

MacVector 17.5

for Mac OS X

Clone Construction Tutorial

MacVector, Inc.
Software for Scientists

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Overview

The tutorial will show you how to use MacVector to create new plasmids by replicating cloning experiments that you might perform in a practical laboratory. This tutorial focuses on the common restriction enzyme based approach, but there are sections discussing Gateway and Gibson cloning techniques too. There are two main approaches that you can use in MacVector to create constructs based on restriction enzyme cut sites;

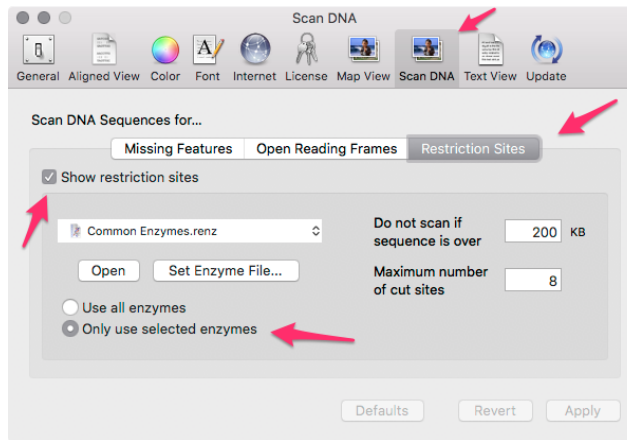
- (a) **Copy and Paste** – this is directly analogous to copying and pasting sentences or paragraphs between Microsoft Word documents. You simply select the source fragment, copy, select an insertion point or section to replace, then choose paste. As MacVector is sticky-end aware, it will never let you create biologically invalid constructs.
- (b) **Cloning Clipboard** – the second approach is that you use the “Digest” function to place selected fragments on the Cloning Clipboard. Once on the clipboard, you can join fragments together by dragging ends onto each other.

Configuring “Auto” Restriction Enzymes

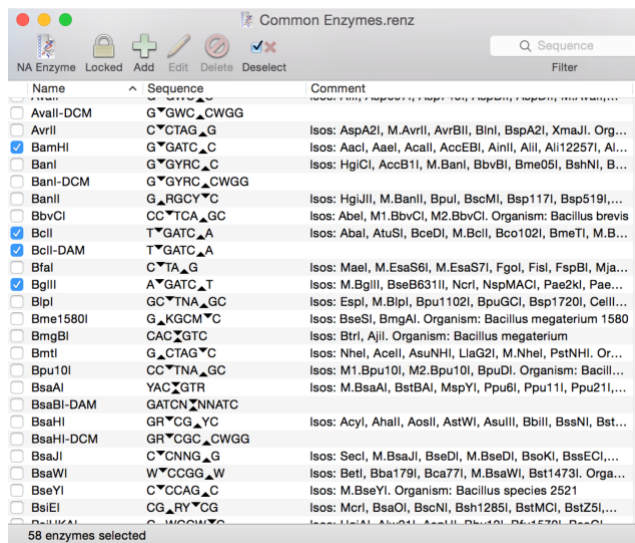
Whenever you open a DNA sequence, MacVector automatically runs a restriction enzyme search on the sequence and displays the results graphically in the **Map** tab. This dynamic or “auto” restriction enzyme search is primarily designed to show you the locations of common enzymes that cut your DNA. There is a more comprehensive restriction enzyme searching function available in the **Analyze** menu that can be used for in-depth analysis of the restriction enzyme sites in DNA. However, the “Auto” restriction enzyme analysis is often more than adequate for most basic clone construction DNA manipulations.

Open the files Gal cosmid and pBR322. You will find these in the /MacVector/Sample Files/ folder. Make sure you have the Gal Cosmid window front-most, and then click on the **Map** tab if it is not already active.

While the default settings are usually sufficient for this tutorial, to ensure the automatic restriction enzyme searching parameters are set correctly, let’s make sure our preferences are set appropriately.



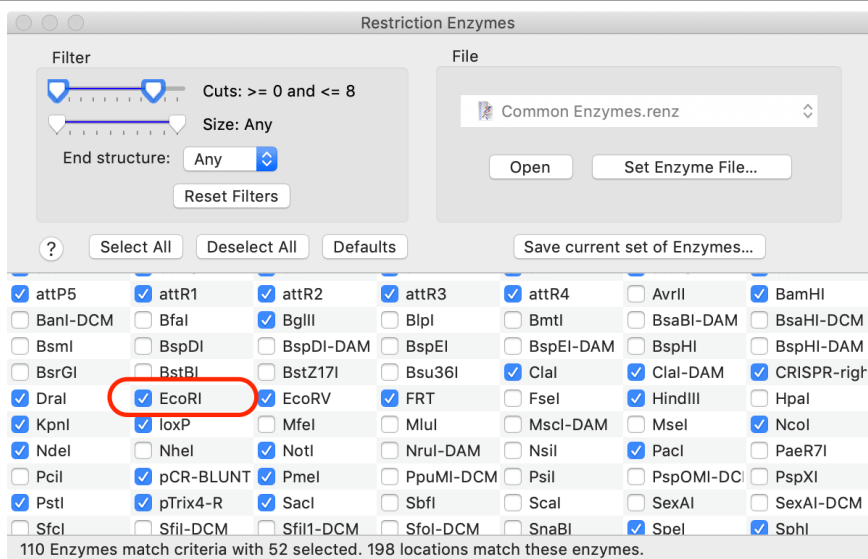
The `Common Enzymes` file opens in a **Restriction Enzyme Editor** window. In this window, you can click on any enzyme name and a check mark appears next to the enzyme indicating it is selected. For example, if you click on the *Bam*HI entry, you will see the checkmark toggle on and off. At the same time, if you look at the pUC19 Map view, you will see the *Bam*HI site turning on and off as you select/deselect the enzyme.



