DNA polymerase will begin to amplify your target sequence using the primer as the site to elongate the DNA from. Select the time that is listed at the bottom of the column, and it will pop up a screen for you to adjust the time as well. A good rule of thumb is, for every 1 kb you need to amplify, multiply that number by 30 seconds, and that is how long you should set the elongation time for.

Column 5 is the number of cycles the PCR will repeat starting from the short denaturing stage. This will increase the number of amplicons you get as the final product. Column 6 is the final extension phase where the DNA is polished off (2). Column 7 holds the temperature at 4°C so that no further enzymatic activity occurs.

Afterwards, select run, block A or B, then select "OK" when asked to confirm the volume (40 µL) and the lid temperature (105°C).

Wait for the lid temperature to preheat to 90°C before adding your PCR tube. This is to prevent the sample from evaporating during the run. Close the lid, then proceed to set up your agarose gel to check the size of the fragment once the run is done.

Analyzing PCR Product on an Agarose Gel

After your PCR run is complete, we always check the size of our fragments via gel electrophoresis on an agarose gel. Most of the time, the gel will be 0.8% agarose; if the fragment you are amplifying is larger than 4 kb however, decreasing the percentage of agarose will allow the fragment to migrate through the gel easier and the bands will be better separated.

Setting Up a Gel

For a 0.8% agarose gel, add to a flask:

0.48 g of PCR specific agarose

60 mL 1x TAE

Note: This agarose can withstand higher temperatures than the agar we use for plates and will not melt during electrophoresis

Stick the flask in the microwave for 1 minute and 30 seconds. Swirl the flask halfway through with the heat protective mitts to ensure the agarose boils into solution completely.

Our gel casting trays, combs, and rubber stoppers are located in the top drawer beneath the microwave. Set up the gel casting tray by wetting the rubber stoppers, then fitting them snuggly to either end of the gel tray. Pour the melted agarose into the gel tray, then add a comb with enough wells to fit your samples plus the ladder. Place the comb in the slot second from the top with the white side facing down. Allow to harden for 20-30 minutes, or until it is firm to the touch.

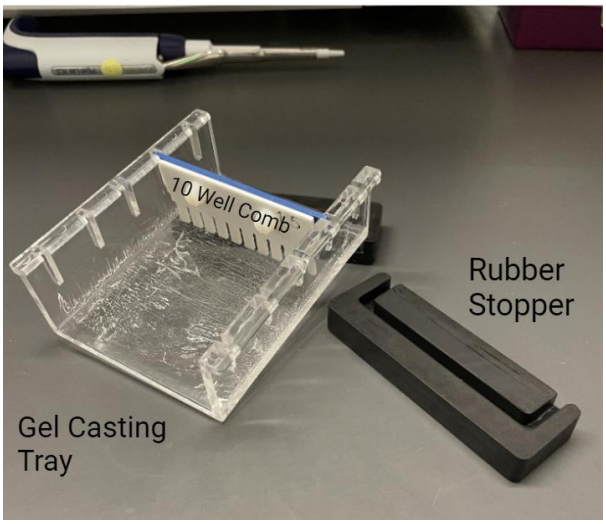


Figure 4. Gel Casting Tray for Electrophoresis of DNA on an Agarose Gel.

When to use a specific comb:

For normal Q5 reactions, you can use any comb from 8 wells to 16 wells.

For gel purification, you want to use the comb with 5 wells. These wells can hold the most sample and will allow you to extract your DNA from the gel all at once in a singular band.

For colony PCR using Sapphire, you want to use the comb with 16 wells. Because we often screen 10 or more colonies at a time, this comb will allow you to check all your samples at once, as well as have enough space for your ladder.

After the gel has hardened, peel off the rubber stoppers and carefully remove the comb by lifting straight out. Be especially careful handing the gel when the agarose percentage is lower than 0.6% as it will be soft. Stick the gel and the gel casting tray into the electrophoresis device so that the wells are closest to the black electrode. Add enough 1 x TAE to cover the surface of the gel. This is crucial for the DNA to migrate through the gel!

Loading a Gel

In order to view the DNA after it has been run on a gel, both the 1 kb Plus reference ladder and your samples need to be treated with 6x DNA loading dye. This dye binds to the nucleic acids in the sample and omits a visible fluorescence when viewed under UV light.

To preserve the integrity of your samples and the ladder, always remove the appropriate amount of sample and mix with the dye on half a strip of parafilm. The exception to this rule however, is if you ran colony PCR with Sapphire, or are loading your entire sample for gel purification. In both these cases, you can add the dye directly to the PCR tube.

Figure 5. Electrophoresis Device Connected to Voltage Box.

Sample Type	Sample/Ladder	6x DNA Loading Dye	Load to Gel
Q5 PCR	4 µL	3 µL	7 µL
Gel Purification	20 µL Ladder 40 µL Sample	5 µL 10 µL	25 µL 50 µL
Sapphire/Colony PCR	20 µL	5 µL	7 µL

To load the gel, pipette up the appropriate amount of sample, and hold the pipette tip perpendicular to the gel. For best control of the pipette, place your elbows on either side of the tray and use both hands to guide the pipette tip straight down into the well. If you feel resistance, stop, and lift the pipette tip up a little so that you do not puncture a hole in the bottom of the well. Pipette down until you reach the first stop. DO NOT pipette further, this will potentially add air to the well and blow out the sample. Lift the pipette tip straight back out, then discard the pipette tip. You should be able to see your sample sitting in the well. If you do not, the well may have been punctured and escaped out the bottom or been washed out while loading. If you are unsure, it is best to prepare a new sample and load it into a different well.

Place the lid on the machine until its snaps shut with black matching black, and red matching red. Black is the negative anode and red is the positive cathode. The electrophoresis unit applies an electric field through the running buffer (1x TAE) so that the DNA migrates through the gel and separates out by size. This is because DNA is negatively charged, and will travel along the electric field towards the red cathode. The DNA will separate out by size as only smaller fragments will be able to pass through the agarose matrix during the set amount of time (3).

Switch the device on, select the mode button, then hit start. Check the machine for bubbles at the anode end. If you see bubbles, this means the lid is shut tightly and the current is flowing correctly.

