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

Instructor Manual

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Instructor Preparation

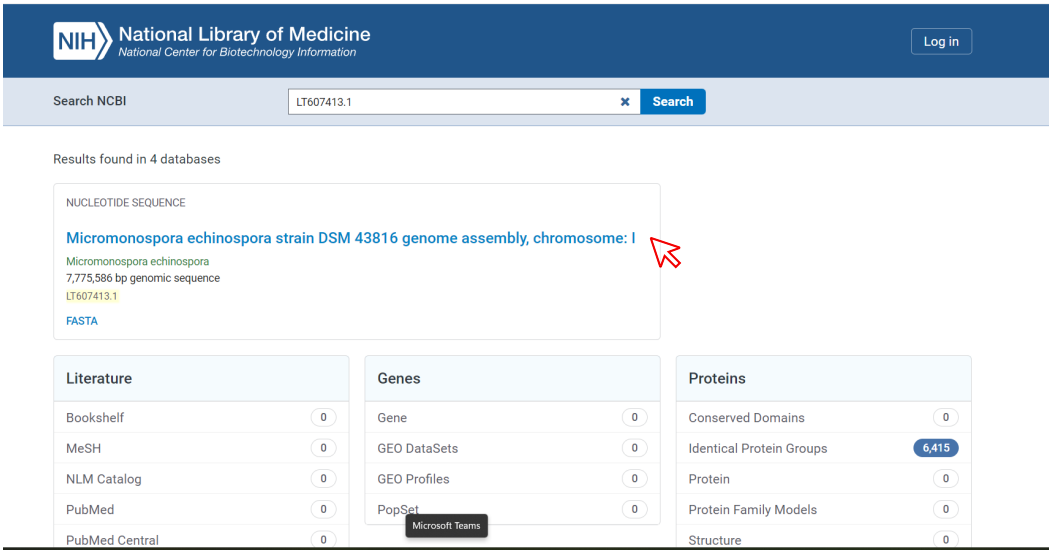
Gibson Assembly Primer Design Using Benchling Assembly Wizard

1. Sign into Benchling or create an account if you do not already have an account.

2. Determine the DNA template source for your gene insert(s), such as genomic DNA, plasmid, synthetic DNA, etc.

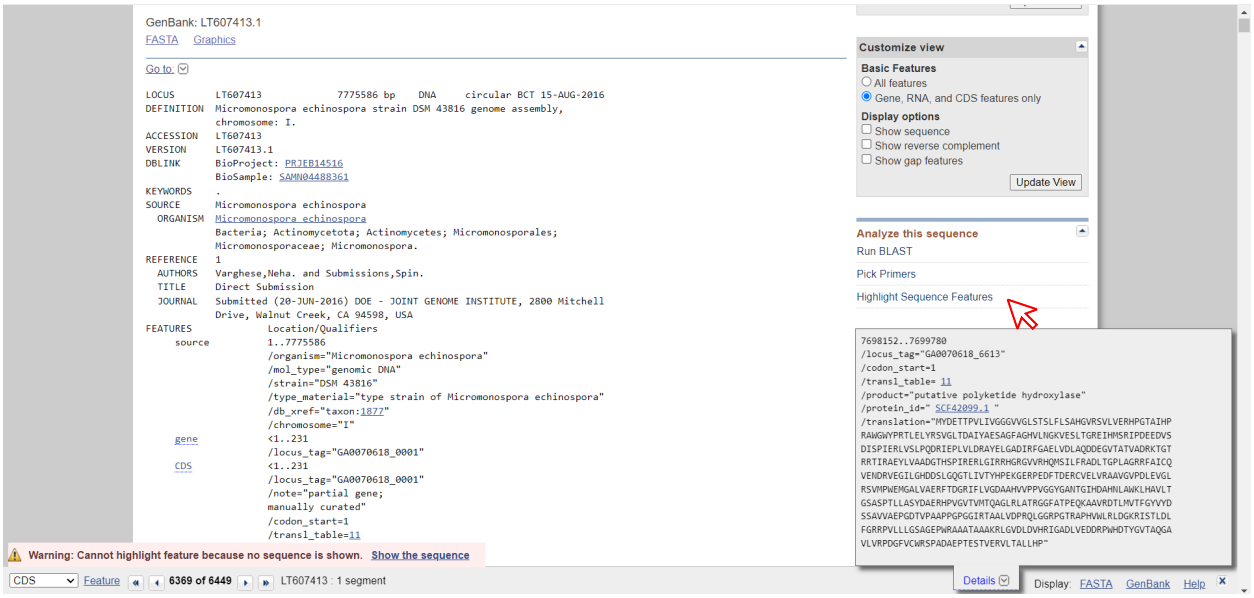
   NOTE: For genes that will be amplified from complex genomic DNA templates, it is advised to first determine unique binding sites for primers within the genomic DNA template that are specific for the gene(s) of interest, using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST)[1], as detailed in the following sub-steps. For simple templates such as plasmids or synthetic DNA, you may proceed to step 3.

   A. Retrieve genome sequence from the National Center for Biotechnology Information database, using the GenBank accession number. Use accession number LT607413.1 for this example.

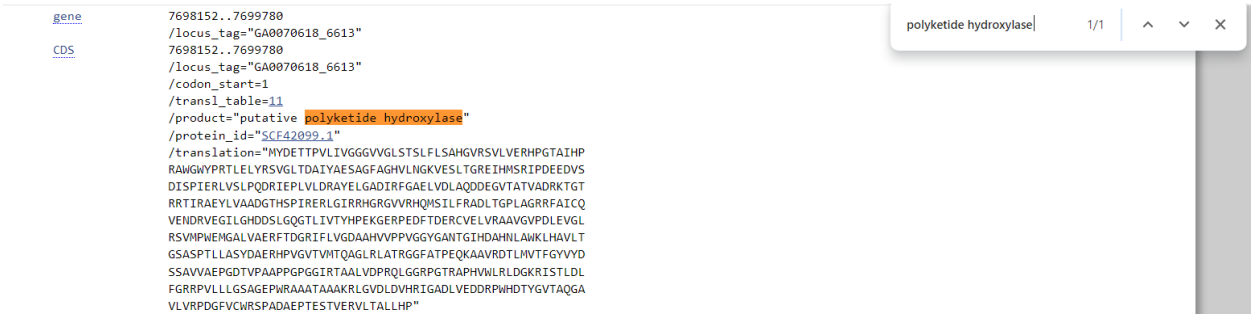


   B. Find the location and size of your gene of interest within the genome, using your browser's 'Find' function and the gene name, or the 'Highlight Sequence Features' tool; for example, the putative polyketide hydroxylase: 7698152..7699780, 1629 nucleotides.