Make sure the enzymes *Bam*HI, *Eco*RI, *Not*I, *Sma*I and *Sal*I are selected (you can leave others selected as well). You can permanently save your favorite selections in the file by selecting **Save** in the **File** menu.

Using the Restriction Enzyme Picker

A new feature added in MacVector 17 is a floating *Restriction Enzyme Picker*. When a sequence is opened, this is populated with the restriction enzymes as defined by the default **Scan DNA** settings.



The interface is dynamic, so if you click on the highlighted *EcoRI* site above, the checkbox will deselect and any *EcoRI* sites in the currently active sequence document will become hidden.

One of the key concepts of the **RE Picker** is that when a sequence is initially opened, ALL of the enzymes in the default file are scanned against the target sequence, not just those that were selected in the original .renz file. So, you can drag the sliders in the **Filter** area and all of the restriction enzymes that are displayed in the **RE Picker** are those that have the selected cuts/size/end structure in the molecule. However, only those that have a checkbox next to them are displayed in the **Map** view.

You can continue to play with the filter controls and selecting/deselecting enzymes to suit your needs. However, there are often times when you just want to get back to the original default set of enzymes.

Click on the **Defaults** button in the **RE Picker** window.

This reverts everything back to the initial state, the same as it would be if you closed the document window and re-opened it.

It should be noted that at any time you can click on the **Save current set of Enzymes** button in the **RE Picker** and the currently displayed enzymes are saved to a new file, complete with their selection status.

Clone Construction Using Copy/Paste

This section will show you how to create constructs using a simple “**Select, Copy, Paste**” approach. The basic concept is similar to how you might approach moving sentences between Microsoft Word documents: First select the source sentence, choose **Copy**, then move to the target document, select the insertion position, or sentence to be replaced, then

choose Paste. MacVector is “sticky-end” aware and will never allow you to create invalid constructs. If you try to paste a fragment into an incompatible site, a dialog will be presented warning about the incompatibility, but also allowing you to modify the ends if appropriate.

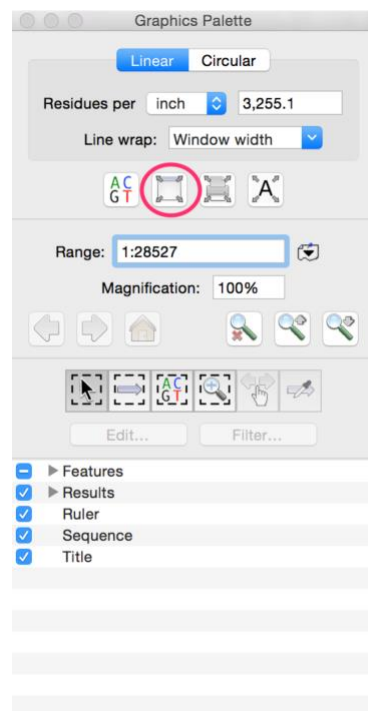
A Simple Single Enzyme Cloning

Lets copy an *EcoRI* fragment from Gal Cosmid and insert it into the *EcoRI* site of pBR322.

Make sure the Gal Cosmid window is frontmost and the **Map** tab is active.

You will see a graphical map of the cosmid with the locations of the cut sites displayed. There should also be a floating palette window with the title **Gal cosmid** available. If you don't see this, look in the **Windows** menu and select **Show Graphics Palette** or click on the **Graphics** toolbar button.

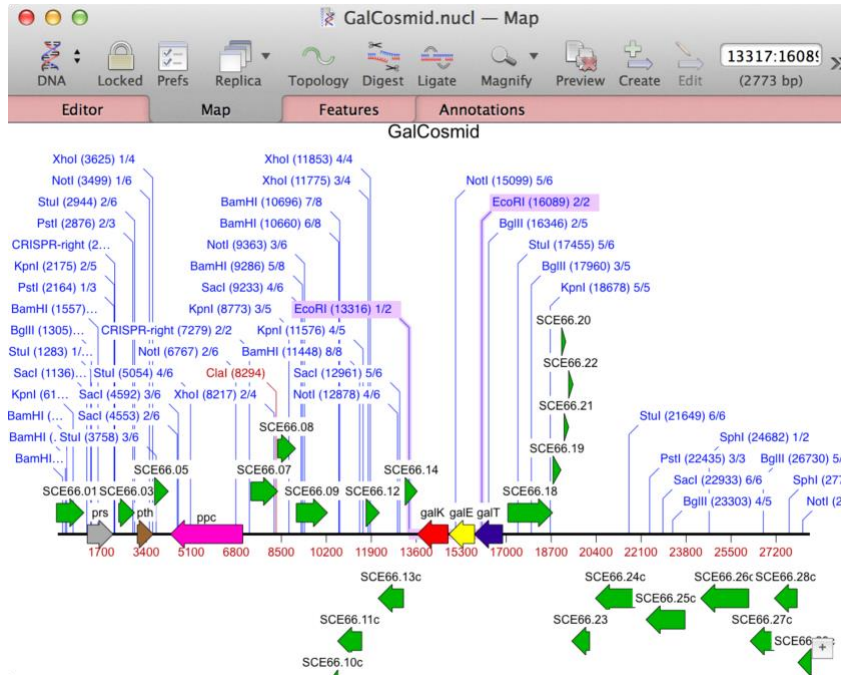
Click on the **Fit to Window** button in the floating palette window.



