Improving Student Outcomes with an Adaptable Molecular Cloning Course-Based Undergraduate Research Experience

Student Manual

Prepared by Christopher B. Cummings, Samuel S. Catania, Eirene M. Q. Ednacot, A.J. Kinsella-Johnson, Claire E. Meeds, Jack W. Reynolds, Ava E. Sanderson, Rachel A. Johnson, and Dr. Katharine R. Watts
Department of Chemistry and Biochemistry, Cal Poly San Luis Obispo

Introduction:

The independent project portion of this class will focus on Gibson Assembly, which is preferred over traditional cloning methods due to its versatility. With Gibson Assembly[1], plasmids can be assembled with fewer steps and fewer reagents than restriction cloning. Additionally, multiple inserts can be combined to form the plasmid of interest. Gibson Assembly uses three enzymes: a 5' exonuclease, a DNA polymerase, and DNA ligase. To prepare for Gibson Assembly, the genes of interest and vector backbone are PCR amplified as linear fragments of DNA, or the linear vector may be obtained via restriction digest. PCR primers used in amplification are designed to add 15 to 20 base pair overhangs at the ends of each linear fragment, which contain complimentary sequences between the linear fragments to be ligated. In the Gibson Assembly reaction, the generated linear fragments are combined in a single tube with the three enzymes, and the reaction proceeds isothermally at 50 °C.

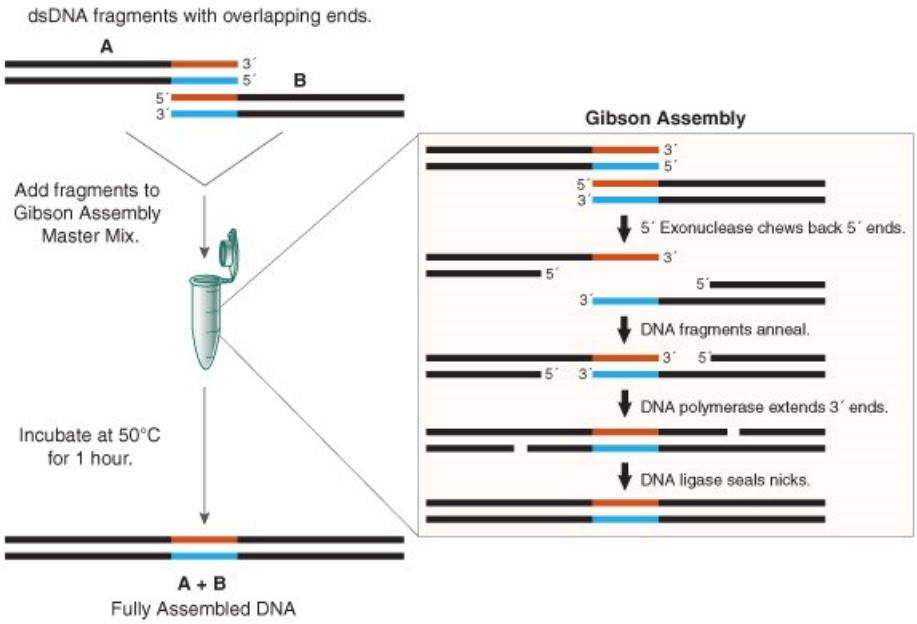


Figure 1: Enzymatic steps of the Gibson Assembly reaction. Complementary overhangs added to the linear fragments of interest are shown in orange and blue. The inset shows the results of each enzymatic step in the Gibson Assembly reaction. The 5' exonuclease chews back the 5' ends of each linear fragment, allowing the complementary overhangs to anneal via Watson-Crick base pairing. Annealing is followed by extension of the 3' ends by DNA polymerase, and remaining gaps in the connected fragments are sealed by DNA ligase. Figure courtesy of New England Biolabs[2].