Highlight the feature with the NBCI tool:

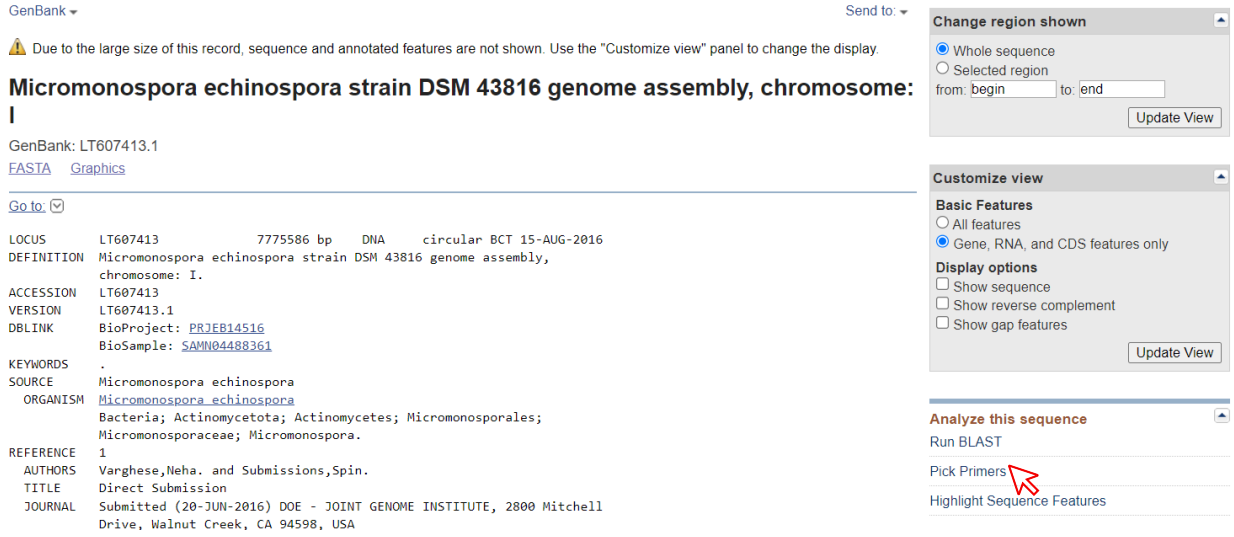


Or use the find function:



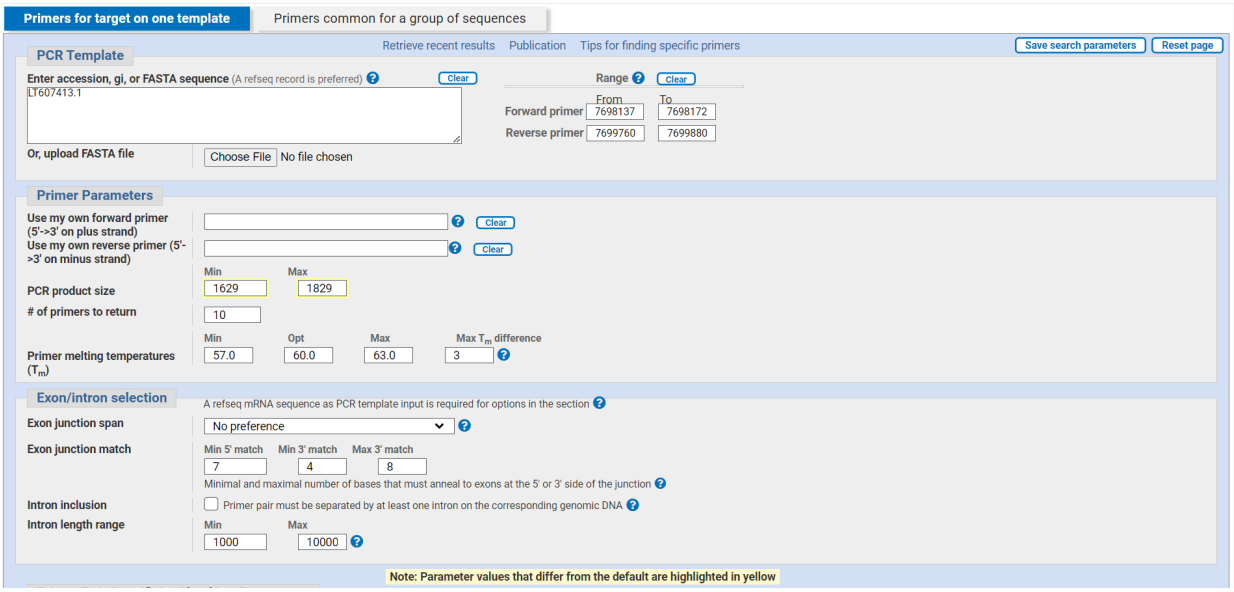
C. Open the 'Pick Primers' tool.

a. In the 'Range' boxes, enter the desired location of your gene specific primers relative to the retrieved gene location in the genome. For example, to design a forward primer that will amplify the 5' end of the gene including the start codon, enter the gene start site minus 100 nucleotides in the 'From' box, and the gene start site plus 20 nucleotides in the 'To' box (ex. 7698052 and 7698172, respectively).

