MacVector 18.8

for MacOS

Gibson Assembly and Ligase Independent Cloning Tutorial

MacVector, Inc.
Software for Scientists

Copyright statement

This version of the Gibson Assembly and Ligase Independent Cloning Tutorial was published in August 2025.

Contents

Introduction

Gibson assembly is a Restriction Enzyme independent cloning method described on this Wikipedia page - https://en.wikipedia.org/wiki/Gibson_assembly. The key for this approach is that the user generates multiple PCR fragments with 15-40nt repeats at the ends of the fragments. The primers used are carefully designed to have a section that matches the left or right end of each target fragment, along with unique tails that will be used to drive the assembly once the fragments are gently treated with a 5' exonuclease to generate complementary 3' overhangs. For a simple two fragment cloning you would have e.g. [rpt1-fragA-rpt2] and [rpt2-fragB-rpt1] where rpt1 and rpt2 are unique 15-40nt oligonucleotides.

Many users have struggled to get this to work consistently (though labs that have succeeded often will use this technique almost exclusively). This resulted in the development of a modified version called Ligation-Independent Cloning (LIC) (https://www.neb.com/applications/cloning-and-synthetic-biology/ligation-independent-cloning). This is very similar in concept to Gibson Assembly except that the repeat "tails" are designed to be completely missing one of A, G, C or T. This allows users to more easily generate single-strand extensions by incubating with T4 DNA Polymerase in the presence of just that dNTP so that the 3' exonuclease activity results in a 5' single-stranded overhang. This means that, practically, the repeat regions can be much shorter (typically only 15 nt) and obviates the need for a ligation step, especially if the single-stranded ends are GC rich. Certain cloning vectors have suitable restriction sites (mostly fortuitously, though some are engineered) that lie in regions missing one residue for 12-15nt on each side. These can be used as a vector backbone with single-stranded overhangs – target fragments would thus need amplifying with primers that would add on these overhangs as a tail, then get cut back to produce complementary single-strand overhangs prior to cloning.

A related approach is the popular "In-Fusion" system from Takara Biosciences. Prom the point of view of designing primers, this can be treated the same as the 3' exonuclease LIC approach described above.

With careful design of primers, users can fairly easily generate cloning experiments with 5 or more fragments. There have been reports of success with 10+ fragments, though that is not typical.

MacVector 17 introduced a new Gibson Assembly interface that provides a whole slew of functions to help in the design and implementation of both Gibson Assembly and Ligase Independent Cloning experiments. You can;

(a) Provide a set of required fragments through a simple drag and drop interface and let MacVector generate a suitable matched set of primers ready for Gibson Assembly.

(b) Design primers suitable for inserting fragments into LIC cloning vectors with T4 DNA Polymerase treatment of ends missing an individual dNTP.

(c) Generate fully annotated circular constructs using the automatically generated primers.

(d) Provide a set of pre-amplified fragments and have MacVector assemble them into a circular construct.

(e) Provide custom primers and have MacVector generate the appropriate PCR fragments and assemble them into a final circular construct.

(f) View the actual predicted translations and fusions at each junction, with the ability to insert spacer residues in the primers to ensure the fusions are in-frame.

This tutorial will show you how to use most of this functionality. After running through the examples, you should be able to adapt the protocols for use in your own cloning experiments.

Sample Files

The majority of the files used in this tutorial can be found in;

`/Applications/MacVector/Tutorial Files/GibsonAssembly/`

Unless otherwise specified, look in this folder for the sample data.