Reading a Gel

After the gel has finished running, turn the machine off and remove the lid. Carefully lift the gel tray out of the machine, keeping a finger on the gel as it will slide around. Rinse the gel with cool water to reduce fogging. Turn off the lights then place the gel and tray on the blue UV reader. Place the orange cover on top then switch the UV light on.

You should be able to see bright crisp bands for the reference ladder, as well as clear bands for your product like that for pAB002 V1 at 0.4 kb, and pAB005 V1 at 1.2 kb (Figure 6). Compare the size of your product to the reference ladder and the expected size of your fragment as designated on the plasmid map.

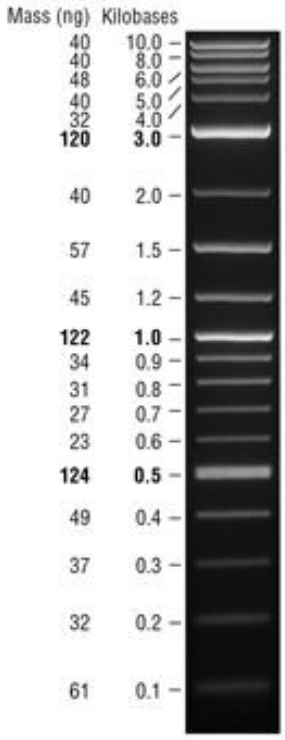


Figure 7. 1 kb Plus DNA Reference Ladder from New England Biolabs, Inc.

Gel Electrophoresis Troubleshooting

If the product is the wrong size or there is smearing in the lane, you may have to repeat the PCR with different primers, annealing temperatures, or elongation times. It is also good practice to include a negative control containing sterile water instead of DNA to ensure the primers are not faulty.

If there are multiple bands like that for pAB002 BK and pAB005 BK, you may have to check that your primers only have one binding site, or rerun your sample on a different gel to extract only the DNA that is the correct size.

If there are no bands in your lanes but the ladder is clear, your primers may have been insignificant to produce a product, or have bound to each other and formed primer dimers that appear as dull bands lower that 0.5 kb.

If there are no bands across the entire gel including that of the ladder, you have not added enough loading dye to see the DNA, or have washed the sample out of the wells.

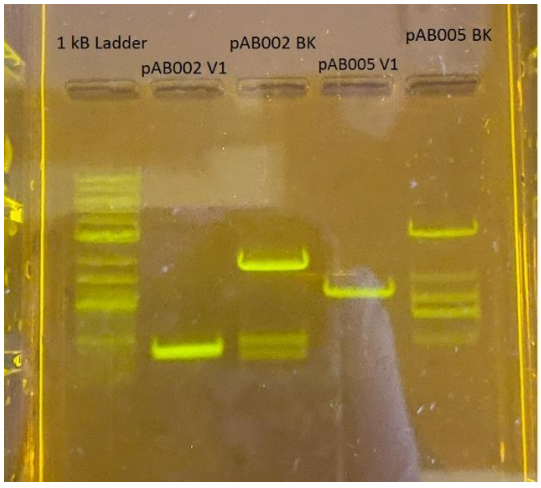
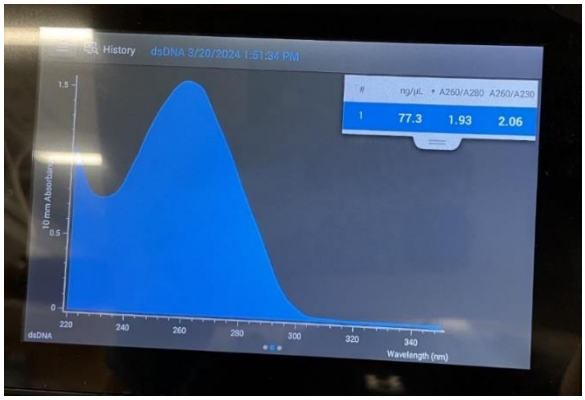


Figure 6. Sample Gel with 5 Wells for Gel Purification.

PCR Purification

If your PCR product has a single band that is the correct size, you can then purify your product. Keep track of how much sample you have left after running your gel as this volume will be important for later.

Protocol:



1. Using the volume of your remaining PCR sample, calculate how much binding buffer (PB) you will need to add by multiplying that volume by 5 µL.
2. Mix this volume with your sample in the PCR tube by pipetting up and down, then immediately load it all into a spin column. Centrifuge for one minute at 13,000 rpm. At this stage, the DNA should be bound to the white filter inside the spin column.
3. Pour off the binding buffer then replace the cap back into the same tube.
4. Wash the sample one time with 750 µL wash buffer (PE). Centrifuge for one minute at 13,000 rpm.
5. Pour off the wash buffer, replace the cap back into the same tube, then centrifuge again for one minute to remove any residual wash buffer from the filter. The wash buffer should have removed any residual proteins and salts from the sample leaving behind pure DNA.
6. Transfer the cap to a clean 1.5 mL microcentrifuge tube and add 50 µL elution buffer (EB) to the center of the filter. Let stand for one minute to increase the yield. Centrifuge for one minute at 13,000 rpm. The elution buffer should have disrupted the DNA bound to the filter and eluted into the 1.5 mL microcentrifuge tube (4).
7. Throw away the spin cap and nanodrop the resulting purified PCR product.

Using the Nanodrop

Tap on the screen to wake up the machine. Select double-stranded DNA. Wait for the machine to self-test and initialize before lifting up the arm. Spray some ddH_2O on a clean Kimwipe and wipe both the pedestal AND the bottom of the arm. Blank the machine with elution buffer (EB) by adding 1 µL to the pedestal, then slowly closing the arm (it is magnetized and with snap shut). After it is blanked, lift up the arm and clean the top and the bottom. Add 1 µL of your sample to the pedestal as before, then close the arm. You want to see a clear peak at 260 nm.

If there is not a clear peak at 260 nm, you can attempt to read the sample again by cleaning the pedestal more thoroughly, or by re-blanking the machine. If there is still not a precise peak but a slope and then a small bump around 260 nm, reduce the amount of DNA by half or so depending on the size of the peak and the value of DNA the machine predicts you have.

Figure 8. Double-stranded DNA with a clear peak at 260 nm.