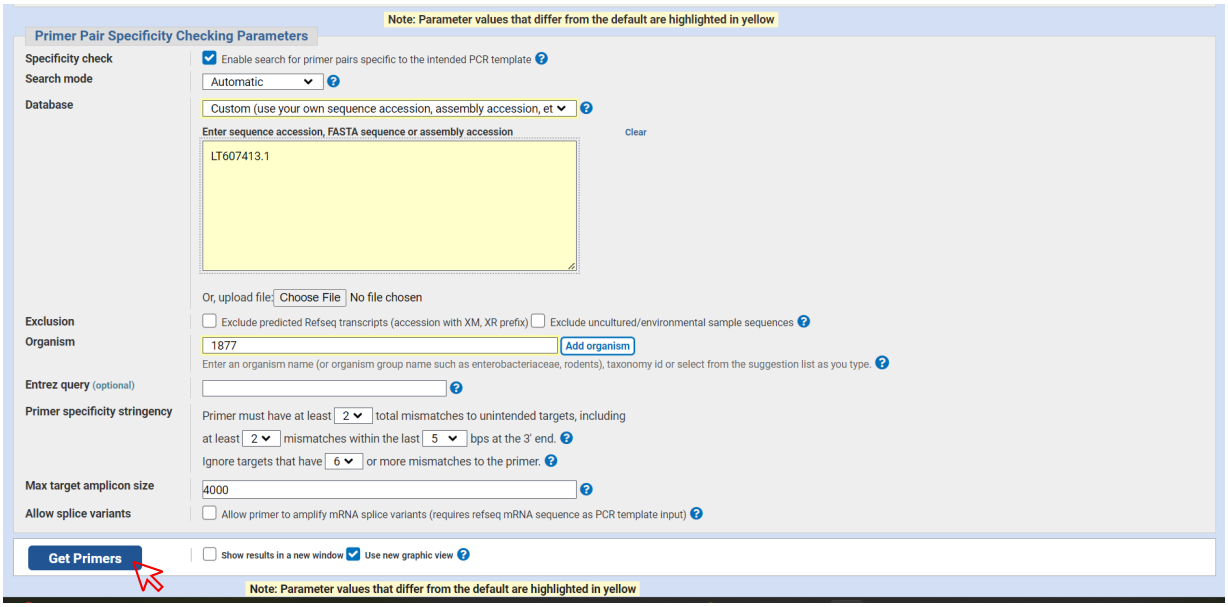


NOTE: If gene of interest is to be cloned into an expression vector, it is recommended to be more stringent on the placement of the forward primer to ensure that the gene start codon will be near a ribosomal binding site within the expression vector backbone. For example, enter the gene start site minus 15 nucleotides in the 'From' box, and the gene start site plus 20 nucleotides in the 'To' box (ex. 7698137 and 7698172, respectively).

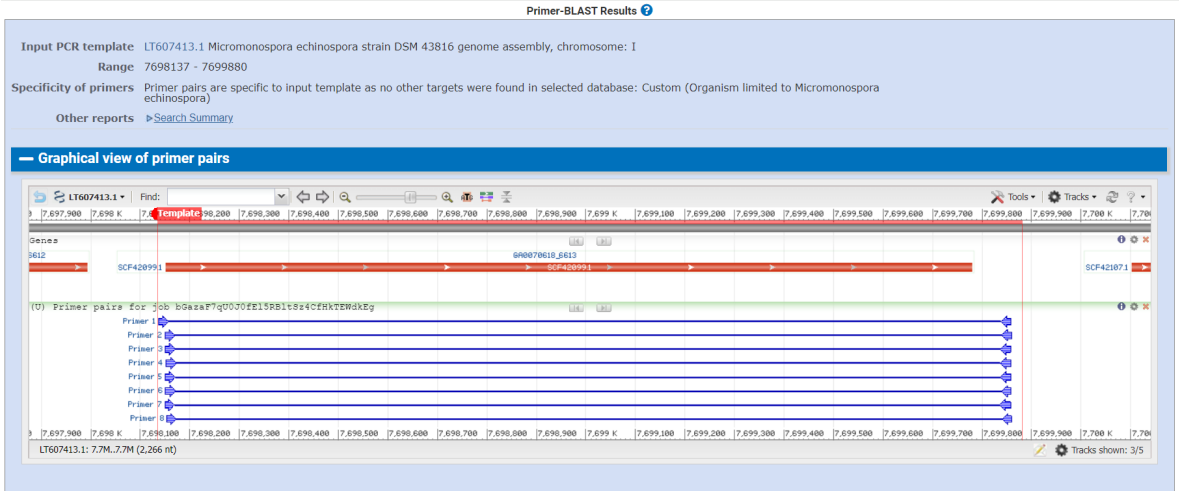
b. For the reverse primer, enter the gene stop site minus 20 nucleotides in the 'From' box, and the gene stop site plus 100 nucleotides in the 'To' box (ex. 7699760 and 7699880, respectively).



c. Under 'Primer Parameters', adjust the PCR product size to match the desired gene size. The minimum product size should be no smaller than the gene of interest (ex. 1629 nucleotides), and the max should be no larger than the gene size plus 200 nucleotides (ex. 1829 nucleotides), based on the primer binding ranges.
d. Under 'Primer Pair Specificity Checking Parameters,' select 'Custom' Database, and paste the GenBank accession number into the text box. Also ensure that the organism number corresponding to your genome is pre-filled (for example, 1877 can be selected from the auto-populated dropdown menu when *Micromonospora echinospora* is typed into the organism box).
e. Click 'Get primers.'

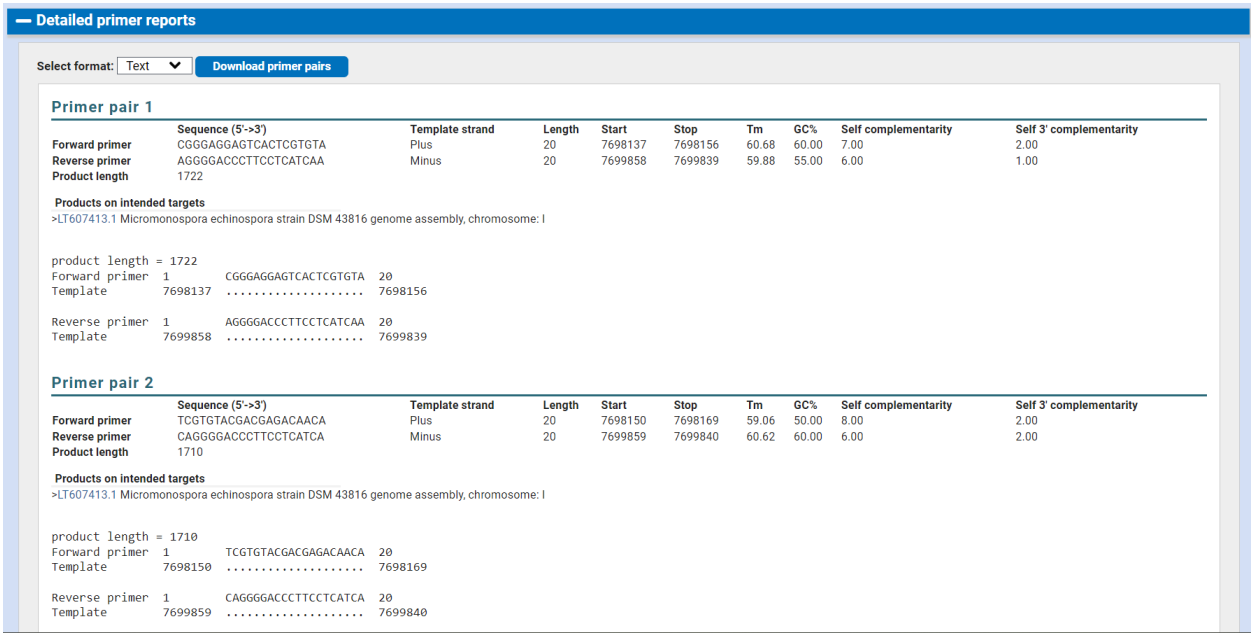


D. Examine the results under the 'Graphical view of primer pairs' and ensure that the entire gene is amplified with the primer pairs produced. In other words, ensure that the primer amplifies the entire gene from start to stop codon.



NOTE: See how primers include the entire gene sequence (red region in template, example gene GA0070618_6613).