Tutorial

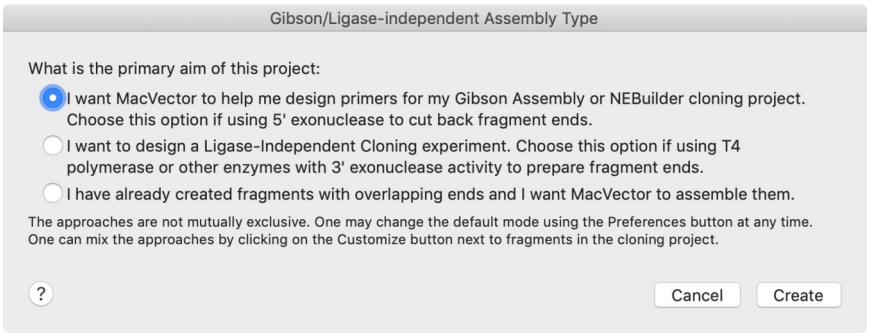
A Simple Gibson Assembly Project

For this example, we will ask MacVector to design a pair of primers so that we can clone a fragment into a vector. While many Gibson Assembly projects might have all of the required fragments be generated by PCR, you can also often just provide a microgram or so of linearized vector as one of the fragments, and thus you just need two appropriate primers to amplify your target fragment.

Creating a Project

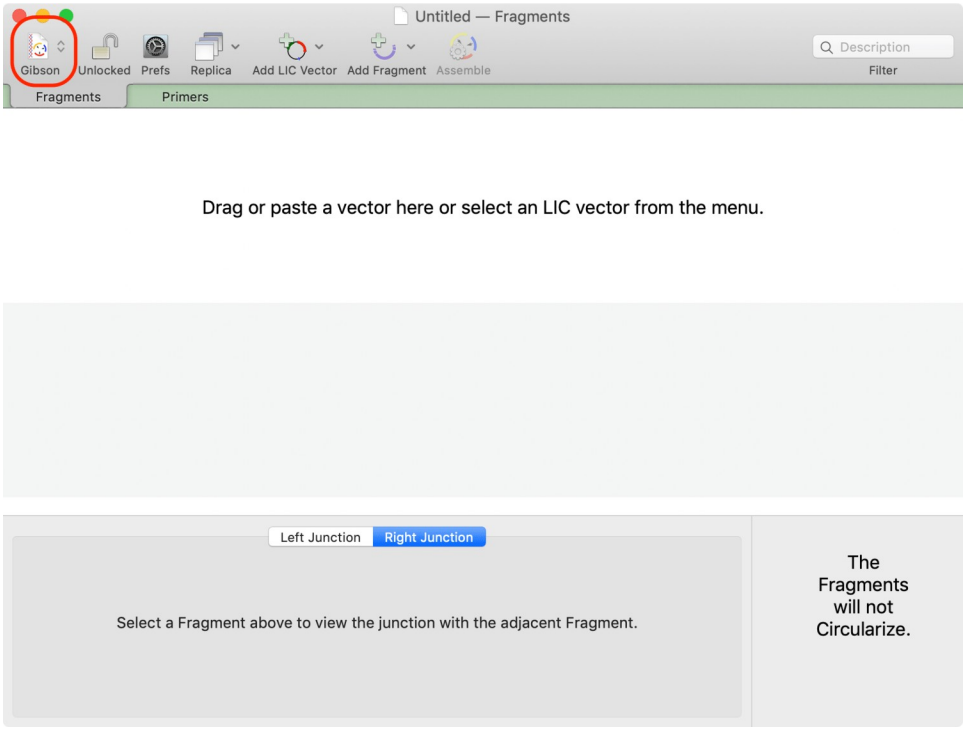
Select **File | New | Gibson/Ligase-Independent Assembly**... to create a new Gibson Assembly project.

You first need to decide what type of project you are planning on. While you can change this later, it usually easier to do this at the beginning.



Make sure you have the first "Gibson Assembly" option selected and click on the **Create** button.

A new *Gibson Assembly Project* window opens. Notice that the **Mode** button is set to **Gibson**.