Record the concentration of DNA in ng/µL on the side of the tube and repeat the process until you have read all your samples. Select end experiment, end again, then thoroughly clean the pedestal and arm for the next person.

Gel Purification

In the event that your PCR product has multiple bands, you will have to cut out the band that is the correct size to recover your DNA fragment.

BEFORE YOU START! Place your 100% isopropanol on ice to chill for at least 10 minutes before starting. This step is important for increasing the yield of your product.

Protocol:

1. Spray both your razor blade and curved tweezers with ethanol and wipe dry.
2. Weigh a 1.5 mL microcentrifuge tube on the scale and record the weight. This weight will be used later after you have extracted your band from the gel.
3. Slide the gel off the tray and carefully cut out only the band you need under the UV light. Try to cut out as little gel as possible as you cut around your band. This will help increase the purity of your product.
4. Take your curved tweezers and gently lift the band out of the gel. Place the band in your weighed 1.5 mL tube and check your gel for any remaining DNA.
5. Once you have collected all of your DNA from the gel, weigh the 1.5 mL microcentrifuge tube and record the weight of the gel minus the weight of your tube.
6. Add 5 volumes of QG buffer to your 1.5 mL tube. Approximately 100 mg of gel is equivalent to 100 µL.
7. Vortex the tube then allow to incubate in the 42°C heating block for 10 minutes or until the gel has completely dissolved. Vortex every 2-3 minutes to help the gel dissolve.
8. Check that the color of the mixture is yellow. The QG buffer contains a pH indicator that is yellow when the pH is less than or equal to 7.5. DNA adsorption to the membrane is only efficient at a pH of 7.5 or less. If the mixture is orange or violet, add 10 µL of 3 M sodium acetate (pH 5.0) and mix until the solution is yellow (5).
9. Add 1 gel volume of ice-cold isopropanol to the sample and mix. Cool on ice for two minutes.
10. Add up to 800 µL of your sample to a spin column and centrifuge for 1 minute at 13,000 rpm.
11. Pour off the flow through and place the cap back in the same tube. If you have remaining sample, add the remainder to the spin column and spin down as before. Pour off the flow through and place the cap back in the tube.
12. Wash the sample twice with 750 µL wash buffer (PE). Centrifuge for one minute at 13,000 rpm, and pour off the flow-through between washes. Place the cap back in the tube and centrifuge once more to remove any residual wash buffer.
13. Place the spin column cap in a clean 1.5 mL microcentrifuge tube and add 30-50 µL of elution buffer (EB) to the center of the white filter. Allow to stand at room temperature for one minute to increase the yield. Centrifuge for 1 minute at 13,000 rpm.
14. Toss the spin column cap and nanodrop the remaining liquid in your 1.5 mL tube. This should now contain your purified PCR product.

Note: The sample will likely have a high background when nano-dropped as salts and other contaminants have carried over from your gel. Record the concentration of DNA as being 1/3 or 1/2 of the given value unless the peak at 260 nm is clear.

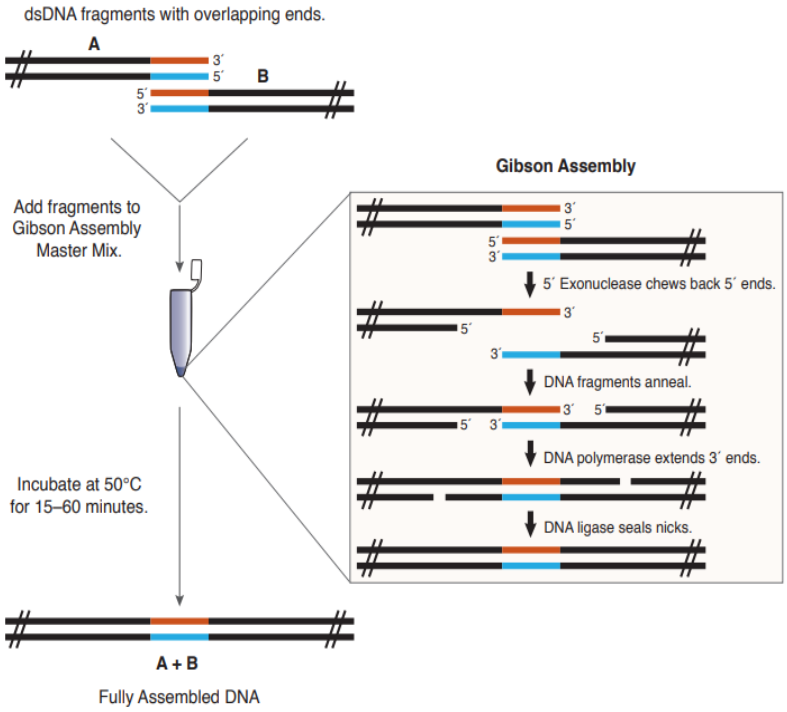


Figure 9. Gibson Assembly of PCR Products in an Isothermal Reaction (New England Biolabs, Inc., 2020).

Gibson Assembly

Once your PCR fragments have been PCR or gel purified, we can use Gibson assembly to combine the fragments to form a circular plasmid.

The 2x Gibson assembly reaction mixture contains:

1. An exonuclease to chew back the 5' ends of our PCR product creating 3' overhangs that the complementary regions on our fragments will bind to.
2. A DNA polymerase to fill in the gaps on the annealed region created by the exonuclease.
3. A DNA ligase to seal the nicks in the phosphate backbone of the DNA (6).

BEFORE YOU START! Get a bucket of ice and the metal PCR tube holder. The Gibson Assembly mixture is highly heat sensitive and needs to remain on ice the entire time it is out of the -20°C.