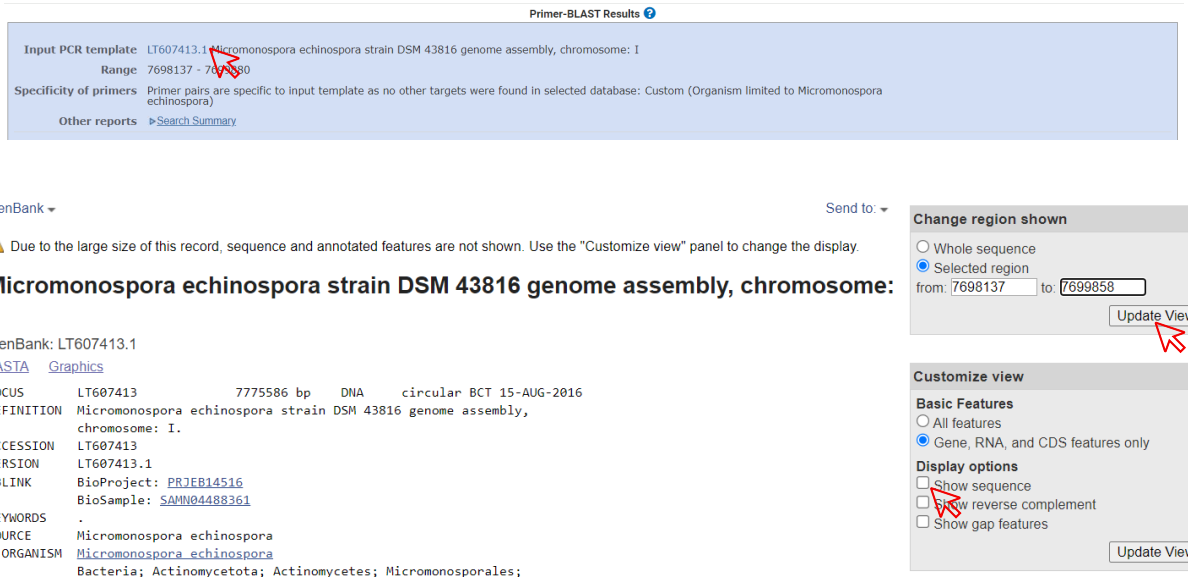
E. Under 'Detailed Primer Reports', view products on intended targets and on potentially unintended templates for each primer pair. Unintended templates are areas with similar sequences in other regions of the genome and can cause non-specific annealing during PCR. Choose a primer pair that has the fewest number of potentially unintended products.



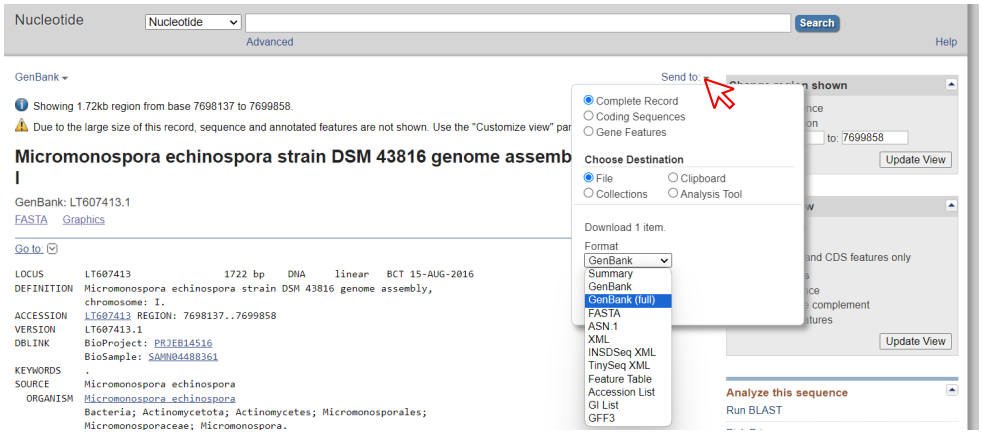
NOTE: If many potentially unintended products arise for all primer pairs, this will be indicated at the top of the page. See the 'help on specific primers' link for hints on how to adjust search parameters to yield more specific primers.

F. Record the nucleotide position for the 5' end of each primer—they will determine the boundaries of the sequence you will retrieve for the following steps (ex. Primer pair 1: 7698137 and 7699858).

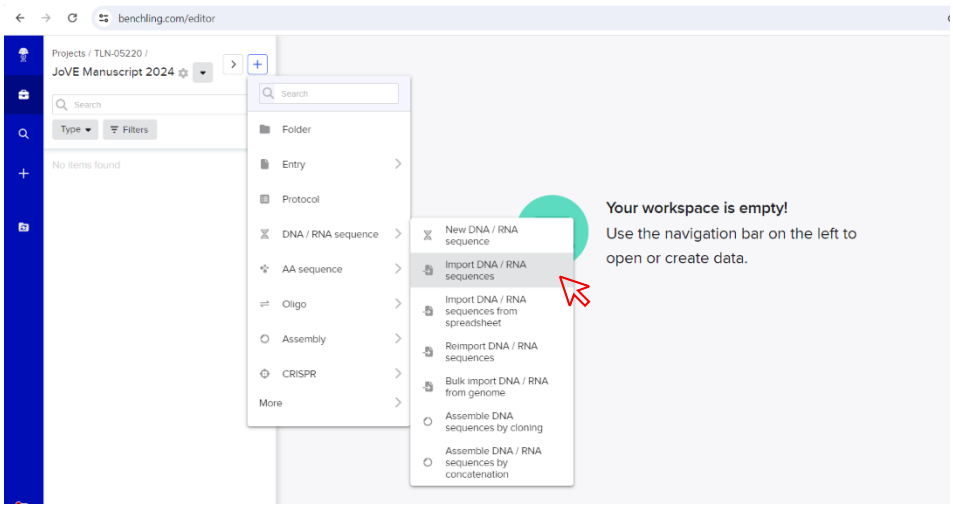
G. At the top of the primer result page, click on the link to the accession number for your genome template sequence. Then, in the 'Change Region Shown' boxes, enter the nucleotide positions recorded in the previous step. Then click 'Update View.'