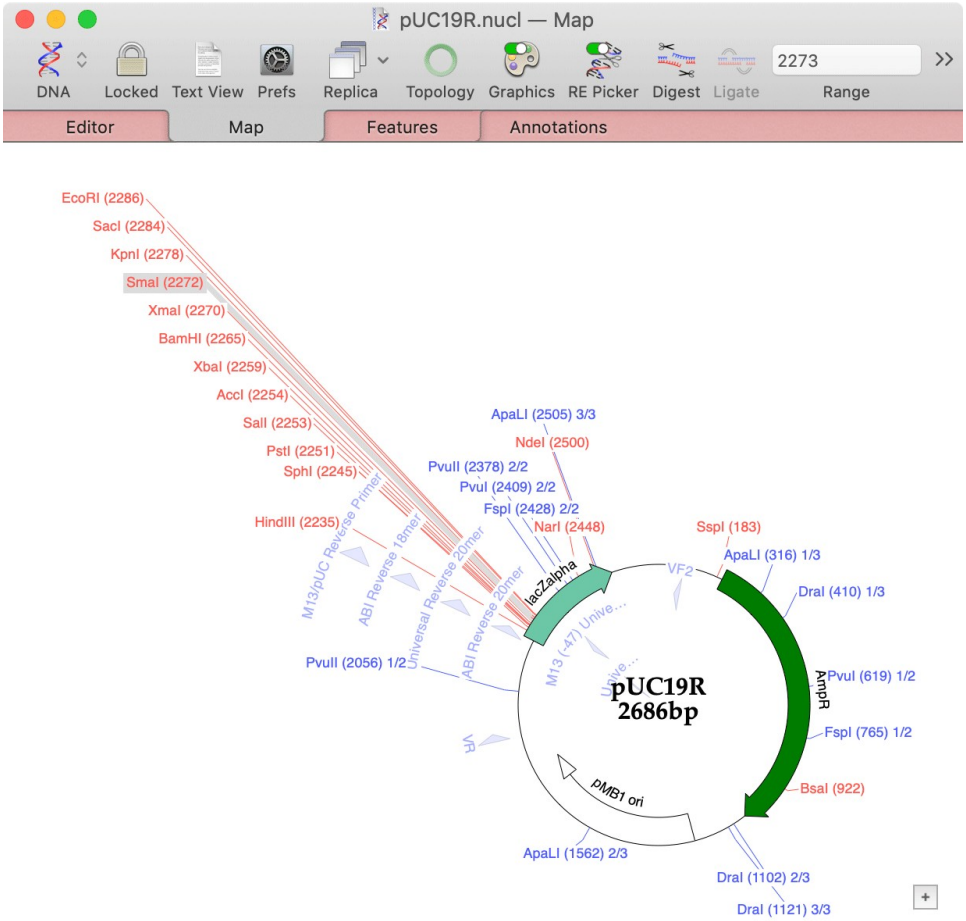


This is a primary document window, meaning it can be saved and opened later with all settings and contents preserved. It is highly interactive – there are many ways you can add fragments you would like to use in the assembly to the window.

Adding Vectors and Fragments

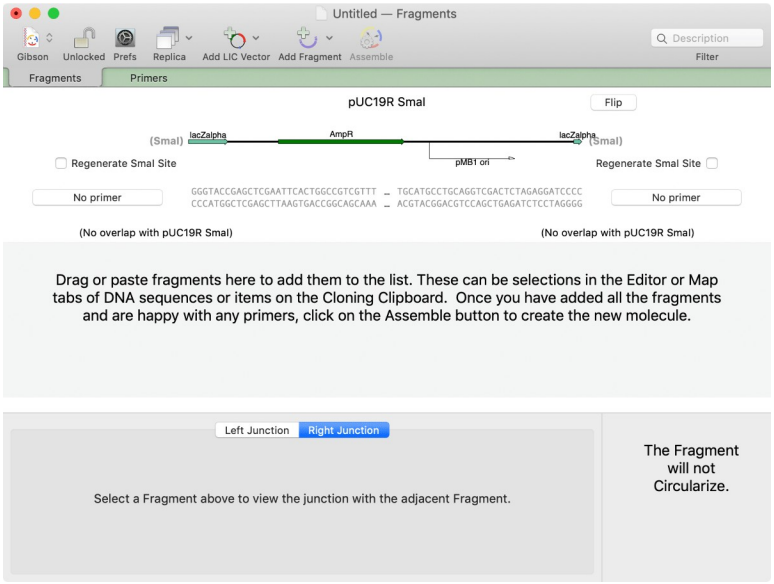
Let's do a vector first;

Open `pUC19R.nucl` (in the `/Applications/MacVector/Tutorial Files/GibsonAssembly/` folder). Switch to the **Map** tab. Select the *Sma I* site.