The display should resize so that you can see the entire cosmid on a single line. If you look closely, you will see that there is a red colored *galK* gene in the middle of the sequence. In addition, there are two *EcoRI* sites in the cosmid and they conveniently lie on either side of the *galK* gene.

Click on the first *Eco*RI site at 13,316. Hold down the <shift> key and click on the second *Eco*RI site at 16,089.

The two sites highlight, along with the section of sequence between them.



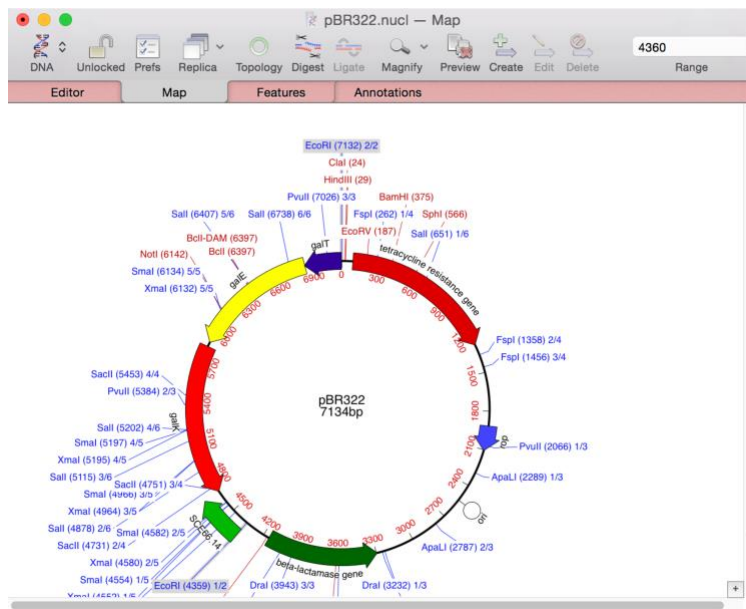
Choose **Edit | Copy**

This copies the selected sequence to the clipboard. It's important to note that the features on that fragment, and information about the structure of the ends of the fragment, are also copied. This is the actual sequence of any overhanging ends, not just the name of the enzyme or the consensus sequence of its recognition site.

Switch to the pBR322 window. Click on the *Eco*RI site at 12 o'clock to select it. then choose **Edit | Paste**.

You should be prompted to unlock the pBR322 sequence. This is to prevent you from accidentally modifying an important sequence file.

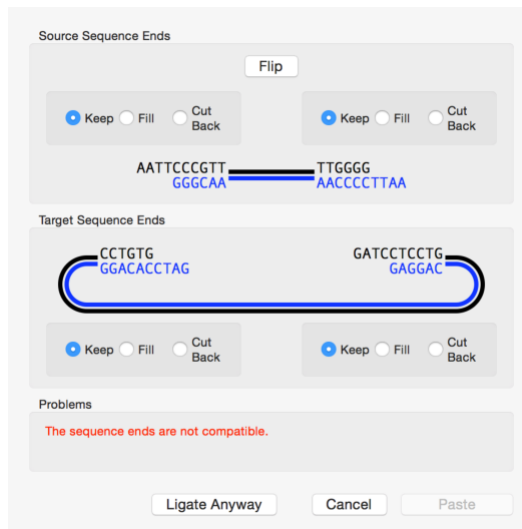
The display updates to show a new molecule with the *Eco*RI fragment from Gal Cosmid inserted into the *Eco*RI site of pBR322. If you look carefully, you can see the two *Eco*RI sites flanking the inserted fragment. Note how all of the features have been included on the copied fragment.



Lets see what happens if we try to insert the copied fragment into an incompatible site.

Choose **Edit | Undo**. MacVector has essentially unlimited levels of undo, so you can undo almost any action you make. Now select the *Bam*HI site at 375 and choose **Edit | Paste**.

A ligation dialog will appear warning you that the fragment cannot be inserted because the sequence ends are not compatible.



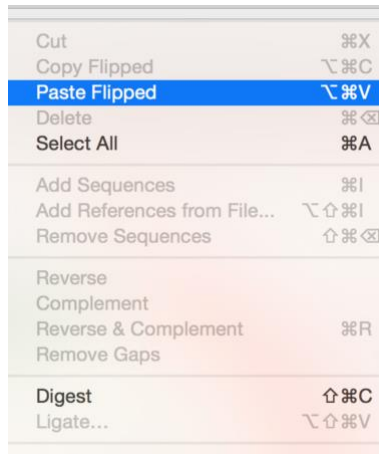
The dialog lets you fill or cut back the ends if you wish so that you can force a blunt-ended cloning. But you can be confident that MacVector will never let you accidentally create an invalid construct using this Copy-Paste approach.