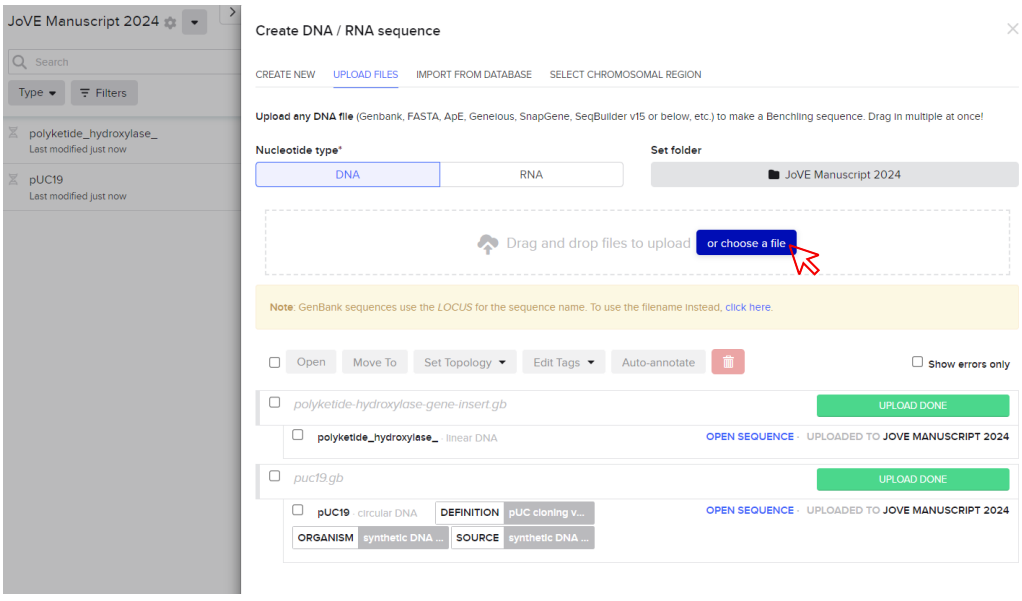
H. Once the page updates with your desired region, you will now download a DNA sequence file of the desired sequence from the genome, that begins and ends with unique primer binding sites. (If the gene sequence is not displayed, click 'Show Sequence' in the Customize view box.) Click on 'Send To' in the upper right corner, then choose 'File' as the destination, and then click Create File. Choose a file type from the drop-down menu. This will automatically prompt a download of the DNA sequence file.



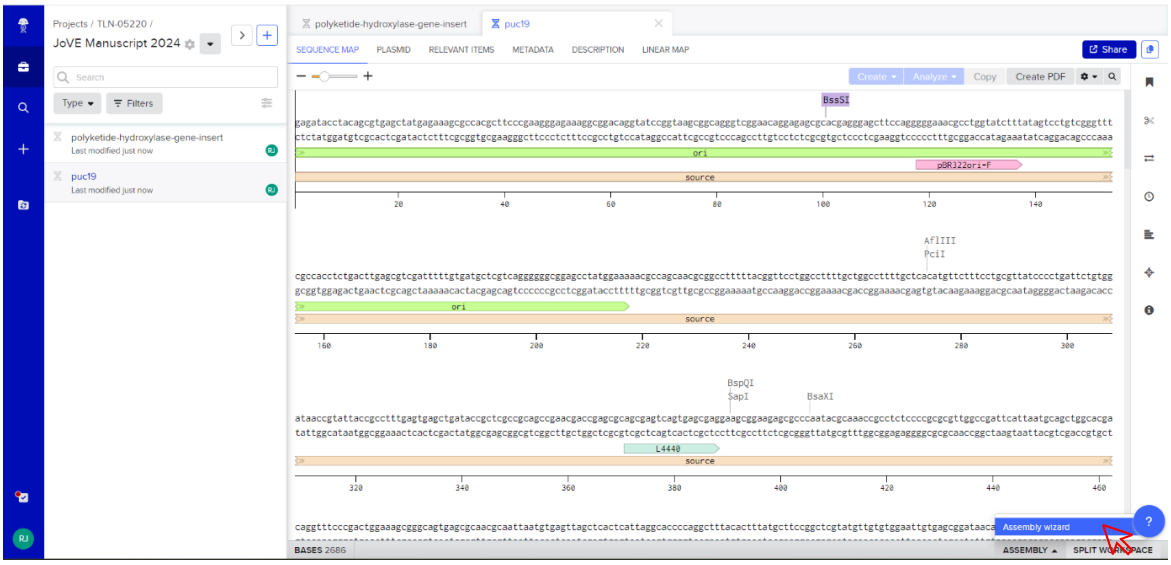
3. Retrieve DNA sequences of your gene insert(s) and vector (for example, see polyketide hydroxylase gene sequence and pUC19 sequence in the Supplementary Material). Import your desired insert sequence(s) and desired vector sequence into Benchling, each as a new DNA sequence file. Open each imported sequence that you wish to include in your Gibson Assembly reaction.



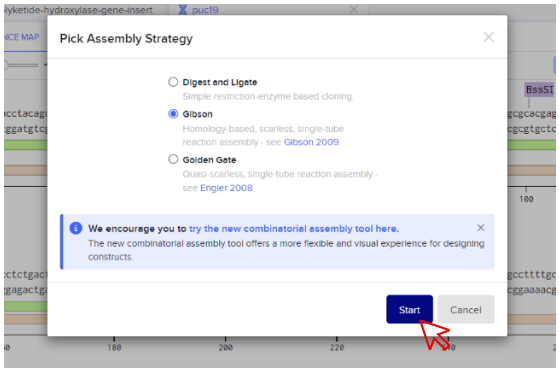
NOTE: You may import DNA sequence files in several formats, including GenBank and FASTA. You may also import a sequence directly from a database with an accession number or paste in a nucleotide sequence copied from another file or viewer.



4. 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.