Click again on the *SmaI* site and carefully drag the selected site over the ***Gibson Assembly Project*** window and release the mouse.

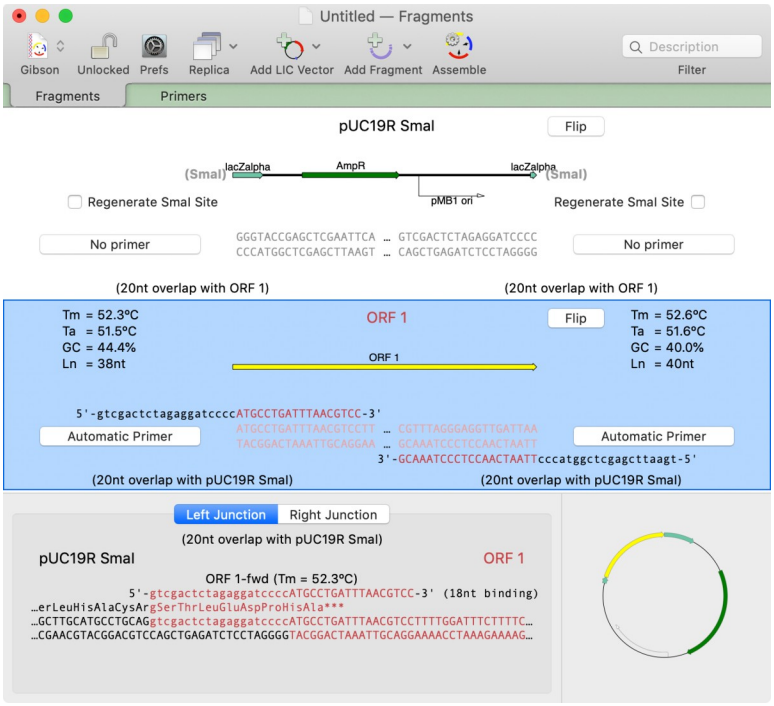
A linearized copy of pUC19, split at the *SmaI* site, appears in the project;



The core backbone of the vector with replication origin and selectable marker is always assumed to be the first fragment in the list. When you add a vector like this, MacVector assumes you will be providing the fragment as a cut vector, rather than as the result of a PCR amplification. Accordingly, each end is set to *No Primer*, indicating that the fragment will be accepted "as is". That means that if you add additional fragments to the project, MacVector will generate primers with extra-long tails to make sure they overlap the ends of the vector with enough residues to drive the assembly. Let's do that now;

Open `SequenceSample.nucl` (in the `/Applications/MacVector/Tutorial Files/GibsonAssembly/` folder). Switch to the **Map** tab. Select the yellow ORF 1 graphic, hold down the mouse button and carefully drag the item onto the second pane in the Gibson Assembly window.

The display updates with the ORF 1 open reading frame in the second pane. However, MacVector has also automatically calculated suitable primers that could be used to amplify the ORF 1 open reading frame and provide an overlap with the *Sma*I digested pUC19R vector backbone.

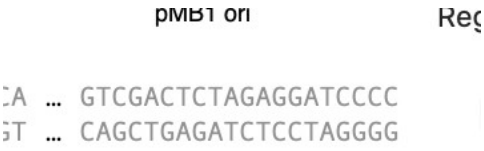


Tails on Primers

MacVector adds overhanging tails to the primers to generate suitable repeats between the ends of adjacent fragments in the final construct. Because in this case the vector backbone will be used "as is", and no primers will be used to amplify it, the primers for the insert fragment need much longer tails (in this case, 20nt) in order to provide sufficient repeats

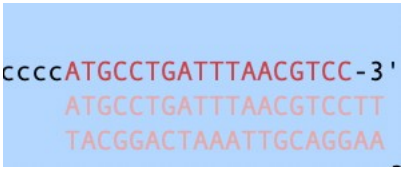
to enable recombination. However, we can see that the “tail” for the forward primer;