Protocol:

1. In a PCR tube on ice, mix:

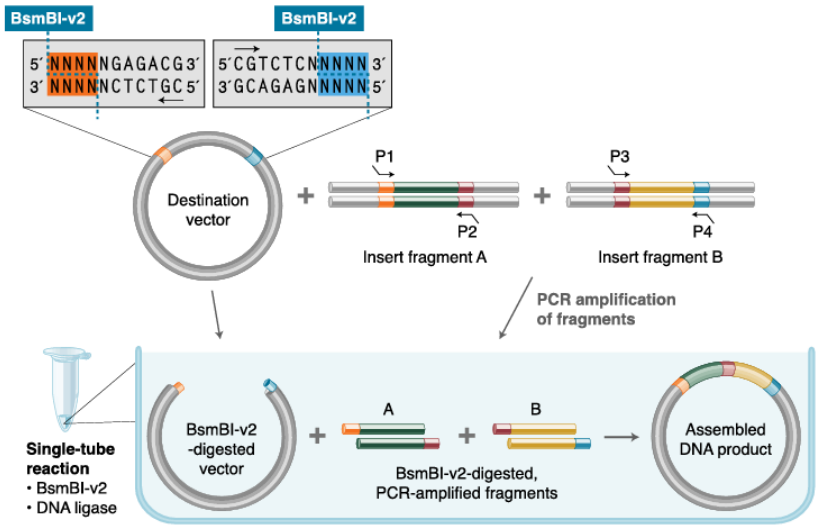
- 1.25 µL Backbone DNA (Approx. 70 ng DNA, adjust volumes as needed)
- 1.25 µL Insert DNA (Approx. 70 ng DNA, adjust volumes as needed)
- 2. 5 µL ddH_20 (Adjust as needed)
- 5.0 µL 2x Gibson Assembly Master Mix

  ———————————————————

  10.0 µL Total Volume

2. Incubate at 50°C for 15 minutes in the thermocycler.

Note: When combining 2-3 fragments, increase the incubation time to 20-30 minutes. When combining 4-6 fragments, increase the incubation time to 45-60 minutes. If the required volume of samples exceeds 5 µL, adjust the volume of the master mix so that the sample to master mix ratio remains 1:1.



3. Place on ice for 5 minutes before proceeding to *Bacterial Transformation with Chemically Competent Cells*, or place in the large -20°C freezer for later use.

Golden Gate Assembly

If the fragments you are combining have several regions of high similarity, Golden Gate Assembly may be necessary to correctly recombine your fragments. Golden Gate Assembly requires the use of a Type IIS restriction enzyme and the T4 DNA ligase. Unlike other restriction enzymes that cut the DNA at their recognition site, Type IIS restriction enzymes bind to their recognition site, but cut the DNA at a nucleotide position downstream of their recognition site (7).

In the lab, we use two different Type IIS restriction enzymes, BsaI and BsmBI. BsmBI has a recognition site of CGTCTC(N1/N5), where it will bind to the sequence CGTCTC, but cut the DNA that is 1 nucleotide downstream on the top strand, and 5 nucleotides downstream on the bottom strand (7). This creates a 4 bp overhang that will join with the 4 bp overhang created on the other end of the destination vector. BsaI on the other hand has the recognition site of GGTCTC(N1/N5) but will have a cut site like that of BsmBI (8).

Taking advantage of these enzymes' natural predilection to cut the DNA downstream of its binding site allows us to design primers containing the recognition site on the 3' end and insert any 6 base pairs we want downstream to create unique overhangs in

Figure 10. Golden Gate Assembly using Type IIS Restriction Enzyme BsmBI-v2 (New England Biolabs, Inc., 2020).

the DNA. This will allow our fragments to recombine based on these 4 nucleotide overhangs, and not through DNA fragments sharing terminal end homology.

BEFORE YOU START! Get a bucket of ice and the metal PCR tube holder. The NEB Golden Gate Enzyme Mix is highly heat sensitive and needs to remain on ice the entire time it is out of the -20°C.

Golden Gate Assembly using BsmBI-v2

Protocol:

1. Thaw T4 DNA Ligase 10x buffer on ice. Vortex to reincorporate precipitate.
2. To a PCR tube on ice, add:

    1.25 µL Backbone DNA (Approx. 70 ng DNA, adjust volumes as needed)
    1.25 µL Insert DNA (Approx. 70 ng DNA, adjust volumes as needed)
    2.0 µL T4 DNA Ligase 10x Buffer
    1.0 µL NEB Golden Gate Enzyme Mix (BsmBI-v2)
    14. 5 µL ddH_20 (Adjust as needed)

    _____
    20.0 µL Total Volume

3. Choose the appropriate assembly protocol:

INSERT NUMBER	SUGGESTED ASSEMBLY PROTOCOL
For 1 Insert	42°C, 5 min (cloning) or 42°C, 1 hr (library preparation) → 60°C, 5 min
For 2–10 Inserts	(42°C, 1 min → 16°C, 1 min) x 30-60 → 60°C, 5 min
For 11–20+ Inserts	(42°C, 5 min → 16°C, 5 min) x 30-60 → 60°C, 5 min

Note: Most reactions can be run using the following protocol:
(42°C, 1 min → 16°C, 1 min) x 20 cycles → 60°C, 5 min → 4°C, ∞

4. Follow with *Heat Shocking with Commercial Competent Cells* or store in the large -20°C freezer for later use.

Golden Gate Assembly using BsaI-HF-v2

Protocol:

1. Thaw T4 DNA Ligase 10x buffer on ice. Vortex to reincorporate precipitate.
2. To a PCR tube on ice, add:

    1.25 µL Backbone DNA (Approx. 70 ng DNA, adjust volumes as needed)
    1.25 µL Insert DNA (Approx. 70 ng DNA, adjust volumes as needed)
    2.0 µL T4 DNA Ligase 10x Buffer
    1.0 µL NEB Golden Gate Enzyme Mix (BsaI-HF-v2)
    14. 5 µL ddH_20 (Adjust as needed)

    _____

20.0 µL Total Volume

3. Choose the appropriate assembly protocol:

Note: Most reactions can be run using the following protocol:

(37°C, 1 min → 16°C, 1 min) x 20 cycles → 60°C, 5 min → 4°C, ∞

4. Follow with *Heat Shocking with Commercial Competent Cells* or store in the large -20°C freezer for later use.

Bacterial Transformation with Chemically Competent Cells

After you have assembled your fragments, you should have a complete, circular piece of DNA that is capable of being inserted into the bacterial cell and replicated without being degraded. If your plasmid has not been constructed correctly, host nucleases will be able to target any free hanging 3'-OH or 5'-phosphates, and degrade any linear DNA (9). This degradation of linear foreign DNA is one of the many defense mechanisms that bacteria have to prevent infection via bacteriophage, as well as from any genetic material that may compromise the bacterial fitness of the cell.