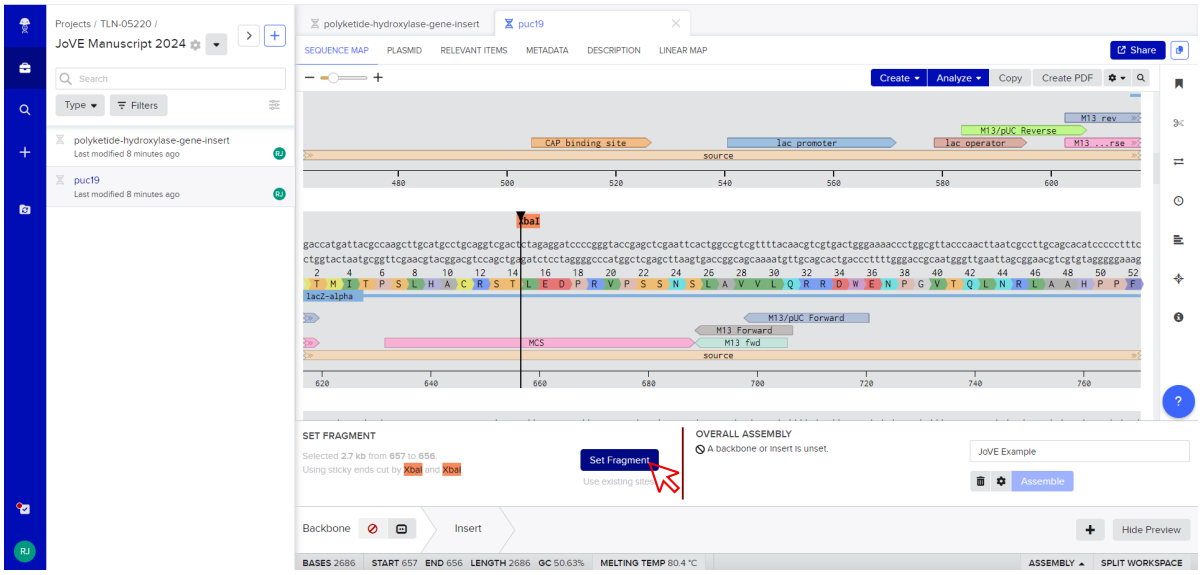


5. In the vector sequence window, select all bases you want to include in your assembly from the vector backbone. Start your selection at the nucleotide position you want at the 3' end of your gene insert (ex. position 657 of pUC19) and select all remaining nucleotides to be included (ex. through position 656 to include entire pUC19 sequence). Once selected, click the 'Backbone' tab at the bottom of the screen, and then click 'Set Fragment'.

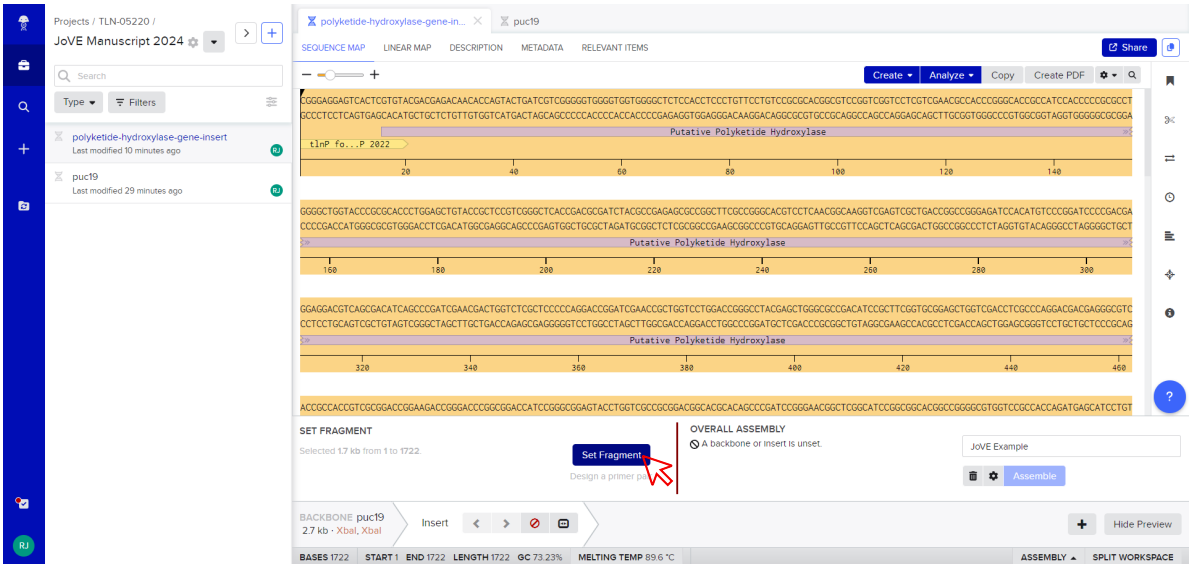
   NOTE: You may want to toggle between the 'Sequence Map' and 'Plasmid' views of the vector to select all desired bases of a circular template.

   NOTE: Alternatively, if you plan to prepare the vector fragment via restriction digest (instead of PCR) for Gibson Assembly, you may linearize the backbone sequence with a restriction enzyme cut site (for ex. XbaI). To do so, click the cut site, hold Shift, and click the cut site again. Then click Set Fragment to set the backbone. Be aware that vector specific primers will not be generated by Benchling if you linearize the

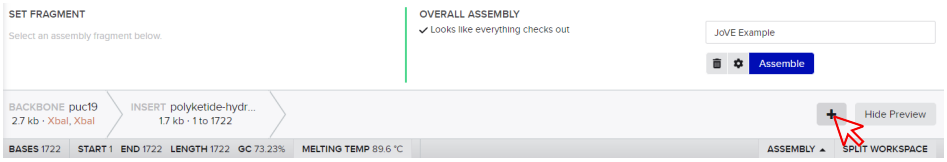
backbone with a restriction enzyme in the Assembly Wizard.



6. In the insert sequence window, select all the insert's bases you want to include in the assembly (for the polyketide hydroxylase ex. select full sequence in file). Once selected, click the 'Insert' tab at the bottom of the screen, and then click 'Set Fragment'.

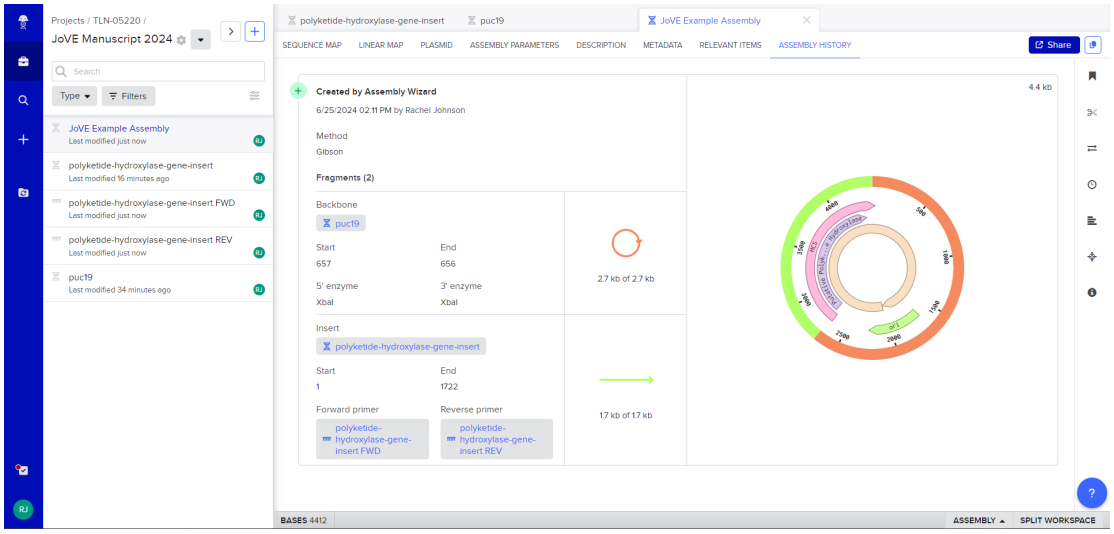


7. If you have multiple gene inserts (not applicable in this example), click the '+' button on the right side of the Assembly Wizard. In the insert sequence window, select all the insert's bases you want to include in the assembly. Once selected, click the 'Insert' tab at the bottom of the screen, and then click 'Set Fragment'.