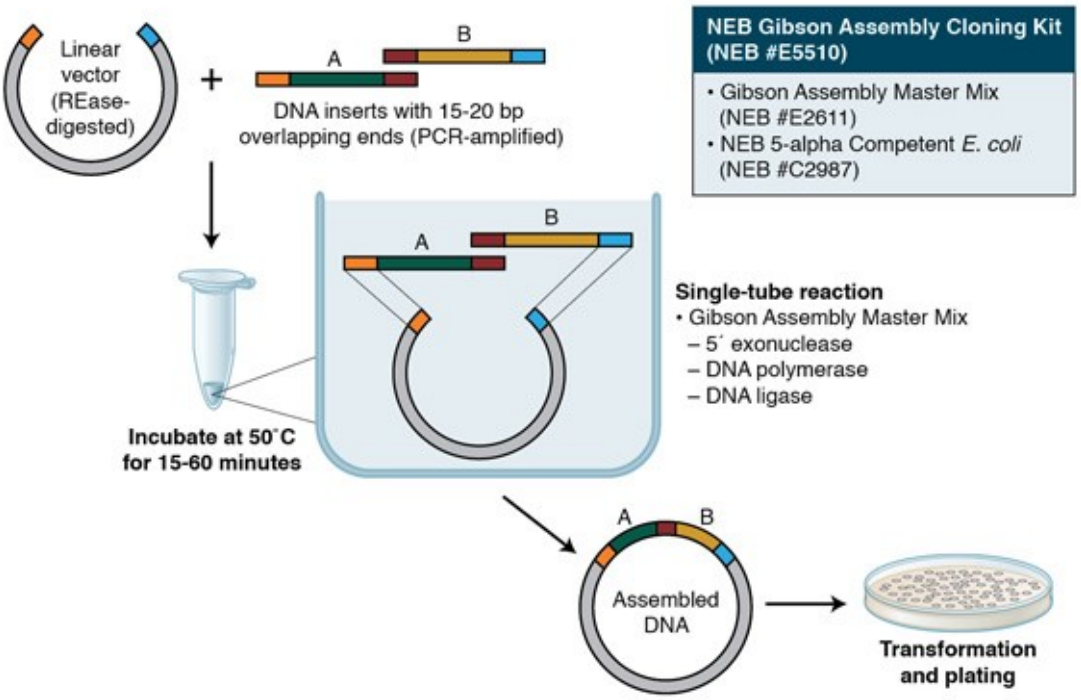


Figure 2: Depiction of the Gibson Assembly process. Linear fragments of the vector backbone and inserts (which have been amplified with complementary overhangs shown in orange, red and blue) are combined *in vitro* along with a Master Mix for Gibson Assembly® consisting of the 5' exonuclease, DNA polymerase, and DNA ligase enzymes. Reactions incubate for 15–60 minutes at 50 °C prior to transformation with chemically competent *Escherichia coli* cells and plating onto selective media. Figure courtesy of New England Biolabs[3].

The Gibson Assembly Module is divided into three separate Experiments (A–C) over six lab periods. In Experiment A, you will obtain all linear fragments of interest for the Gibson Assembly reaction through PCR or restriction digest. PCR(s) or restriction digests will be analyzed via agarose gel electrophoresis to confirm successful isolation before proceeding to Gibson Assembly. Linear DNA fragments of insert(s) and vector will be purified using a commercially available kit. In Experiment B, you will design a Gibson Assembly recipe and set-up the reaction with the linear fragments you obtained in Experiment A. To determine if any plasmids were created from the Gibson reaction performed in Experiment B, you will transform your reaction into chemically competent *Escherichia coli* cells and select for clones via growth on an antibiotic-containing media. Successful transformants will be inoculated into liquid media and grown with the selective antibiotic. In Experiment C, you will 1) isolate the Gibson Assembly clones from your liquid cultures by plasmid DNA miniprep and 2) screen these plasmids through either PCR or restriction digest to determine if cloning was successful.

Protocols:

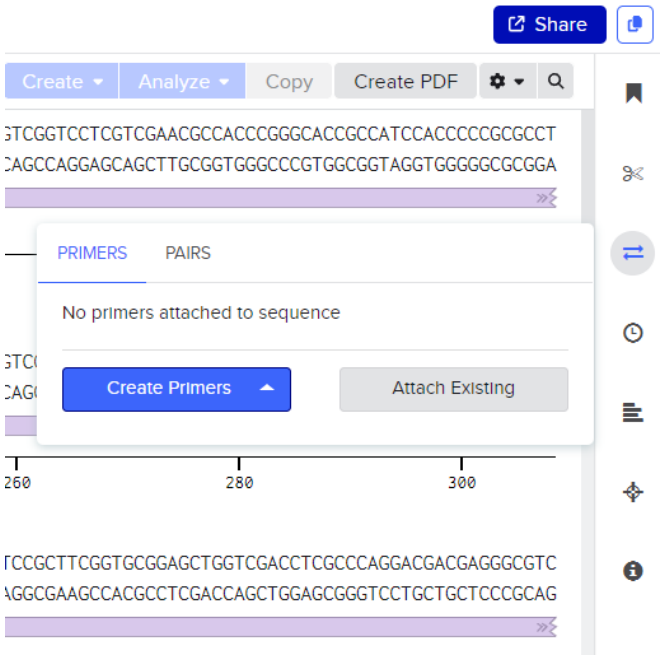
The following protocols will cover each experiment completed, and each experiment will be broken into two days.

Experiment A: Obtain Insert and Vector PCR and Restriction Digest.