…exactly matches the sequence for the 3’ end of the vector;

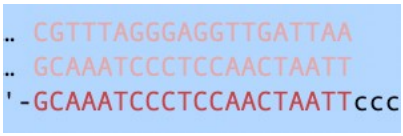


Balanced Primer Binding Tms

MacVector also tries to ensure that the forward and reverse primers have a closely matched Tm to help ensure efficient PCR amplification. In this case, the forward primer has 18nt that bind to the 5’ end of the insert fragment giving it a predicted Tm of 52.3 ºC;



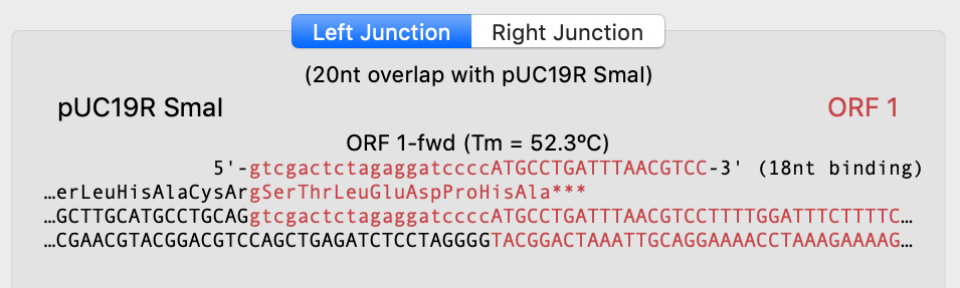
…whereas the reverse primer has been given a longer 20nt binding region, but that gives a predicted Tm of just 52.6ºC, very close to that of the forward primer;



Junction Structure

The lower pane displays the details of the fragment junctions.

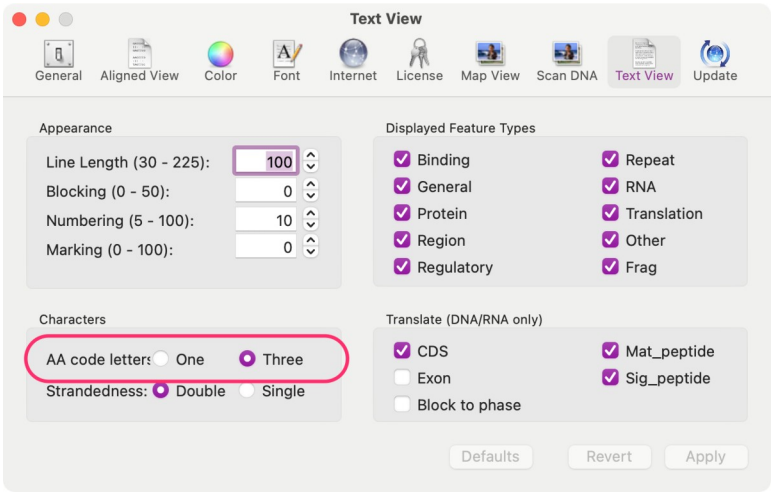
Click in the *ORF 1* panel to select it, then click on the **Left Junction** tab.



The junction shows the primer(s) used to generate the overlap (only one in this case) and color codes the residues so that you can see where the different sequences are derived from. By convention, for the duplicated sequence regions, MacVector shows the upper strand colored according to the fragment that provided the 5' sequence and the lower strand in the other color, so that the overlap can be viewed as the region with the complementary colors. The primer(s) used are shown above the sequence for the forward primer and below for the reverse primer (not used in this example). "Tails" are shown in lower case.

Finally, translations are shown immediately above the DNA sequence. It is important to understand that these key off existing CDS annotations in the fragment sequences, with preference given to CDS features coming into the junction from the 5' direction. So, in this case, the *lac*Z alpha CDS feature from pUC19 has precedent over the ORF 1 CDS. You can clearly see that the junction between lacZ alpha and the ORF 1 CDS is not in frame and the predicted translation terminates shortly after it passes the ATG start codon of ORF 1. Note how the tails are in lower case, so we can clearly see the upper case ATG at the start of the ORF 1 CDA, and there is not a methionine Met amino acid in-frame underneath that codon.