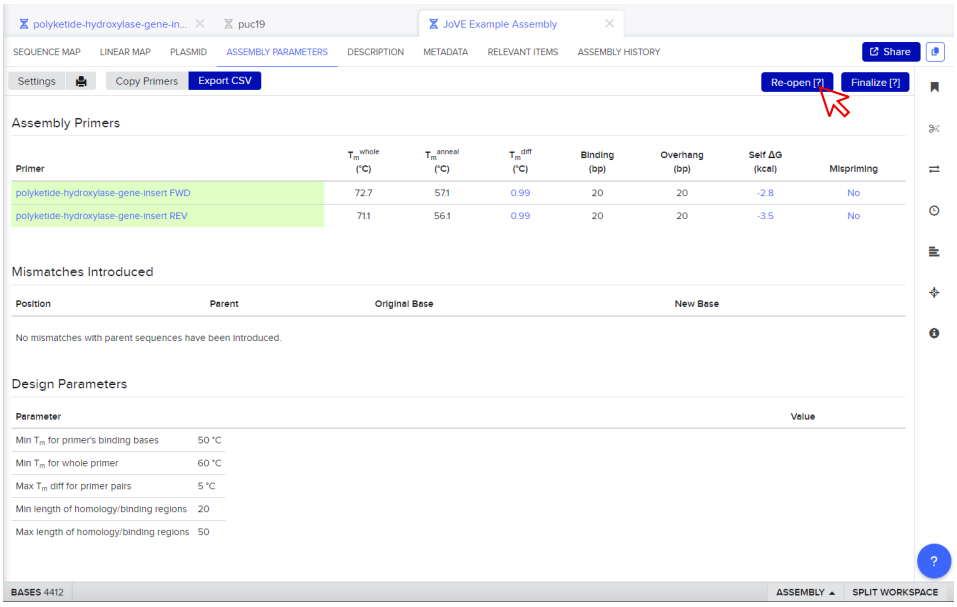
8. Once all fragments are set, rename the assembly to your desired plasmid name and press Assemble on the right of the Assembly Wizard. Select the desired folder location for both the Sequence Folder and Primer Folder. Press Create to assemble the recombinant plasmid sequence.

9. 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.

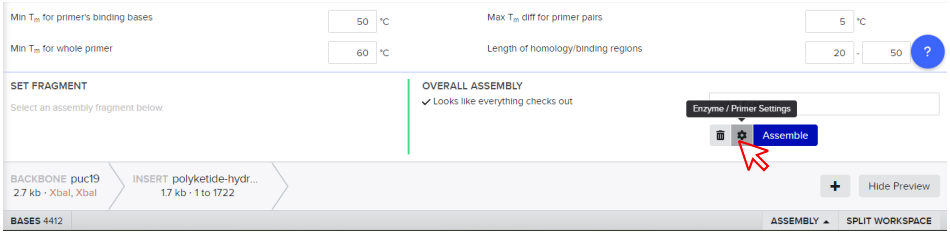


10. In the 'Assembly Parameters' tab, you can view names of the primers designed for your assembly. Note that there will be two primers designed for each insert (a forward and a reverse), and the primer names will be derived from the titles of your DNA sequence files. Also ensure that primer melting temperatures and suggested annealing temperatures are compatible in this window.

    NOTE: If primer melting temperatures or annealing temperatures are not ideal, you can manually adjust primer sequences (ex. removing nucleotides to reduce melting temperature) within the primer sequence files. You can also click on 'Re-open' and adjust the primer settings by clicking the Enzyme/Primer Settings button next to the 'Assemble' button' in the Assembly Wizard. Then repeat the Assembly step and re-examine primer sequences.



Adjust primer design parameters here:



NOTE: If you plan to clone a library of plasmids with many different genes cloned into the same vector backbone, you can utilize the same primer pair for your vector in each case. To do so, manually remove all the nucleotides from the vector primers that would install the overhangs that are specific to the insert. These nucleotides would be found at the 5' end of the primer. (See Supplementary Material, Representative Primers.) After removal, ensure that primer melting temperatures and annealing temperatures are compatible.

NOTE: If you plan to clone a library of plasmids with many different genes cloned into the same vector backbone, it is advised that all insert primer pairs have very similar melting temperatures and annealing temperatures. This will reduce the need for annealing temperature optimization of every pair and enables all student PCR reactions to be cycled at the same time using the same thermocycler program.

11. Once primer design is complete for desired plasmids, click 'Finalize' in the Assembly Wizard.

Experimental Preparation

1. Order your primers from a DNA synthesis company and retrieve samples of your DNA templates for insert(s) and vector.

2. Test both insert- and vector-specific primer pairs for optimal annealing temperatures using a gradient PCR experiment with your DNA templates. PCR conditions should be modified based on the DNA polymerase used in the reaction; the New England Biolabs website can serve an excellent resource for PCR thermocycler methods and other troubleshooting. In the initial screen, test the Benchling recommended annealing temperatures ± 3 °C. Move onto step 3 once successful PCR conditions are determined.

3. Prepare aliquoted solutions of primers (10 µM) and DNA templates (1–100 ng/µL).

4. Make digital sequences of primers and DNA templates available to students on a course web page. Students can download sequences from the course website and upload the sequences into a sequence analysis tool. We recommend the free web-based tool Benchling for *in silico* work.

5. In two iterations of the course where students were ligating two linear fragments (one insert and a vector), we utilized a 'homemade' Gibson Assembly Master Mix[2] instead of a commercially available MasterMix. If you would like to prepare a homemade MasterMix, follow the recipe in **Table 1** and reference the notes on the following page.

Table 1. Recipes for 'homemade' Gibson buffer and Gibson Assembly MasterMix[2].

4X Gibson Buffer (4000 µL)	[Stock]	Vol./Amount	[Final]
Tris-HCl (pH 7.5)	1 M	2000 µL	500 mM
PEG-8000	----------	1 g	25.0% (w/v)
$MgCl_2$	1 M	200 µL	50 mM
DTT	1 M	200 µL	50 mM
dNTPs	10 mM	400 µL	1 mM
NAD^+	100 mM	200 µL	5 mM
Water		1000 µL	
2X Master Mix (1250 µL)	[Stock]	Vol (µL)	[Final]
Taq ligase	40 U/µL	250	8 U/µL
T5 exonuclease	10 U/µL	5	0.04 U/µL
Phusion polymerase	2 U/µL	31.25	0.05 U/µL
Gibson Buffer	4X	625	2X
Water		338.75	

NOTES: First prepare the 4X Gibson buffer, then the 2X MasterMix in the Gibson Buffer. Aliquot into 50 µL volumes and freeze at −20 °C for use in Experiment B. For projects in which >2 linear fragments will be combined, we recommend using a commercially available MasterMix to improve cloning outcomes[3].