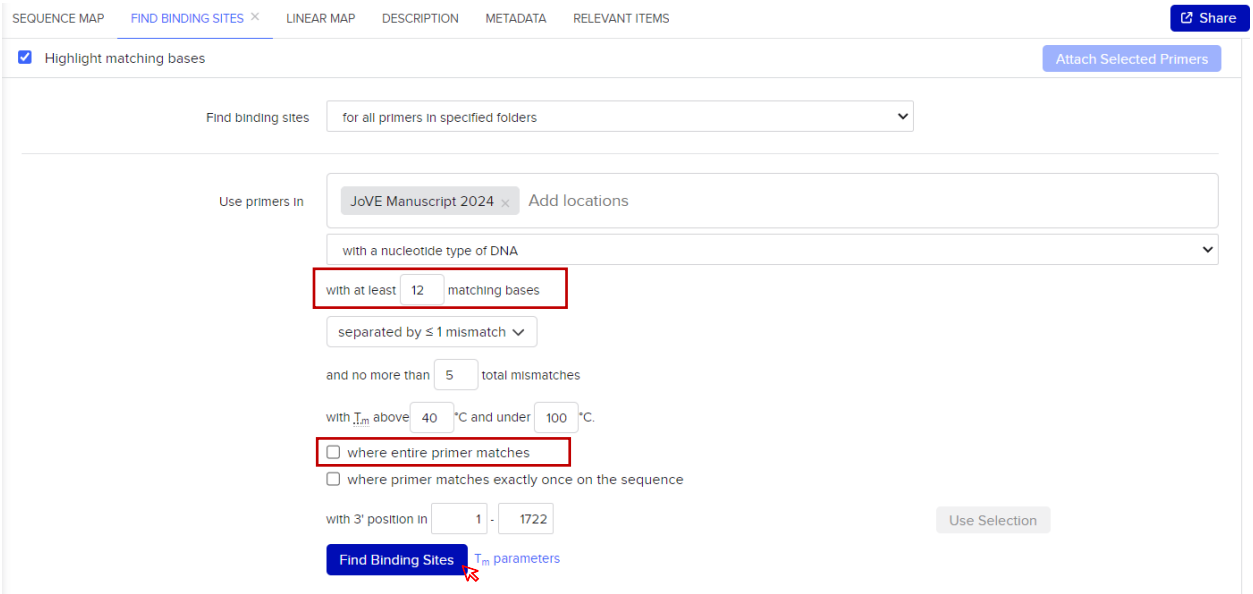
You will be assigned a set of primers from which you will design a PCR to obtain linear fragments of interest—gene(s), gene fragment(s) and/or your vector backbone. Alternatively, your vector backbone may be generated by a restriction digest and gel purification from another plasmid. If this method is employed, it will be completed and provided to you by your instructor. You will purify your PCR amplicon(s) and determine the concentrations of your linear fragments using a microvolume spectrophotometer. You will then calculate the volume of insert(s) and vector that must be combined for the Gibson Assembly reaction and formulate a recipe.

Prior to the Day 1 lab section, the Experiment A Planning Worksheet must be completed to determine the proposed annealing temperature, extension time, and amplicon size for your PCR(s).

1. To determine primer annealing temperatures:
    a. Determine which nucleotides of the primer will anneal to your template in the first cycle of PCR. These are the nucleotides that are complementary to your DNA template. They do NOT include the nucleotides that will add complementary overhangs to the vector or to another insert.
    b. Copy the nucleotide sequence of the annealing portion of each primer. Paste the sequences into a web-based annealing temperature (T_m) calculator, such as the New England Biolabs T_m calculator. If using this tool, be sure you select the appropriate DNA polymerase that will be utilized in your PCR reaction.
    c. Record the annealing temperature recommended for the primer pair of interest.
2. To determine your amplicon size:
    a. Retrieve the DNA sequence file of the template DNA to be used in your PCR reaction and import into Benchling.
    b. Import your primer sequences as 'Oligos' into Benchling.
    c. With your DNA template sequence open, click on the primer tool symbol on the right sidebar, then click on 'Attach Existing.'

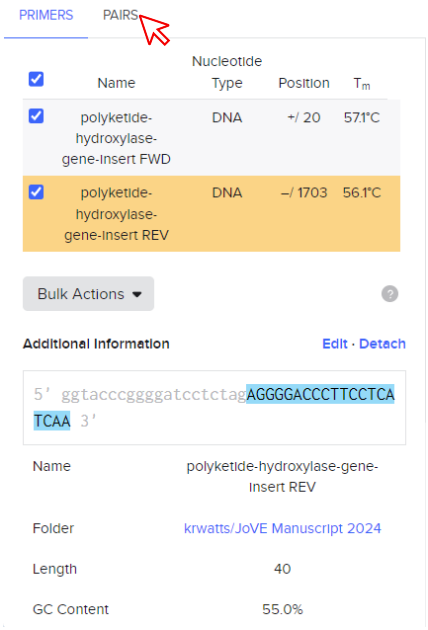


d. Choose the folder that contains the primer sequences you uploaded to Benchling. Adjust the number of matching bases to at least 12, and ensure the box is unchecked for 'where entire primer matches.' Click 'Find Binding Sites.'



3. Click the boxes next to your forward and reverse primers in the list that appears below. Then click 'Attach Selected Primers' at the top right of the screen.

4. Click once again on the 'Sequence Map' tab and find the region in which your primers bind. Locate the forward primer above the template DNA and click on the sequence so that it selects all primer bases. Hold the 'Shift' key on your keyboard and click on the reverse primer sequence. This should highlight all bases in between the two primers.
5. Next, click the primer tool, and open the 'Pairs' tab.



If primers were selected appropriately in the previous step, the 'Link Primers' button will be illuminated under the 'Pairs' tab. Click 'Link Primers' and then click 'Create PCR Product.' In the pop-up window, ensure the box for 'use primer bases instead of sequence (includes overhang if any) is checked. Then click 'Copy.' Select the folder in which the new amplicon sequence will be saved.

6. In the new sequence window, note the number of bases in the sequence on the bottom left corner. This is equal to your amplicon size.
7. To determine what your extension time should be, ascertain what the extension rate is of the DNA polymerase to be used in your reaction from the manufacturers site (for example, 30 seconds per kilobase pair for Q5 DNA Polymerase). Consider the extension rate to your amplicon size to calculate extension time for your PCR cycling protocol.