Flipping The Inserted Fragment

Of course, there may be times when you want the fragment inserted in the opposite orientation. MacVector has a simple trick for this;

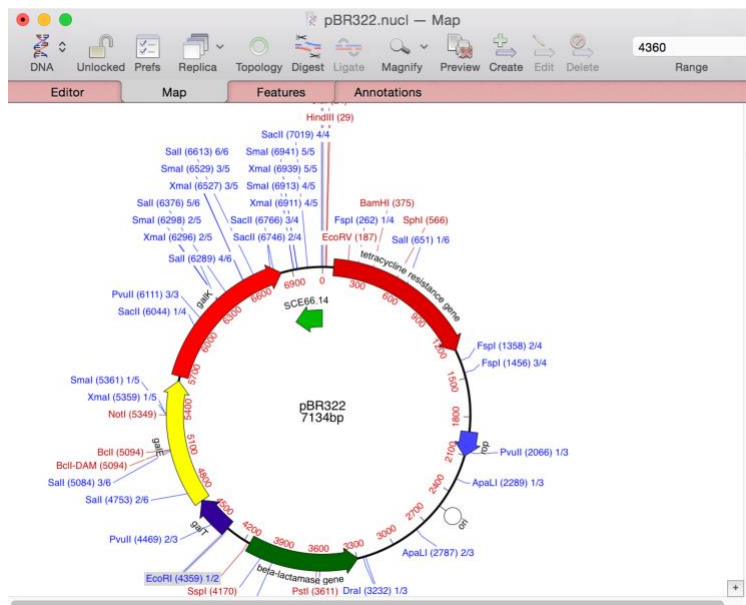
Click on the **Cancel** button to dismiss the ligation dialog. Click back on the *EcoRI* site, hold down the `<option>` key and click on the **Edit** menu.

Note how that while you hold down the `<option>` key, the **Paste** menu item changes to **Paste Flipped**. If you release the `<option>` key the menu title reverts to **Paste**.



While holding down the `<option>` key, choose the **Edit | Paste Flipped** menu item

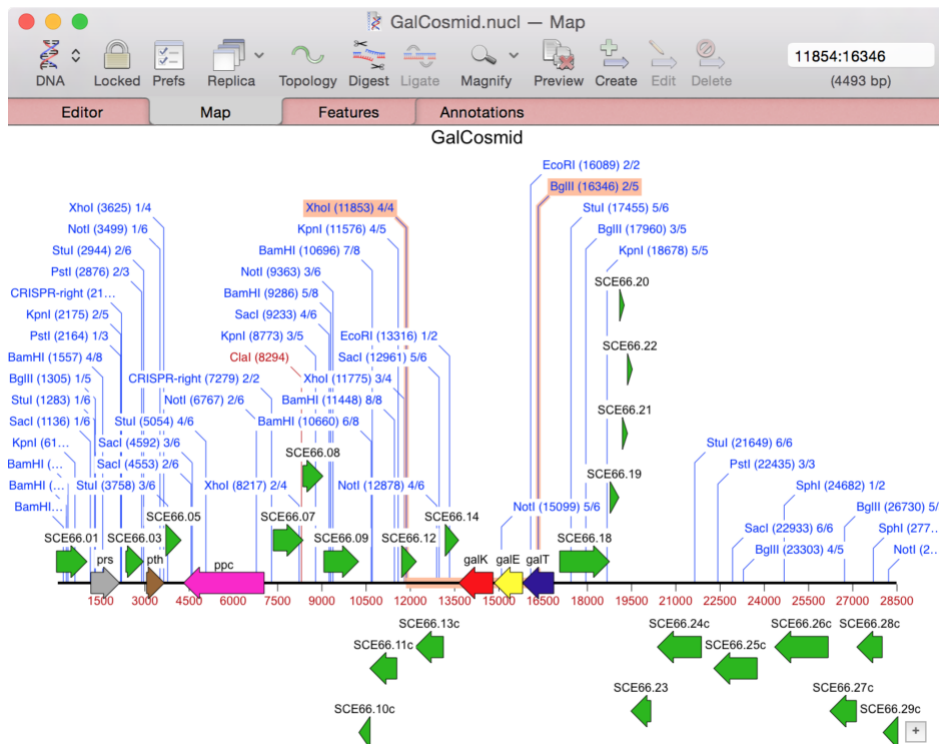
The display updates to show the fragment inserted in the opposite orientation. Compare the image below with the equivalent image above.



Two Enzyme Directed Cloning

Quite often, you will find it convenient to perform cloning experiments using two different enzymes. In these cases, the fragment can typically only be inserted in one orientation, but MacVector understands this and will automatically “flip” the fragment if required to maintain compatibility between ends.

Choose **Edit | Undo** to restore pBR322 then switch back to the GalCosmid window. This time, click on the *XhoI* site at 11,853, hold down the <shift> key and click on the *BglIII* site at 16,346.