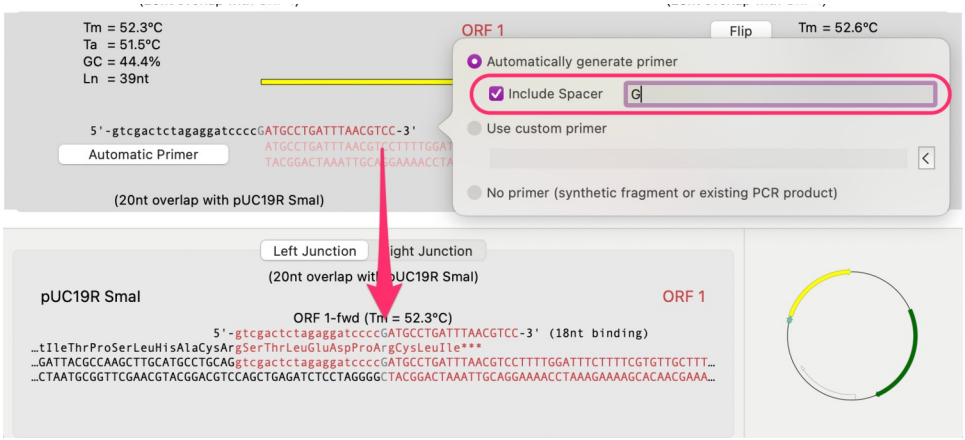
NOTE: You can control whether amino acids are shown using a single letter or three letter code using the **MacVector | Preferences | Text View** pane;



Inserting Spacer Residues

As the ORF 1 coding region is not in-frame, we need to edit the primers to add additional residues so that the ATG start codon of ORF 1 is in-frame with the lacZ-alpha coding region.

Click on the left hand **Automatic Primer** button, select the **Include Spacer** checkbox and type a “G” into the edit box.



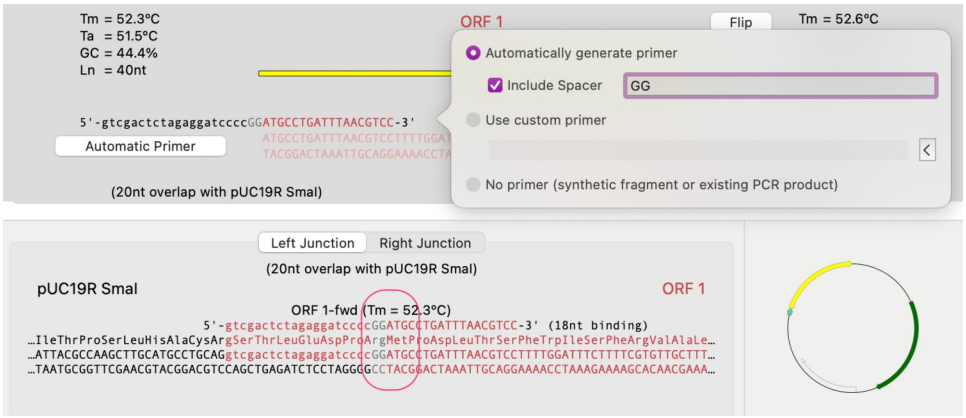
The display immediately updates to show the new G residue inserted into the primer in a gray color. The coding regions update in real-time to show the effects of the insertion.

Choose the **Edit | Undo** menu item.

As with most functions in MacVector you can undo editing changes. And the window will revert to the previous state.

Choose the **Edit | Redo** menu item. Then type another G into the **Include Spacer** edit box.

Once again, the display updates to show the effect of adding two residues into the primer.



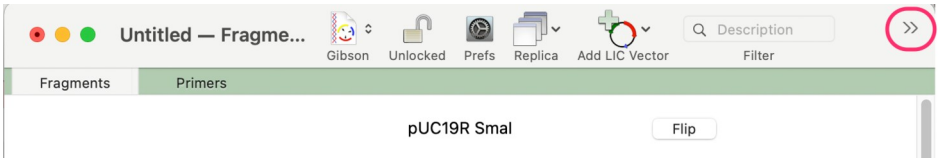
In this case we can see that adding two G residues has adjusted the reading frame so that we now have an in-frame fusion between the lacZ-alpha coding region and ORF-1, with the ATG start codon of ORF 1now being directly over a Met amino acid.