Experiment A, Day 1

PCR Amplify Linear Amplicons

1. Obtain chosen set of primers and template DNA for PCR(s). Your set of primers and template DNA will be determined by the instructor.
2. Follow the recipe in Table 1 to create a 25 µL PCR mixture:

Table 1: PCR Recipe

Reagent	Volume	Final Concentration/Amount
Q5® 2X Master Mix**	12.5 µL	1X
Chosen Forward Primer (10 µM)	1.25 µL	1 µM
Chosen Reverse Primer (10 µM)	1.25 µL	1 µM
Chosen DNA Template	Varies	1 – 100 ng*
Nuclease-free Water	Varies	N/A
Total Volume	25 µL	

*DNA amount will vary with template type. For genomic DNA, 100 ng is recommended; for purified plasmid DNA, 1–10 ng is recommended.

**PCR recipe is dependent on the DNA polymerase used. Please see manufacturer guidelines if a different polymerase is available.

3. Cycle your PCR in a thermocycler according to Table 2 below.

Table 2: PCR Cycling Program

Step	Process	Temperature (°C)	Time
1	Initial Denaturation	98	30 s
2	Denaturation	98	10 s
3	Annealing	*Specific to amplicon	30 s
4	Extension	72	**Specific to amplicon
5	Repeat Steps 2-4, 24 times		
6	Final Extension	72	10 m
7	Hold	4	Hold

*Annealing temperature can be determined theoretically based on primer T_m's and/or will be determined experimentally by your instructor prior to running your reaction.

**Extension time is determined by the extension rate of the polymerase of choice. Q5® Polymerase has an extension rate of 30 seconds per 1 kb. Use the size of your expected amplicon to calculate an extension time.

4. Store PCR(s) at 4 °C or −20 °C until the following lab period.

Experiment A, Day 2

Agarose gel electrophoresis and DpnI digest

1. Weigh out 0.5 g of agarose and add to an Erlenmeyer flask. Add 50 mL of 1X TAE buffer and microwave until agarose is fully dissolved, intermittently swirling the flask to mix the solution and prevent the solution from boiling over. CAUTION: Flask will be hot; please use proper thermal gloves to avoid burns.
2. Add 5 µL of GelRed to the solution and pour into gel cast. Insert the comb to create wells in the gel. Incubate at room temperature until agarose is set, about 15–20 minutes.
3. Add 1 µL of 6X Loading dye to a 5 µL aliquot of PCR. Pipette the molecular weight standard into one well, and the PCR + loading dye mixture into another well. After connecting the gel apparatus to the power source, run the gel at 120 V.
4. If PCR template was a plasmid: While the gel is running, add 1 µL of DpnI to the remaining PCR. Incubate this mixture for 1 hour at 37 °C.
5. For gel imaging, consult instructor for how to set up and image your gel. Confirm with the gel image that PCR was successful and the correct amplification was achieved by comparing the size of your band to the known size of your amplicon (See Experiment Planning Worksheet A).

PCR Purification

After digesting the reaction with DpnI to digest any template, plasmid DNA into small fragments, purify the linear PCR product from the reaction components and small template fragments.

Note: this protocol was adapted from the Thermo Scientific GeneJET PCR Purification Kit.

1. Add a 1:1 volume of Binding Buffer to completed PCR mixture (e.g. for every 100 µL of reaction mixture, add 100 µL of Binding Buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
2. Transfer up to 800 µL of the solution from step 1 to the purification column. Centrifuge at 14000 × *g* for 30–60 seconds. Discard the flow-through.
3. Add 700 µL of Wash Buffer to the purification column. Centrifuge at 14,000 *xg* for 30–60 seconds. Discard the flow-through and place the purification column back into the collection tube.
4. Centrifuge the empty purification column at 14,000 × *g* for an additional 1 minute to completely remove any residual wash buffer.
5. Transfer the purification column to a clean 1.5 mL microcentrifuge tube. Add 50 µL of Elution Buffer to the center of the purification column membrane, let Elution Buffer incubate on the column for 1 minute and centrifuge at 14000 × *g* for 1 minute.
6. Measure the concentration of purified PCR product using a microvolume spectrophotometer (ng/µL) for use in subsequent Gibson assembly calculations.

Preparing Gibson Assembly Reaction Mixture

1. Before the next class meeting, design your reaction recipe for Gibson Assembly. The directions in this subsection of the lab manual accompany the Experiment B Planning Worksheet. Fill out the worksheet to prepare for Experiment B. The type of MasterMix and which recipe to design (see Table 3 or Table 4 below) will be determined by your instructor. The following tips apply to either:
    a. A helpful calculator for converting ng/µL concentrations to molar concentrations can be found here. To use the calculator, you will need the correct molecular weight of your amplicons. Review your Experiment A planning worksheet to find these values.
    b. You may need to dilute your purified amplicon(s) to be able to pipette a reasonable volume (at least 1 µL).
    c. Include a protocol for any necessary dilutions as part of your procedure.
2. Fill in either Table 3 or Table 4 below and check your recipe with the instructor at the beginning of next class.