Chemically Competent Cells

Chemically competent cells are bacterial cells that have been treated with a divalent cation like Ca^{2+}, to make the cell membrane more permeable. In the lab, we treat our cells with 1x TSS, which contains $MgCl_2$ and DMSO, both substances that can increase the permeability of the cell membrane (10). Bacteria have the ability to uptake foreign DNA from the environment naturally (natural competence), but at extremely low levels (11). By pretreating cells with divalent cations, the negative charges on the cell lipid bilayer and extraneous DNA are neutralized, allowing foreign DNA to better pass through the membrane (12).

To stretch our stock of competent cells, we can sacrifice a tube of commercial competent cells and get 10-20 tubes of homemade competent cells with reduced transformation efficiency. These cells are best used with simple plasmid constructs, retransformations into different bacteria strains, or when using high copy plasmids. The risk for contamination and mutations down the line is much

INSERT NUMBER	SUGGESTED ASSEMBLY PROTOCOL
For 1 Insert	37°C, 5 min (cloning) or 37°C, 1 hr (library preparation) → 60°C, 5 min
For 2–10 Inserts	(37°C, 1 min → 16°C, 1 min) x 30 → 60°C, 5 min
For 11–20+ Inserts	(37°C, 5 min → 16°C, 5 min) x 30 → 60°C, 5 min

higher when making our own cells, so aseptic technique is absolutely critical! Preparing homemade competent cells is unlikely something that you will be tasked with doing, however it is important to inform someone if the stock of competent cells is running low so that we can prepare new ones.

Heat Shocking with Commercial Competent Cells

The commercial competent cells are *highly* competent, meaning they are able to uptake a lot of foreign DNA with ease. They can withstand a lot more environmental stressors without dying off, however it is crucial that you DO NOT touch the bottom of the tube! Even touching your hand to the bottom of the tube or inserting the pipette tip is enough to prematurely heat shock the cells and lose competency. Always bring a bucket of ice with you when you go to retrieve your competent cells.

Quick Protocol:

1. Thaw one tube of commercial competent cells on ice for 5-10 minutes. Put your DNA sample on ice while your cells are thawing.
2. Add 950 µL SOC or NEB outgrowth recovery medium to a 1.5 mL microcentrifuge tube and place in the 42°C heating block. Place your LB antibiotic plate in the 37°C incubator to warm. Prewarming the recovery media reduces the stress placed on the bacteria and allows them to grow better.
3. Cool your pipette tip by pipetting up and down in your cold DNA sample. Add 2 µL of your DNA sample to the competent cells. Pipe up and down one time to mix. Incubate on ice for 2 minutes. *Note: For Golden Gate Assemblies, incubate on ice for 30 minutes before heat shocking.*
4. Heat shock for 30 seconds in the 42°C heating block, then immediately place your cells back on ice for 2 minutes.
5. Mix your warmed 950 µL SOC into your heat shocked cells then place it back on the heating block for one minute. *Note: For Golden Gate Assemblies, recover for 60 minutes in the 37°C shaker before plating.*

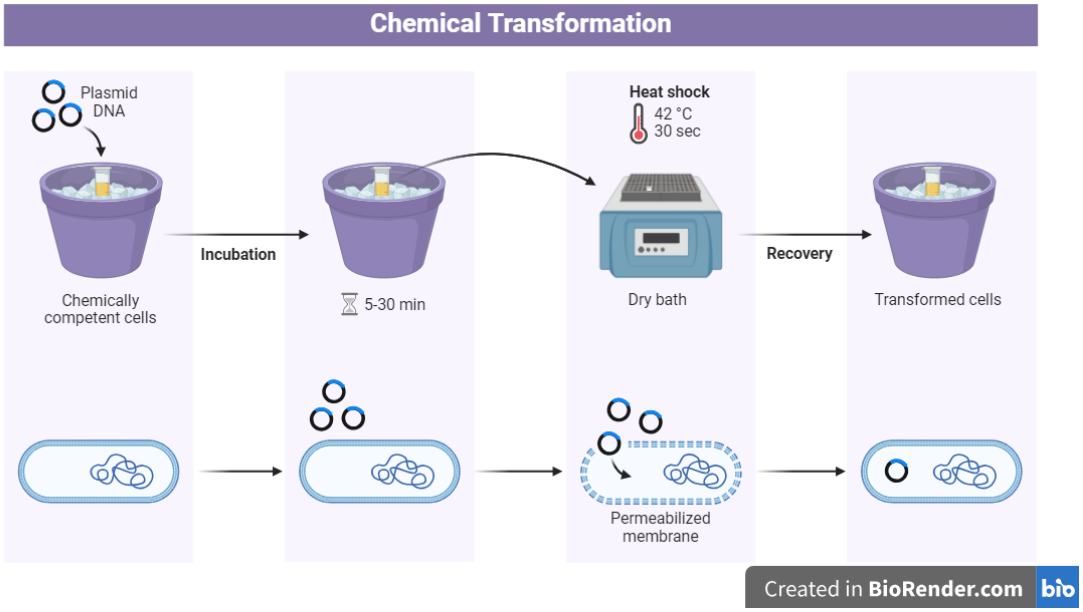


Figure 11. Heat Shock Transformation of Plasmid DNA into Chemically Competent Cells.

6. Label your plate on the agar side. Add 6-15 sterile glass beads to your LB antibiotic plate. Plate 25 – 300 µL of your transformed cells onto the plate, then swirl the plate on the bench to fully cover the plate with cells. The volume you plate will depend on the copy number of your plasmid and how many fragments you had to assemble.
7. Incubate agar side up in the 37°C incubator overnight.

Heat Shocking with Homemade Competent Cells

Our homemade competent cells have been grown in SOC to mid to late exponential phase from the commercial strains. Because they are less competent, we need to incubate the DNA and the cells together on ice prior to heat shocking them, as well as recover them in SOC for longer.