One thing to point out is that although we have extended the left hand primer by 2 residues, we have not actually changed the region that binds to the target sequence. So the right hand “Tm balanced” primer should remain the same.

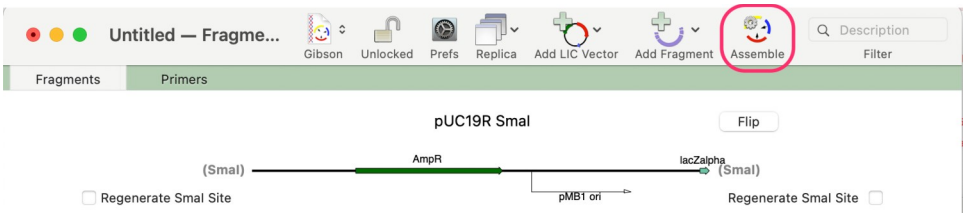
Circularizing the Construct

We could continue to add fragments to be included in the final construct, each of which would generate another pair of primers. MacVector has no limit to the number of fragments that you can add, but, practically, you probably don’t want to add more than four or five. For this simple example, we will stop with this two-fragment construct.

If the fragment can be circularized, the **Assemble** button will be active. It is possible that the window may be too small to display the **Assemble** button, in which case you may see something like this;

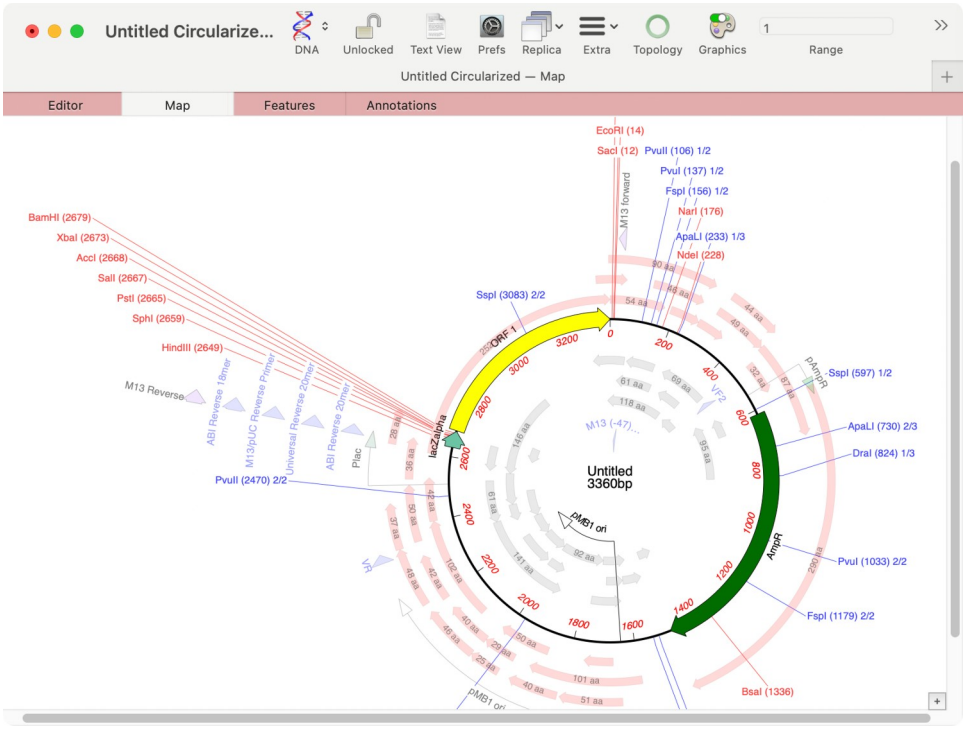


The double arrows indicate that additional menu buttons are available but cannot be shown. You can click on those and see the additional buttons displayed in a pop-up menu, or you can resize the window so that all of the buttons are shown;



Click on the **Assemble** toolbar button.