Choose **Edit | Copy** to copy the fragment to the clipboard. Then switch to pBR322, click on the *BamHI* site at 375, hold down the <shift> key and click on the *SaI* site at 651.

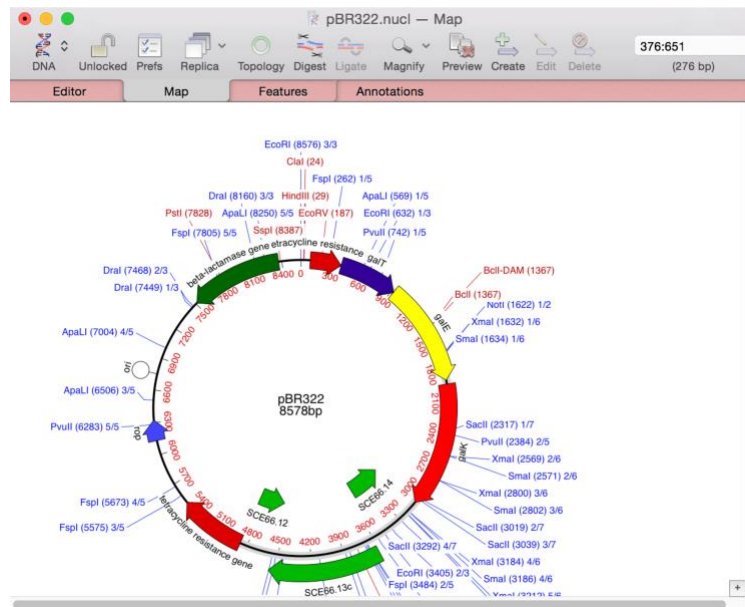
There are a couple of things to note here;

- (i) The sticky end produced by *BamHI* cleavage (5' GATC – 3') is identical to that generated by *BglIII* cleavage. Because MacVector looks at the actual structure of the overhang, not the enzymes that created the overhang, it will recognize that the two ends are compatible. Similarly, the ends produced by *SaI* and *XhoI* are compatible.

- (ii) The order that you click the two sites is very important. MacVector always selects moving clockwise around the sequence. In this case we want to select the segment of the vector that we want to replace, so we must select *Bam*HI first, followed by *Sal*I. If we select *Sal*I first, we would be selecting around the main core of the vector containing the replication origin, so the resulting construct would not be what we require.

Choose **Edit | Paste**.

The display updates, inserting the *Xho*I to *Bgl*II fragment between the *Sal*I and *Bam*HI sites of pBR322.



Note that MacVector has automatically flipped the fragment as it can only be inserted in one orientation. Also, no sites are regenerated at the *Bam*HI/*Bgl*II or *Sal*I/*Xho*I junctions.

You can use this simple **Copy-Paste** approach for most of your restriction enzyme based clonings, safe in the knowledge that MacVector will not let you create invalid constructs. However, MacVector has a second set of functions for clone construction that are even more flexible and are discussed in the next section.

Choose **Edit | Undo** to restore pBR322 back to its original state.

Clone Construction Using the Cloning Clipboard

The **Cloning Clipboard** is a unique MacVector window that maintains a history of your clone manipulations. You can view the window by

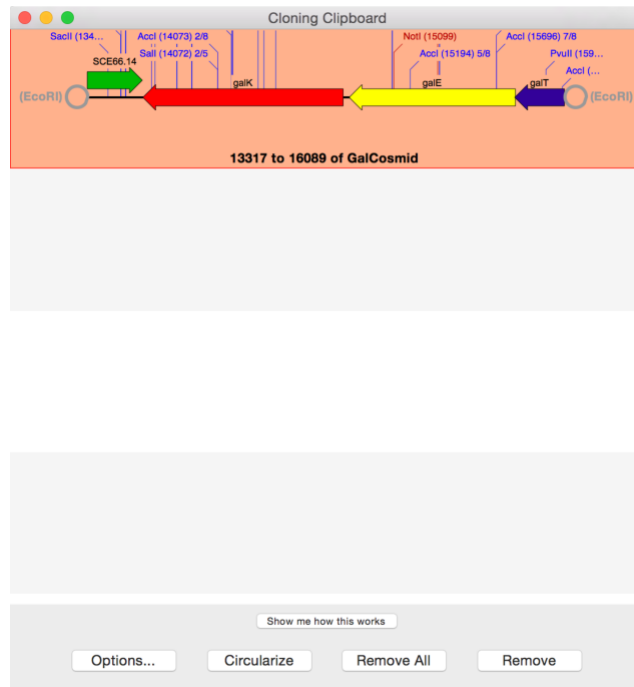
choosing **Window | Show Cloning Clipboard**. This works in conjunction with the **Digest** and **Ligate** toolbar buttons and menu items to simplify many aspects of clone construction, particularly for multi-fragment clonings.

A Simple Single Enzyme Cloning

Lets replicate our *EcoRI* fragment cloning, but now using the **Cloning Clipboard**.