Homemade Protocol:

1. Thaw one tube of homemade competent cells on ice for 5-10 minutes. Put your DNA sample on ice while your cells are thawing.
2. Add 950 µL SOC or NEB outgrowth recovery medium to a 1.5 mL microcentrifuge tube and warm in the 42°C heating block.
3. Cool your pipette tip by pipetting up and down in your cold DNA sample. Add 2 µL of your DNA sample to the competent cells. Pipe up and down one time to mix. Incubate on ice for 30 minutes.
4. Heat shock for 30 seconds in the 42°C heating block, then immediately place your cells back on ice for 2 minutes.
5. Mix your warmed 950 µL SOC into your heat shocked cells then place it back on the heating block for one minute.
6. Recover your cells in the 37°C shaker for one hour (or longer if necessary).
7. Label your plate on the agar side. Add 6-15 sterile glass beads to your LB antibiotic plate. Plate 100 – 300 µL of your transformed cells onto the plate, then swirl the plate on the bench to fully cover the plate with cells. The volume will depend on the copy number of your plasmid and how many fragments you had to assemble.
8. Incubate agar side up in the 37°C incubator overnight.
9. If colonies have grown on the plate, proceed to *Streaking for Isolation and Media Inoculation*. If colonies have not grown on the plate, check that you have plated your cultures on the correct LB antibiotic plate, and that all fragments were included in your Gibson Assembly.

Streaking for Isolation and Media Inoculation

Once you have transformed your plasmid into your competent cells and they have successfully grown on your LB antibiotic plate, we can then “pick” single colonies, inoculate fresh LB antibiotic media, and streak them out for isolation.

A single isolated colony on a plate can be made up of millions or even billions of cells that are all exact copies of the same parent cell (13). By picking a single colony with a sterile loop, we can both inoculate fresh LB antibiotic media to grow up new cultures overnight for minipreps, and make a backup plate of that colony for future experiments. By streaking for isolation, we can confirm that the colony we have chosen truly is a single colony and will give us the purest plasmid DNA as a result.

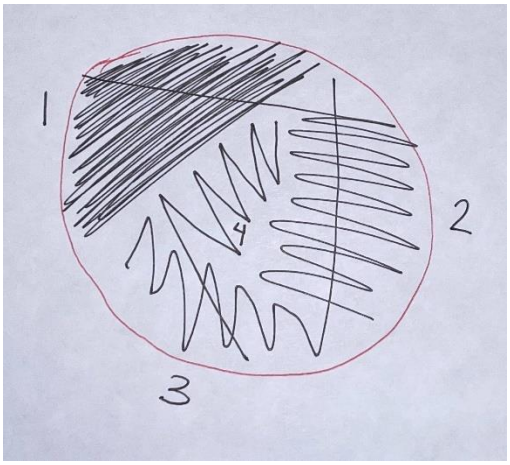


Figure 13. Path of Loop for Quadrant Streak Plate Technique 2. Loop passes only once through previous quadrant. The distance between zigzags is increased along the length of the quadrant.

Quadrant Streak Plate

When picking a colony and streaking for isolation on a plate directly, a quadrant streak plate may be necessary to thin out the cell density on your loop to get isolated colonies. This is also the case when pulling from our -80°C freezer stock.

BEFORE YOU START! If you are streaking out a culture from the freezer stock, get a bucket of ice. Do not let the cells thaw! Bring the bucket with you to the -80°C freezer and keep the freezer stock on ice at all times. Immediately return the freezer stock to the -80°C freezer once finished with it.
While the cells have been frozen in their exponential growth phase, extreme changes in temperature will result in the stock dying off.

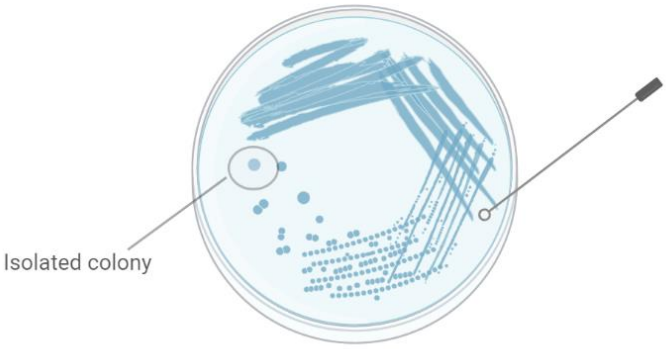


Figure 12. Well Isolated Colony from a Quadrant Streak Plate (Technique 1). Loop passes multiple times through previous quadrant to make up the streaks of the subsequent quadrant

Protocol:

1. Label your LB antibiotic plate on the agar side and place in the 37°C incubator to warm for 10 minutes.
2. Under UV light, identify and circle a well isolated colony from your plate. See Figures 12 and 15 for reference.
3. Lightly tap your loop to the surface of the colony. Do not scoop or swipe to pick up the colony. This can push the colony into surrounding colonies and will no longer give a pure culture.
4. On your new LB antibiotic plate, repeatedly pass your loop across one third of the plate to get off as much colony as possible. If your culture is from the freezer stock, lightly touch the surface of your loop to the top of the frozen culture and streak as before. This region, quadrant 1, will have the densest growth.

5. Unless there is a lot of colony left on your loop, use the same loop for each subsequent quadrant.
6. *Technique 1*. Pass your loop 5-6 times through quadrant 1 and drag the lines out into adjacent empty space on the plate. These streaks will be your quadrant 2. Repeat the process 3-4 more times until you have filled your plate with streaks (Figure 12).
7. *Technique 2*. Pull a singular line through quadrant 1 and begin streaking quadrant 2 in a zigzag pattern. DO NOT pass over your previous streaks. Increase the distance between zigzags as you streak along the length of the quadrant. Repeat for quadrants 3 and 4 (Figure 13).
8. Dispose of your loop and incubate agar side up overnight in the 37°C incubator.

Inoculating Media for Overnight Growth and Back Up Streak Plates

Growing a single colony overnight (15-18 hours) will achieve a higher cell density than when left to grow to mid to late exponential phase. This will allow for the recovery of more plasmid DNA when miniprepped. After you inoculate your media, immediately streak that same loop on your agar plate. This will ensure the DNA you recover from your plasmid matches that of the colonies on your backup plate!