Table 3: Recipe for NEBuilder® HiFi DNA Assembly MasterMix* reaction (20 µL reaction, 1 per plasmid)

Reagent/Tool	Volume to Pipet	[Final] or Amount
PCR Amplicon(s)**	varies	0.05 pmol
Linearized Vector	varies	0.05 pmol
2X Gibson Assembly® Master Mix	10 µL	1X
Nuclease-Free Water	Varies	N/A
Total Volume:	20 µL	

*This commercially available MasterMix is recommended when more than one insert is to be combined with a vector.

**The number of inserts will depend on your project. You may have multiple amplicons to ligate into your vector, but there should be at least 0.05 picomoles of each insert. The '0.05 pmol' of each fragment is the final quantity of moles per fragment (i.e. $0.05 \cdot 10^{-12}$ mol) in the reaction. This can also be written as 50 femtomoles (i.e. $50 \cdot 10^{-15}$ mol). This value is an amount, not a final concentration.

You will be combining 0.05 pmol of each fragment with 2X Gibson Assembly® Master Mix in a total volume of 20 µL. Thus, you need to figure out how to get 0.05 pmol of each fragment to be assembled (2 or more) in a volume that will allow all required solutions to be added.

Suppose you want to pipette 1 µL (i.e. $1 \cdot 10^{-6}$ L) moles containing the 0.05 pmol (i.e. 50 fmol) of insert. The concentration of a solution that would give you this would be:

$$\frac{(50 \cdot 10^{-15}\, mol)}{(1.0 \cdot 10^{-6}\, L)} = 50 \cdot 10^{-9}\, \frac{mol}{L}$$

Using the online calculator linked above, determine the nanomolar concentration of each linear fragment solution from the measured ng/µL concentrations. If your solution is more concentrated than 50 nM (i.e. $50 \cdot 10^{-9}$ M), you must dilute that solution to give a concentration of 50 nM or less. This necessary dilution is in order to avoid pipetting less than 1 µL of any solution into your Gibson Assembly reaction.

Table 4: Recipe for 'Homemade' Gibson Assembly MasterMix* reaction (6 µL reaction, 1 per plasmid)

Reagent/Tool	Volume to Pipet	Final Concentration/Amount
PCR Amplicon*	Varies	0.017 pmol
Linearized Vector	Varies	0.017 pmol
2X Gibson Assembly Master Mix	3 µL	1X
Nuclease-Free Water	Varies	N/A
Total Volume:	6 µL	

*This 'homemade' MasterMix[1] is recommended when only one insert is to be combined with a vector.

You will be combining 0.017 pmol of each fragment with 2X Gibson Assembly Master Mix in a total volume of 6 µL. Thus, you need to figure out how to get 0.017 pmol (i.e. $0.017 \cdot 10^{-12}$ mol) of each of the 2 fragments in a volume that will allow all required solutions to be added to the reaction.

Suppose you want to pipette 1 µL containing the 0.017 pmol (i.e. 17 fmol) of insert. The concentration of a solution that would give you this would be:

$$\frac{(17 \cdot 10^{-15}\, mol)}{(1.0 \cdot 10^{-6}\, L)} = 17 \cdot 10^{-9}\, \frac{mol}{L}$$

Using the online calculator linked above, determine the nanomolar concentration of each linear fragment solution from the measured ng/µL concentrations. If your solution is more concentrated than 17 nM ($17 \cdot 10^{-9}$ M), you must dilute that solution to give a concentration of 17 nM or less. This necessary dilution is in order to avoid pipetting less than 1 µL of any solution into your Gibson Assembly reaction.

Experiment B: Gibson Assembly, Transformation, and Selection for Positive Clones.

You will combine the vector and insert(s) you obtained in Experiment A with a Gibson Assembly Master Mix, which contains a 5'-exonuclease, a DNA polymerase, and a DNA ligase. You will follow the Gibson Assembly reaction recipe you designed in Experiment A. A small portion of your Gibson Assembly mixture will be transformed into chemically competent *E. coli* cells. In the following lab period, you will select for positive clones and inoculate cultures for plasmid isolation.

Experiment B, Day 1

Gibson Assembly and Transformation

NOTE: Steps 8 and 10–12 may be conducted by flame or in a sterile hood, if available.

1. Pipette your Gibson Assembly reaction according to your designed recipe (Reference your completed Experiment B Planning Worksheet).
2. Incubate the reaction at 50 °C for 15 minutes. If you are combining more than 2 fragments, incubation for up to 60 minutes may increase your success.
3. While reactions incubate, prepare for transformation by thawing chemically competent *E. coli* cells on ice.
4. Add 2 µL of the Gibson Assembly reaction mixture to the competent cells and gently flick to mix.
5. Incubate the mixture on ice for 30 minutes.
6. Heat-shock the tube containing the mixture by placing it in a 42 °C water bath for 30 seconds.
7. Incubate the tube on ice for 5 minutes.
8. Add 950 µL of SOC media to the tube, being careful to keep the stock media sterile.
9. Place tube in the shaking incubator at 37°C for 60 minutes (250 rpm).
10. Pipette 100 µL of the cells on 2 selection plates and spread with sterilized beads.
    a. To use beads, add 3–4 beads onto the plate, put on the lid, and gently move the plate back and forth on the benchtop to spread the cells.
11. Prepare 10^{-4}, 10^{-5}, and 10^{-6} dilutions of the cells in LB by serial 1:10 dilutions.
12. Plate 100 µL of the 10^{-4}, 10^{-5}, and 10^{-6} serial dilutions on LB (no selection) and spread with sterilized beads.
13. Incubate plates overnight at 37 °C.