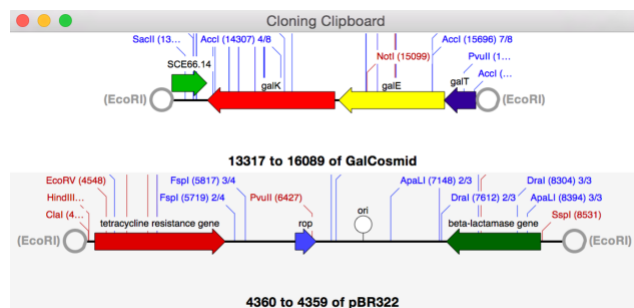
Select the two *EcoRI* sites in Gal Cosmid and then click on the **Digest** button on the toolbar. You can also use the **Edit | Digest** menu item.

The **Cloning Clipboard** window opens with a schematic of the digested fragment. Note the ends of the fragments have a label with the enzyme used to cut the fragment.



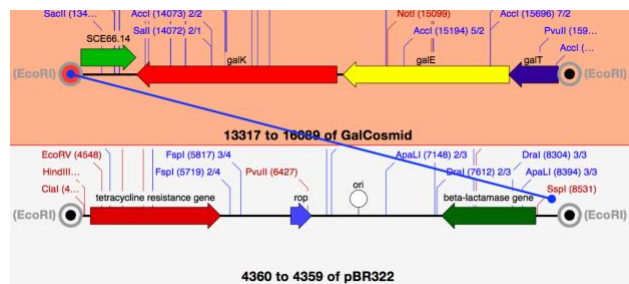
Switch to pBR322, select the *EcoRI* site and then click on the **Digest** button.

A second fragment appears on the **Cloning Clipboard**.

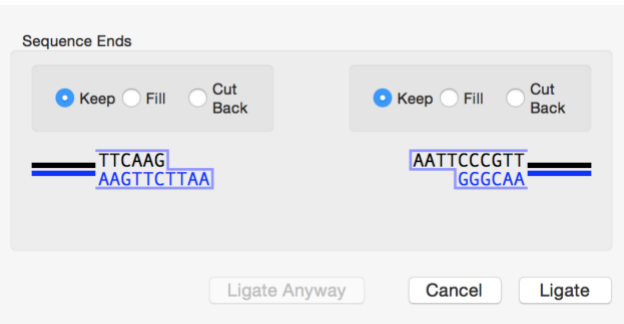


Every time you click on **Digest**, the resulting fragment is saved on the **Cloning Clipboard**. The fragments are remembered permanently – if you shut down MacVector and re-open it, you will find the same fragments remain on the clipboard. You can of course clear the clipboard by clicking on the **Remove All** button, or by selecting individual fragments and clicking on **Remove**.

Click in the circle at the left hand end of the upper Gal Cosmid fragment. The circle highlights and “target” dots appear within the circles at the ends of the other fragment. Hold down the mouse button and drag towards the right hand end of the pBR322 fragment. Let go when the circle there becomes highlighted.

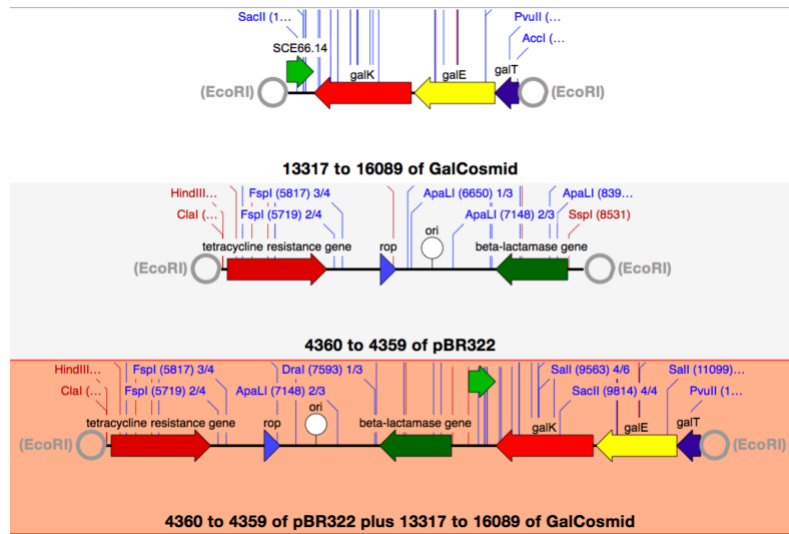


A ligation dialog sheet appears. The staggered “sticky ends” are clearly shown. In addition, they are outlined in blue, indicating that the ends are compatible. This is very similar to the ligation dialog we saw earlier with the copy/paste cloning, except that in this case only two ends are involved.