A new window will appear, containing the circularized construct;

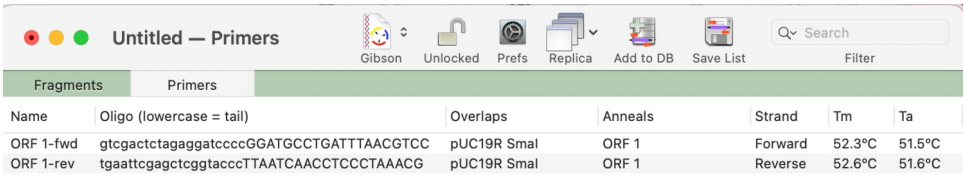


Viewing and Saving/Exporting the Primers

The sequences of the primers can be viewed in the **Primers** tab.

Click on the **Primers** tab.

The primers tab displays information about the primers, organized as a spreadsheet;



Name	Oligo (lowercase = tail)	Overlaps	Anneals	Strand	Tm	Ta
ORF 1-fwd	gtcgactctagaggatccccGGATGCCTGATTTAACGTCC	pUC19R SmaI	ORF 1	Forward	52.3°C	51.5°C
ORF 1-rev	tgaattcgagctcggtacccTTAATCAACCTCCCTAAACG	pUC19R SmaI	ORF 1	Reverse	52.6°C	51.6°C

Note how the tails are shown in lower case to distinguish them from the "core" of the primer that will actually bind to the target molecule. In addition, the predicted melting temperature (Tm) is shown for each primer as well as the recommended annealing temperature (Ta) that should be used in the PCR experiment.

If you want a permanent record of the primers, there are several options;

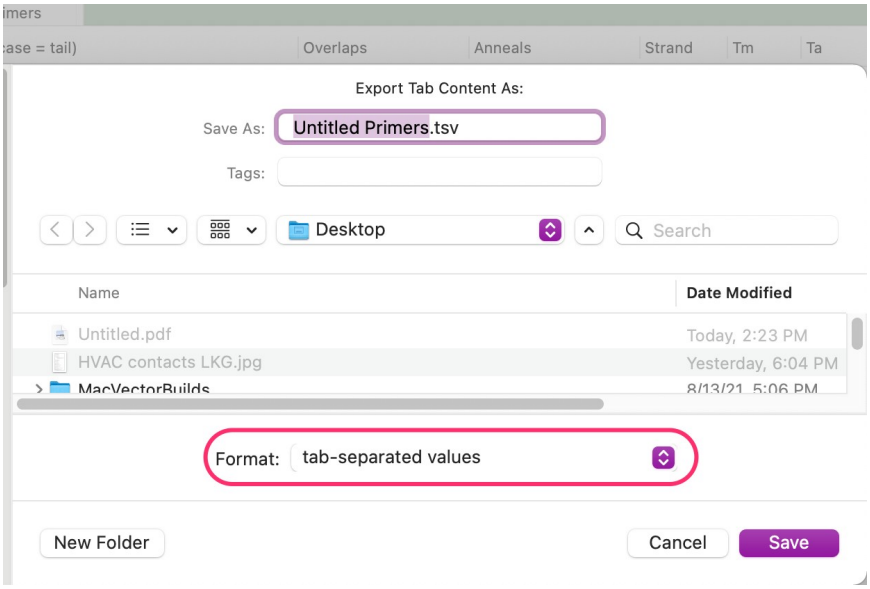
Add to DB

This button adds the primers to the current MacVector primer database. By default, this is the `PrimerDatabase.nsub` file located in the

`/Applications/MacVector/Subsequences/` folder, but you can set any `.nsub` file as the default using the **MacVector | Preferences | Scan DNA | Primers** pane.

Save List

This toolbar lets you save the primers as a tab separated or comma separated text file, suitable for printing or for import into *Microsoft Excel* or other spreadsheet application;



Copy/Paste

You can select the rows you are interested in, choose **Edit | Copy**, switch to *Microsoft Excel* and paste the copied data into a spreadsheet.