Instruction Notes

Lecture Day 1

1. Instruct students to predict PCR amplicon size and sequence from DNA template and primer sequences using Benchling. Challenge students to think about how primers are incorporated into PCR amplicons, including the bases that are not homologous to the template (i.e. the overhangs). To gauge understanding, have students submit the size of each expected amplicon and/or its sequence.
   NOTE: You can quickly grade sequences by comparing student submissions to the expected amplicons using the sequence alignment tool in Benchling.
2. Ask students to design PCR reaction recipes and a thermocycling program for each of the amplicons to be used in the Gibson Assembly reaction of interest. Facilitate determination of theoretical primer annealing temperatures from the primer sequences, and extension times based on expected amplicon size and the polymerase manufacturer's information.
3. Assess student understanding with a paper worksheet and/or online assignment for grading before the next class period (See Experiment A Planning Worksheet).

Experiment A, Day 1

1. Give feedback on students' PCR reaction recipes and thermocycling programs before supplying reagents for PCR reactions.
2. Provide students with solutions of DNA templates (1–100 ng/µL), both forward and reverse primers (10 µM), nuclease-free water, and DNA Polymerase either as a 2X MasterMix containing dNTPs and buffer or as separate solutions.
3. Instruct students to pipette their PCR reaction recipes according to pre-determined recipes and place them directly in the thermocycler or in a PCR tube holder for transport.
4. Before the next lab meeting, cycle the PCR reactions according to polymerase manufacturer's instructions using the optimal annealing temperature from the PCR screen and extension times that were previously determined (Experiment Preparation, Step 2). Store between 4 °C and −20 °C until the following meeting.
5. Ask students to summarize their expectations of a successful experiment before the next meeting.

Experiment A, Day 2

1. Assess the outcomes of PCR experiments through agarose gel electrophoresis. Have students prepare agarose gels, considering agarose percentage for optimal resolution of amplicon size, and stain with GelRed. Students will load PCR reactions onto gels (usually 5 µL of a 25 µL reaction) and run gels, agreeing on what voltage and running time will be utilized for the group.
2. While the gel is running: If the PCR template was a plasmid, direct students to perform a DpnI restriction digest at 37 °C on the remainder of their PCR reactions to ensure that circular DNA templates are degraded and are not transformed into *E. coli* in the subsequent experiment.
3. Help students image their gels and analyze results according to their expected amplicon sizes. Discuss the expectations and/or results for a successful or failed PCR reaction. Students should select their successful PCR reactions to purify in the next step.
4. Instruct students to purify the selected PCR reactions from previous the step once the DpnI digest is complete (i.e. after 1–2 h) using a commercially available kit. Instruct students to measure concentration of purified products on a Nanodrop instrument and discuss the meanings of each value provided by the measurement (A260, A260/280, etc.).
5. Challenge students to calculate a recipe for Gibson Assembly reaction with purified insert(s) and vector for the next laboratory meeting. These calculations will be dependent on insert(s) and vector concentration, volume available, and DNA fragment size. Provide an online calculator to convert concentrations from the Nanodrop instrument to molar concentrations to assist students in calculating appropriate volumes. Provide instructions for diluting vector and insert solutions, ensuring no less than 1 µL of any solution is pipetted for accuracy (See Experiment B Planning Worksheet).

Experiment B, Day 1

1. Assess Gibson Assembly recipes prepared by students.
2. Once recipes are approved, provide Gibson Assembly Master Mix and instruct students to pipette their Gibson Assembly reactions according to approved recipes.
3. Ensure all reactions incubate at 50 °C for 15 minutes.
4. Oversee students perform transformation of commercial chemically competent cells with 2 µL of Gibson Assembly reaction. After transformation, ensure students perform rescue and outgrowth in 950 µL of SOC media, in the absence of antibiotics.
5. After outgrowth, direct students to plate 100 µL of cell suspension on LB agar plates containing the appropriate antibiotic for selection, depending on the vector backbone utilized. Have students prepare serial dilutions in LB (10^{-4}, 10^{-5}, 10^{-6}) for calculating transformation efficiency, label plates with student information, and return the remaining cell suspension to the 37 °C incubator.
6. Incubate all transformant plates at 37 °C overnight (12–16 h).
7. The following day, evaluate the plates. If any selection plates lack colony forming units (CFUs), inoculate additional plates (with appropriate antibiotic) with the remaining cell suspension and incubate overnight at 37 °C. Store all plates containing CFUs at 4 °C until the following class period.

Experiment B, Day 2

1. Direct students to count CFUs on all plates and calculate transformation efficiency.
2. Instruct students to select CFUs from plates containing antibiotic and inoculate 5 mL of LB media (with appropriate concentration of respective antibiotic) from up to 4 CFUs.
3. Ensure cultures are incubated overnight at 37 °C and store at 4 °C until the next class period.

Lecture Day 2

1. Challenge students to design a diagnostic screen to determine successful cloning of plasmid of interest. This includes directing students to perform an *in silico* Gibson Assembly on Benchling and to design either a restriction digest or PCR screen to determine if they have successfully cloned the plasmid of interest.
2. Provide a worksheet for students to summarize the design of their diagnostic screen, including incubation times and temperatures, and expected outcomes for agarose gel electrophoresis. The designed experiment should include both positive and negative controls (See Experiment C Planning Worksheet.)

Experiment C, Day 1

1. Have students isolate plasmid DNA from the previously inoculated liquid cultures using a commercially available plasmid DNA miniprep kit and have students measure concentration of purified products on a Nanodrop instrument.
2. Consult with students and provide feedback before students set up their screen.
3. Incubate restriction digest and/or PCR reactions at appropriate temperatures and store at −20 °C until the next class period.

Experiment C, Day 2

1. Direct students to perform agarose gel electrophoresis and provide insight as students analyze their restriction digest and/or PCR results.
2. Keep any plasmids that match the expectations for successful clones and confirm the sequence by DNA sequencing by your preferred vendor. We recommend full plasmid sequencing by plasmidsaurus.
3. Have students consider the identity of any undesired or ambiguous screening results, such as re-circularized vector, and whether the screening experiment was successful based on their positive and negative controls. Keep the plasmids that had uncertain screening results as backups for further screening or DNA sequencing analyses.

References:

1. Camacho, C. *et al.* BLAST+: architecture and applications. *BMC Bioinf.* **10** (1), 421 (2009).
2. 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. *Nat. Methods*. **6** (5), 343–345 (2009).
3. New England Biolabs Gibson Assembly Cloning Kit. https://www.neb.com/en-us/products/e5510-gibson-assembly-cloning-kit.