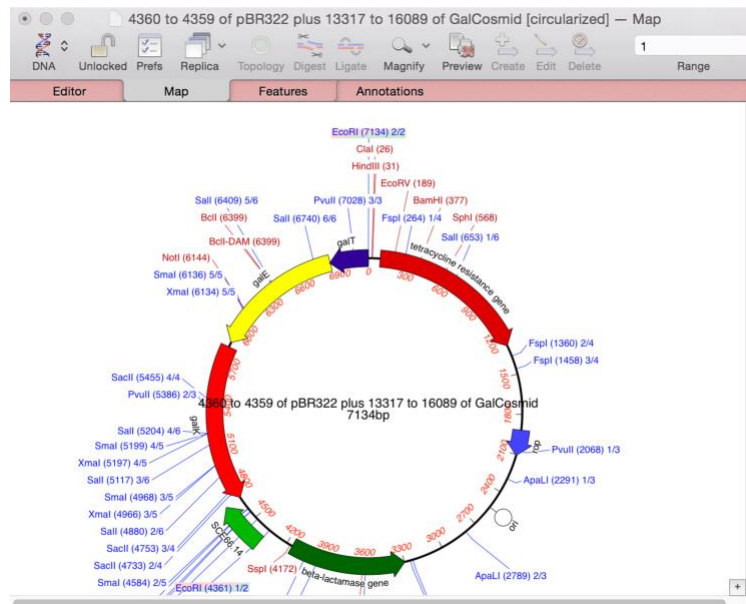
Click on the **Ligate** button.

A new fragment appears in the window, with the two molecules joined together.



Either (a) click on the **Circularize** button or (b) click in the circle at one end of the new fragment and drag the line onto the other end.

A new window appears with the selected fragment circularized.

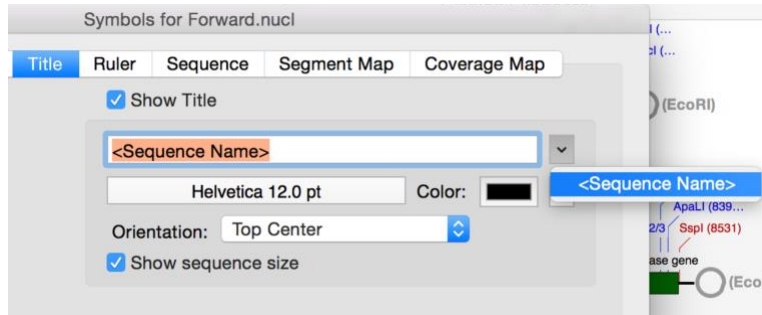


The default title is a description of the way that the molecule was constructed. Lets clean it up a little.

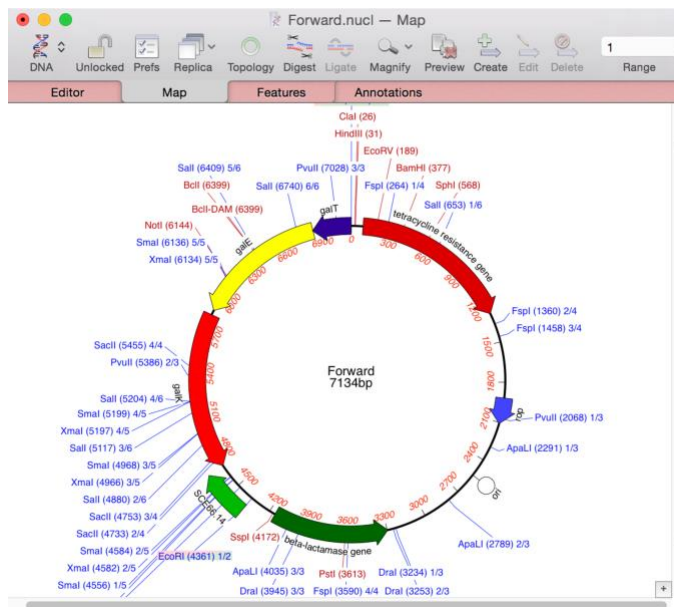
Choose **File | Save** and save the sequence to a suitable location (e.g. your desktop) under the name "Forward".

Depending on the version of MacVector you are using, the center label may not change to reflect the new name of the sequence. If that happens;

Double-click on the label in the center of the circle. This will open up the **Symbol Editor** at the **Title** tab. Click on the down arrow at the right end of the **Title** edit box and select the **<Sequence Name>** item.



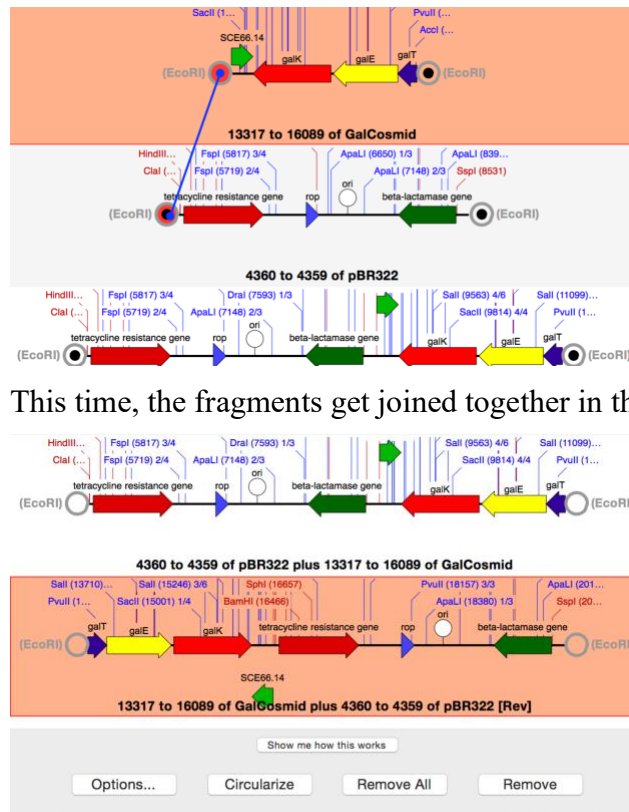
You should end up with a nicely formatted construct;



Flipping The Inserted Fragment

Now let's make a construct with the fragment inserted into pBR322 in the opposite orientation.