Experiment B Day 2

Transformation Results, Inoculate Cultures

1. For each plate (both selection and non-selection), count colony forming units (CFUs).
2. Calculate CFUs/mL for each plate using Equation 1:

$$\left(\frac{\#\ CFU}{volume\ plated}\right) \times \left(\frac{1}{dilution\ factor}\right) = \frac{CFU}{mL}$$

   Where the volume plated should be in mL's, and the dilution factor is the factor by which your cells were diluted for that plate. For example, if you prepped a 10^{-2} dilution solution by pipetting 10 µL of cell solution into 990 µL of LB media (1000 µL total), your dilution factor would be 100.
3. Calculate the average CFU/mL on selective plates and non-selective plates.
4. Calculate percent transformation according to Equation 2:

$$\%\ transformation\ = \left[\frac{\left(average\frac{CFU}{mL}\ on\ selective\ media\right)}{\left(average\frac{CFU}{mL} on\ non\ selective\ media\right)}\right] \cdot 100$$

5. Using a marker, select, circle, and label 4 distinct colonies on your selection plate with your initials and a number (ex. ABC1). Retrieve a new LB-agar plate containing antibiotic for selection and use a marker to divide the plate into quadrants (i.e. fourths). Use ~1/2 of each colony to re-streak onto the respectively labeled quadrant. NOTE: This serves as a backup culture of your positive clones in case the liquid culture does not successfully grow.
6. Use the remainder of the colony to inoculate a 5 mL liquid LB culture for each of the 4 colonies selected for plating. Be sure to label your tubes with the respective colony identity (ex. ABC1) and add 5 µL of a 1000X stock of the correct antibiotic for plasmid selection.
7. Incubate liquid cultures from step 6 in a shaking incubator at 37 °C overnight (225 RPM), and the re-streaked agar plate from step 7 in a static incubator at 37 °C.

Experiment C: Isolate Plasmid DNA, Screen for Insert.

The positively transformed competent *E. coli* cells in your liquid cultures will replicate many copies of the plasmid they were transformed with. Copy number of plasmid ranges from roughly 5 to 500 per cell, depending on the cell genotype as well as the origin of replication within the vector backbone. After *E. coli* cells have replicated the plasmid, you will lyse these

cells and isolate the plasmid DNA using a commercially available kit. Next, you will verify if the plasmid contains the insert(s) of interest using either a restriction digest or PCR screen.

Experiment C, Day 1

Miniprep Your Sample

If following the GeneJET Plasmid Miniprep Kit (adapted from Thermo Scientific™):

1. Place glass culture tubes in the tabletop centrifuge and spin for 10 minutes at no more than 3700 RPM.
2. The supernatant should be discarded from the cell pellet, now present in the bottom of the glass culture tube. Add 250 µL of resuspension buffer to the tube. Vortex the tube to resuspend the cell pellet.
3. Transfer the resuspended bacteria to a microcentrifuge tube containing 250 µL of lysis buffer. Then add 350 µL of neutralization buffer. Close tube lid and flip tube several times gently to mix.
4. Place tube in microcentrifuge and spin for 10 minutes at the maximum speed.
5. Transfer supernatant to a spin column from the kit.
6. Spin for 1 min at maximum speed. Discard the flow-through in an appropriate waste container.
7. Add 500 µL of wash buffer. Spin for 1 min at maximum speed. Discard the flow-through in an appropriate waste container.
8. Repeat step 7.
9. Spin for an additional 1 min at maximum speed to remove excess wash buffer. Discard the flow-through in an appropriate waste container.
10. Place spin column in clean, labeled microcentrifuge tube.
11. Add 20–30 µL elution buffer to the center of the spin column. This is a small volume, so be sure that the buffer contacts the membrane. Let sit for 1–2 minutes. Spin for 1 min at maximum speed.
12. Repeat Step 11, with 10 µL of elution buffer.
13. Using the microvolume spectrophotometer, measure the concentration (in ng/µL) of isolated plasmid.