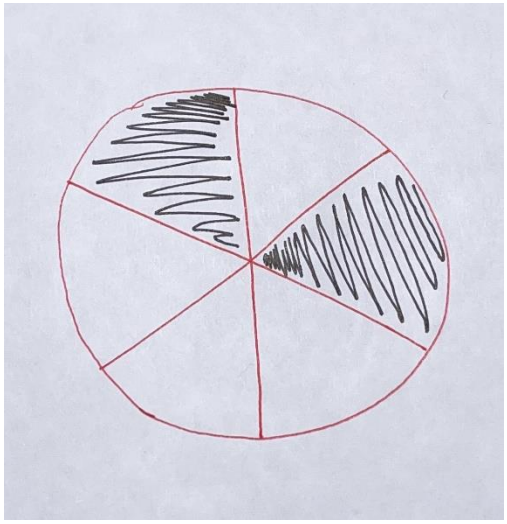


Figure 14. Path of Loop for Backup Streak Plate. The left wedge is streaked from out to in with the densest region of growth being on the outer right corner. The right wedge is streaked from in to out with the densest region of growth at the center of the plate.

Protocol:

1. Gather and label the appropriate number of 12 mL culture tubes and LB antibiotic plates. Always label and divide plates up on the agar side. Using a marker, divide the plate up for as many colonies you pick up to 6 sections a plate. Place your LB antibiotic plate agar side up in the 37°C incubator to warm.
2. Fill each tube with 3 mL of the appropriate LB antibiotic media. You can prewarm your media in the 42°C water bath for better growth conditions.
3. Under UV light, circle 4-8 colonies that match the fluorescence you expect to see with your specific plasmid, and are well isolated from other colonies. If your plasmid does not have a fluorescence protein, circle 4-8 colonies that have the colony morphology of *E. coli*, milky white with a more opaque center, round colonies with potentially wavy (undulate) edges, mucoid in appearance, and flat.
4. Using a sterile loop, lightly touch the loop to the surface of your colony. Do not scoop or swipe to pick up the colony. This can push the colony into a surrounding colony or contaminant, and will no longer be pure.

5. Swirl the loop into your media to get off as much colony as possible. If the colony you pick is small, swirl the loop in the media less so that there are sufficient enough cells left to streak on your plate.
6. Using the same loop, immediately streak the culture on your backup streak plate. Start by touching the loop to one corner of the slice to remove excess liquid, then streak back and forth towards the center of the plate, broadening the distance between streaks each time. You can also do this method with the center having the densest growth, and streaking outward for isolation.
7. Dispose of the loop and place your liquid cultures in the 37°C shaker overnight. Loosen the caps to increase the oxygen available to the bacteria while they grow.
8. Place your plate agar side up in the 37°C incubator. If the plate is fresh, place it in a plastic bag to prevent too much moisture building on the plate. If the plate is older than two months, seal the plate with parafilm to keep in moisture.
9. Follow with *Plasmid Miniprep* or *Making Freezer Stock*.

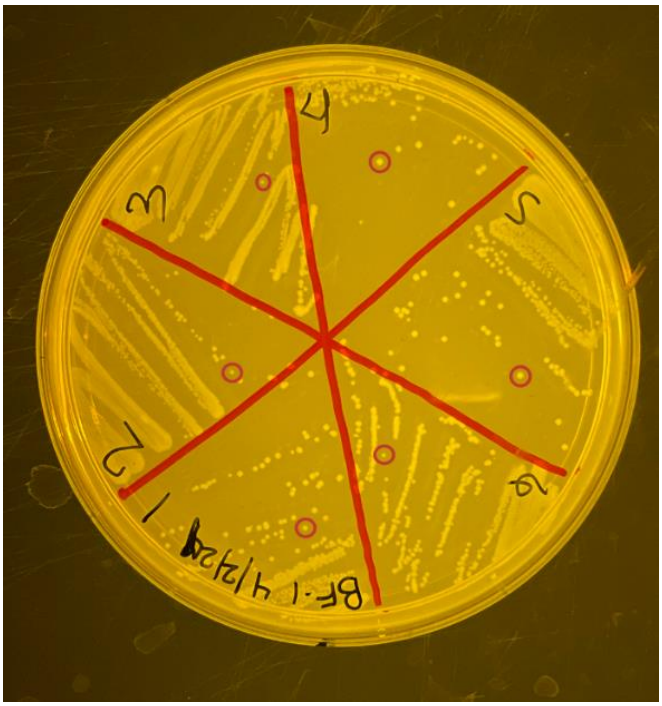


Figure 15. Well Isolated Colonies on a Backup Streak Plate.

Plasmid Miniprep

After your colonies have been streaked for isolation on your backup plate and have been grown overnight in 3 mL of your LB antibiotic media, we then need to recover the plasmid DNA from the bacteria. We use nonpathogenic *E. coli* in the lab to act as a growth vector for our plasmids. Each cell should contain approximately 5-35 copies of our plasmid depending on whether the plasmid has a low copy origin of replication (pSC101), or a high copy origin of replication (ColE) (14). Given that an increase in the number of copies a cell carries increases the metabolic burden on the cell, the bacteria that will grow best on our plates probably only carry a maximum of 20 copies each. These bacteria will continue to divide and exponentially increase the number of our plasmid until we have a dense culture of bacteria carry millions to billions of copies of our plasmid. Once the bacteria have successfully amplified our plasmid, we can extract the plasmid DNA by lysing open the cell, aggregating the bacterial proteins, and filtering out the plasmid DNA from the larger genomic DNA.

Protocol:

1. Spin down ON cultures in the large centrifuge at 4,000 rpm for 6 minutes. The temperature will automatically be set to 4°C for other experiments.

2. While samples are spinning down, get out twice as many 1.5 mL microcentrifuge tubes as you have samples and label them accordingly. One set will be used to pellet the bacterial proteins, and one will be used for your final DNA (and should also be labeled with the date).
3. Pour off the supernatant careful not to dislodge the pellet. At this stage you can check the pellet for fluorescence under UV light.
4. Add 250 µL resuspension buffer (P1) to each tube. Vortex until homogenous. If the entire pellet will not vortex into solution, you can add 250 µL more of P1.
5. Add 250 µL lysis buffer (P2) to each tube. Carefully cap the tube and invert 6 times to mix. DO NOT VORTEX! This can degrade the genomic DNA into small pieces that will elute from the spin column with your plasmid and give a false reading.
6. Let stand 2-5 minutes or until clear enough to see through. If not clear after 5 minutes, add an additional 250 – 350 µL of P2 as needed.
7. Add 350 µL neutralization buffer (N3) to each tube. Invert to mix until as homogenous as possible. The white aggregates are the bacterial proteins and cell membranes that have been lysed.
8. Pipette up 1 mL from each tube and add to their respective 1.5 mL microcentrifuge tube. Spin down for 6 minutes at 13,000 rpm in the tabletop centrifuge. This is to pellet the unwanted proteins leaving the DNA in the remaining supernatant.
9. While the samples are centrifuging, get out enough spin column tubes for as many samples as you have and label them accordingly.
10. Pipette 800 µL of the remaining supernatant into the spin column, careful not to pull up any white proteins floating in the sample. To avoid this, press the pipette down to the first stop. Insert the pipette into the sample until the pipette tip is at the base of the tube opposite the aggregated proteins. Pull up the 800 µL, then remove the pipette tip following the same path out. You can wipe off any proteins stuck to the outside against the rim of the tube. When dispensing the 800 µL into the spin column, do not touch the pipette tip to the spin column. This could transfer residual proteins into the spin column that can skew your results later.
11. Spin down for one minute at 13,000 rpm. Pour off supernatant, then replace the cap back into the same column.
12. Wash the sample one time with 750 µL wash buffer (PE). Centrifuge for one minute at 13,000 rpm.
13. Pour off the wash buffer, replace the cap back into the same tube, then centrifuge again for one minute to remove any residual wash buffer from the filter. The wash buffer should have removed any residual proteins and salts from the sample leaving behind pure DNA.
14. Transfer the cap to a clean 1.5 mL microcentrifuge tube and add 50 µL elution buffer (EB) to the center of the filter for low copy plasmids, or 100 µL to the filter for high copy plasmids. Always check the plasmid map if you aren't sure! Let stand for one minute to increase the yield. Centrifuge for one minute at 13,000 rpm. The elution buffer should have disrupted the DNA bound to the filter and eluted into the 1.5 mL microcentrifuge tube (15).
15. Throw away the spin cap and nanodrop the resulting plasmid DNA. See *Using the Nanodrop* for instructions.

Sending Samples for Sanger or Full Plasmid Sequencing

After you have nano-dropped your plasmid DNA, we will send it out for Sanger Sequencing to confirm that the plasmid has been made correctly. Sanger sequencing, also known as chain termination method, was developed by Frederick Sanger and his colleagues in 1977. This method was considered the gold standard for sequencing for decades due to its high levels of accuracy, and its cost effectiveness for sequencing single genes.

The reaction mixture includes the DNA polymerase, deoxynucleotide triphosphates (dNTPs), the DNA template, and a primer for DNA synthesis to build off from. The reaction mixture also includes fluorescently tagged dideoxynucleotide triphosphates (ddNTPs) that are missing an oxygen atom at the 3' OH group. Without this oxygen atom, an additional nucleotide cannot be incorporated, and synthesis is terminated. These ddNTPs are incorporated at random giving short pieces of DNA that can be separated by size through gel electrophoresis to determine the sequence of DNA. The downside to this method is that Sanger sequencing has a limited read length of approximately 800-900 bps from a single reaction mixture (16). Check out this resource, or the Sarpeshkar Wet Lab google drive to learn more about how sanger sequencing works.

If we want to sequence our whole plasmid, we have to send our samples out for Next-generation, or high throughput sequencing. There are a variety of Next-generation sequencing methods available on the market today, several of which are best explained here. Next-generation sequencing (NGS) can be used for: quickly sequencing whole entire genomes, analyzing and profiling RNA molecules (transcriptomics), and to study the epigenetic modifications on DNA (epigenomics) (17).

Sanger Sequencing Prep

To a PCR tube, add:

0.5 µL Primer

14.5 µL DNA Sample (500 ng DNA total, adjust volume with ddH_2O if high concentration)

If you have several samples to send out, normalize your DNA concentration to your sample with the lowest DNA concentration. Using that, determine how much water you need to add to get a final volume of 14.5 µL. Multiply that by the number of samples you have plus one tube extra. Repeat for the primer. Mix, then aliquot the appropriate amount of water/primer mixture into a PCR strip. Add your DNA accordingly.

Next-Generation Sequencing Prep

To a PCR tube, add:

12 µL DNA Sample (600 ng DNA total, adjust with ddH_2O as needed)

If your sample is less than 50 ng/µL, the concentration may not be high enough to send for sequencing. Regrow cultures ON and miniprep again to see if you can get a higher concentration.

Making Freezer Stock

Once your plasmid has been sequenced and confirmed to be correct, you can then make freezer stock. Our freezer stock is kept at -80°C which is cold enough to "flash freeze" the bacteria in their most viable state while still allowing us to recover them at any point down the line. It is key however to not allow your freezer stock to thaw once it has been frozen as it causes too much stress on the bacteria, and they will not regrow. To combat this, always make one working stock, and one backup stock.

Protocol:

1. Select a single colony from your backup plate and grow overnight (15-18 hrs) at 37°C with shaking in 3 mL of selective LB media.
2. Label a 2 mL purple cap tube with the name of your plasmid, the strain of bacteria it is in, the date, and any important info you need for that plasmid.
3. Aseptically add 0.5 mL of sterile 60% glycerol to the tube.
4. Mix in 1 mL of your overnight culture.
5. Repeat the process for your back up freezer stock.
6. Store in the -80 by number in the appropriate freezer box.

Practice Plasmid

Using this guide, see if you can make DA475 from start to finish. Access the file on SnapGene to check your annealing temperatures and fragment sizes.

Primers and Template DNA:

475 bk: 475-bk-F and 475-bk-R → DA122

475 V1: 460-R1 and 248-R1 → DA473

Useful Calculations

Calculating how much sample you need to get a set concentration of DNA

If I need 25 ng of DNA in my PCR reaction, I want to divide 25 ng by the concentration of my DNA to get the volume in µL I need to add to my reaction.

$$\frac{25\ \text{ng}}{45\ \text{ng/µL}} = 0.56\ \text{µL DNA}$$

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