If Following the Zyppy® Plasmid Miniprep Kit (adapted from Zymo Research™):

1. Add 600 µL of bacterial culture grown in LB medium to a 1.5 mL microcentrifuge.

2. Add 100 µL of 7X Lysis Buffer (Blue) and mix by inverting the tube 4–6 times. Proceed to step 3 within 2 minutes. After addition of 7X Lysis Buffer the solution should change from opaque to a clear blue, indicating complete lysis.
3. Add 350 µL of cold Neutralization Buffer (Yellow) and mix thoroughly. The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form. Invert the sample an additional 2–3 times to ensure complete neutralization.
4. Centrifuge at 11,000–16,000 × g for 2–4 minutes.
5. Transfer the supernatant (~900 µL) into the provided Zymo-Spin™ column. Avoid disturbing the cell debris pellet.
6. Place the column into a collection tube and centrifuge at 11,000–16,000 × g for 15 seconds.
7. Discard the flow-through and place the column back into the same collection tube.
8. Add 200 µL of Endo-Wash Buffer to the column. Centrifuge for 30 seconds. It is not necessary to empty the collection tube.
9. Add 400 µL of Zyppy™ Wash Buffer to the column. Centrifuge for 1 minute.
10. Transfer the column into a clean 1.5 mL microcentrifuge tube then add 30 µL of Zyppy™ Elution Buffer directly to the column matrix and let stand for one minute at room temperature.
11. Centrifuge for 30 seconds to elute the plasmid DNA.
12. Measure concentration of isolated plasmid using the microvolume spectrophotometer (in ng/µL).

<u>Screening your Plasmid: Restriction Digest or PCR Screen</u>

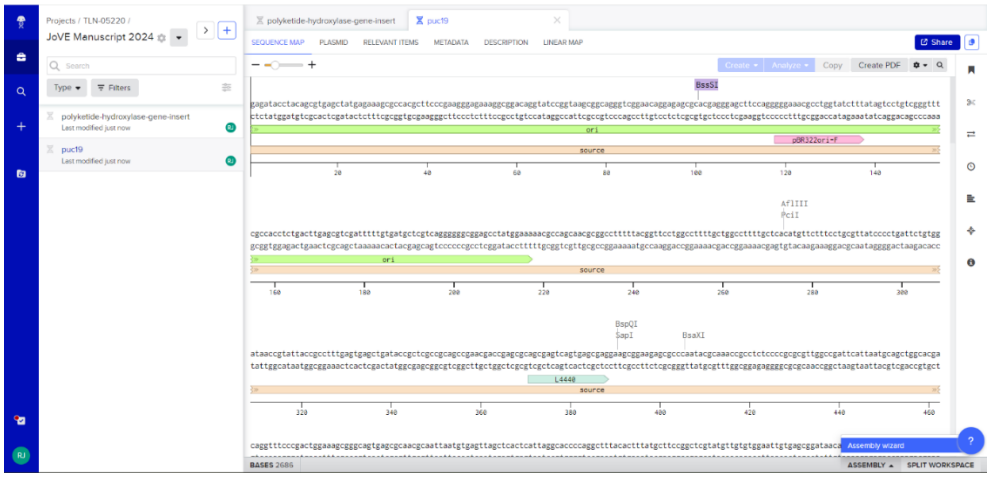
There are multiple ways to screen your plasmid to determine that you have the correct, that is, desired, isolated plasmid. Two possible screening methods are detailed below. Use this information alongside what you know about your desired plasmid sequence to design a screen that assesses whether your construct was assembled as expected.

Your goal in the restriction digest screen or PCR screen is to distinguish your plasmid of interest from other potential sequences that could have been cloned and transformed, resulting in colonies on your selective plate. You want your experimental expectations (i.e. band sizes on an agarose gel) to differ between your desired plasmid and other unwanted DNA sequences, such as empty vector.

First you will need to create DNA sequence files of your desired plasmid and the empty vector.

1. Import your desired insert sequence(s) (i.e. the PCR amplicons you produced *in silico* in Experiment A) and desired vector sequence to Benchling, each as a new DNA sequence file. Open each imported sequence that you wish to include in your Gibson Assembly reaction.

2. At the bottom of your screen, locate the Assembly Wizard tool. Click Assembly Wizard, then select Create New Assembly. From the options provided, select Gibson and click Start to begin the assembly.



3. In the vector sequence window, select all bases you want to include in your assembly from the vector backbone. Once selected, click the 'Backbone' tab at the bottom of the screen, and then click 'Set Fragment'.

NOTE: In this assembly, you will not include any overhangs for an insert that would be installed during PCR. If they were included, they will be duplicated in the final plasmid using this tool.

NOTE: You may want to toggle between the 'Sequence Map' and 'Plasmid' views of the vector to select all desired bases of a circular template.

4. In the insert sequence window, select all the insert's bases you want to include in the assembly. Once selected, click the 'Insert' tab at the bottom of the screen, and then click 'Set Fragment'.

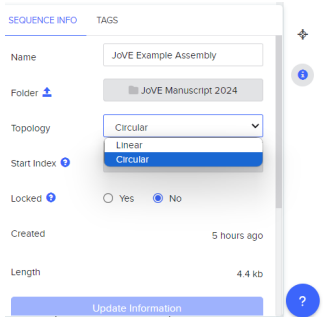
NOTE: In this assembly, you will not include any overhangs for a vector or another insert that would be installed during PCR. If they were included, they will be duplicated in the final plasmid using this tool.

5. If you have multiple gene inserts, click the '+' button on the right side of the Assembly Wizard. In the insert sequence window, select all the insert's bases you want to include in the assembly. Once selected, click the 'Insert' tab at the bottom of the screen, and then click 'Set Fragment'.

6. Once all fragments are set, rename the assembly to your desired plasmid name and press Assemble on the right of the Assembly Wizard. Select the desired folder location for both the Sequence Folder and Primer Folder. Press Create to assemble the recombinant plasmid sequence.

7. Open the assembled plasmid and click 'Assembly History' to view a basic plasmid map and an overview of the sequences from which it was derived. Ensure that the nucleotide positions for each fragment match your desired design. If correct, click 'Finalize' in the Assembly Wizard.

8. Be sure that your *in silico* plasmid and vector are circular. You can find 'Sequence info' by clicking on the lower case i symbol on the right-hand side of the screen with a DNA sequence file open. Be sure that in the 'Topology' drop down menu, your sequence is circular. If it is circular, you will have the option of looking at the sequence in either a linear or plasmid form by clicking on either tab at the top of the screen.



Option A: Restriction Digest Screen

1. Determine and obtain restriction enzyme(s) and necessary reagents.

NOTE: You need to choose an enzyme that will allow you to check if your insert has been correctly inserted into the plasmid backbone. You will be looking for a specific pattern of DNA band sizes that you expect to occur when your desired plasmid is digested with the

restriction enzyme(s) you choose. Therefore, you need to know ahead of time how many times the enzyme(s) you select cut the plasmid of interest, and what the expected DNA base pair lengths are of the digested fragments. It is recommended to choose an enzyme that makes at least two cuts, or two enzymes that make at least one cut each. The expected DNA digest fragments need to be able to be resolved on an agarose DNA gel for confident analyses.

To aid in your decision, you can use Benchling to help determine which enzyme to use.

a. To perform a restriction digest *in silico* on the map of your assembled plasmid, click on the scissor icon on the right-hand sidebar of the screen in Benchling. You can search for an enzyme by typing its name in the search box. You should choose enzymes that are available to you in lab. Once you have selected the enzyme(s) of interest, scroll down within this window until you can see the 'Run Digest' button, and click on this.

b. Another window will appear under a 'Digest' tab that shows the size of fragments expected in this digest. If you click on the 'Virtual Digest' tab, it will also show what the fragments look like when run out on a gel, and you can even choose different DNA molecular weight standards (aka DNA ladders) in the drop-down menu. Perform the same digest on the empty vector sequence and ensure that they are distinguishable on a gel. The empty vector digest will serve as a control.

2. Determine a recipe for your reaction(s) according to the table below.

Table 5: Restriction Enzyme Digest Recipe (for New England Biolabs HF enzymes)

Reagent/Tool	Volume Added	[Final]/Amount
Isolated Plasmid DNA	Varies*	500 ng
10X rCutsmart Buffer	1 µL	1X
Restriction enzyme(s)	0.5 µL	10 U
Nuclease-Free Water	Varies**	N/A
Total Volume:	25 µL	

*Depends on the concentration of your isolated plasmid DNA. Add a volume of your plasmid solution to get 500 ng of DNA total.

**Add enough water so that the total reaction volume is 25 µL.

NOTE: These recipes are for high fidelity (HF) New England Biolabs enzymes (e.g., BamHI-HF). If you are not using these enzymes, we recommend modifying the recipe based on the manufacturer recommendations.

3. Incubate the digest at the recommended temperature for maximum activity for one hour for the enzyme(s) you selected. To access this information, hover over the enzyme you chose in the digests side bar (i.e. scissor symbol). Most often, Benchling provides activity information and a link to the manufacturer's webpage for this product.

4. Analyze restriction digest via agarose gel electrophoresis as in Experiment A. Add 5µL 6X loading dye to your reaction(s) and analyze the maximum volume that you can load in a single lane of an agarose gel. Adjust agarose percentage, voltage, and running time as necessary.

Option B: PCR Screen

1. Determine the PCR reaction(s) necessary to screen for the correct plasmid. Questions to consider include:
   - *What primers have you already used that could determine successful assembly?*
   - *How much template DNA should you use?*
   - *What controls could you include to confirm that the PCR was successful?*
   - *What band sizes do you expect if…*
     - *your assembly was successful?*
     - *the vector re-ligated with itself?*
     - *the original plasmid carried through and was transformed?*

2. To design your PCR screen, reference the following:

   a. Your completed Experiment A Planning Worksheet (if utilizing the same primers you used in Experiment A).

   b. If using different primers, determine expected amplicon sizes for desired product versus empty vector. Depending on the primer sequences, the empty vector may be able to serve as a positive or negative control in your reaction.

      You can use the same strategy as Experiment A of linking primers to the sequence of your desired plasmid in Benchling and creating an amplicon *in silico*. You may also reference the PCR recipes and cycling programs from Experiment A and, relative to your new primers, determine expected amplicon sizes to adjust your recipe and cycling program accordingly.

3. Pipette PCR reactions according to your designed recipe.

4. After completing the PCR, mix 5 µL PCR with 1 µL 6X Loading Dye. Analyze reactions via agarose gel electrophoresis as previously described in Experiment A. Note that as before, you need to know the expected size of your amplicon to analyze your gel.

References:

1. Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., Smith, H.O. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*. **6** (5), 343–345 (2009).
2. New England Biolabs Gibson Assembly Cloning Kit. https://www.neb.com/en-us/products/e5510-gibson-assembly-cloning-kit.
3. New England Biolabs Gibson Assembly Application Overview. https://www.neb.com/en-us/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/gibson-assembly.