Click in the circle at the left hand end of the upper Gal Cosmid fragment. Now hold down the mouse button and drag towards the LEFT hand end of the pBR322 fragment. Let go when the circle there becomes highlighted.



This time, the fragments get joined together in the opposite orientation.

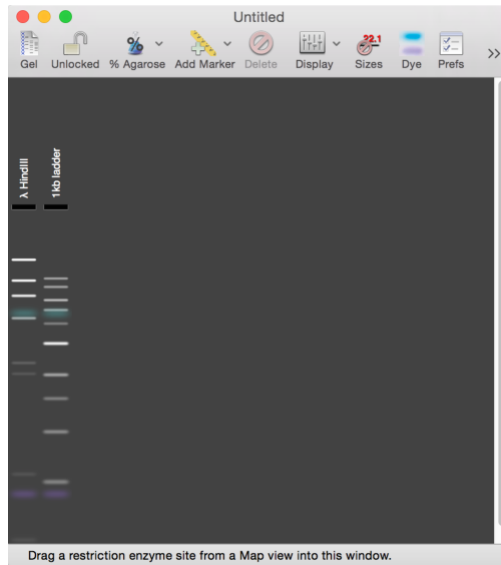
Repeat the circularization of the fragment and save the new circular molecule under the name “Reverse”.

Using the Agarose Gel Simulation Interface To Determine Orientation

In a practical experiment, you cannot directly control the orientation that the *EcoRI* fragments gets inserted into the vector, so you have to screen the clones to determine which have inserts in the correct orientation. The typical approach to do this is to cut with appropriate restriction enzymes and run an agarose gel and analyze the resulting pattern to identify the correct clones.

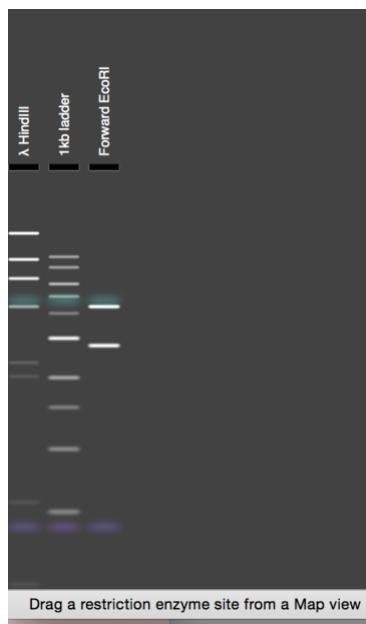
MacVector has a unique Agarose Gel Simulation interface that lets you view photo-realistic gels so you can quickly and easily determine which restriction enzymes will give you a distinctive pattern so that you can identify clones with the insert in the preferred orientation.

Choose the **File | New | Agarose Gel** menu item.

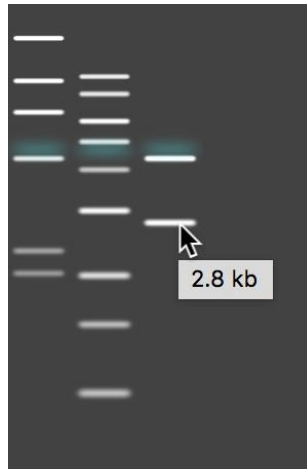


A window opens containing a graphic of a simulated agarose gel. By default, two marker tracks will be already visible. You can change the preferred marker tracks by clicking on the **Prefs** toolbar item. You can also add any other preferred markers by clicking on the **Add Marker** toolbar item and selecting a suitable marker from the popup menu.

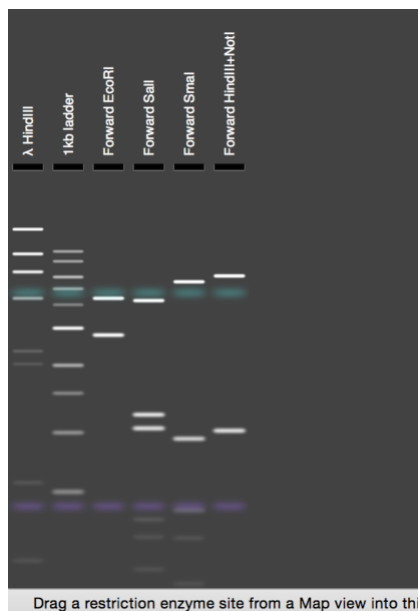
Arrange the windows so that you can see both the `Untitled` agarose gel window and the `Forward` sequence window (set to the **Map** tab) at the same time. Click and hold on one of the *EcoRI* sites in the `Forward` window, then drag the selected site onto the agarose gel window and release the mouse button.



A new track will appear on the gel, showing the predicted banding pattern resulting from digesting Forward with *EcoRI*. If you “mouse-over” the bands you will see that a tooltip appears with the size of the bands and the status bar at the bottom of the window gives more information on the fragment under the mouse.

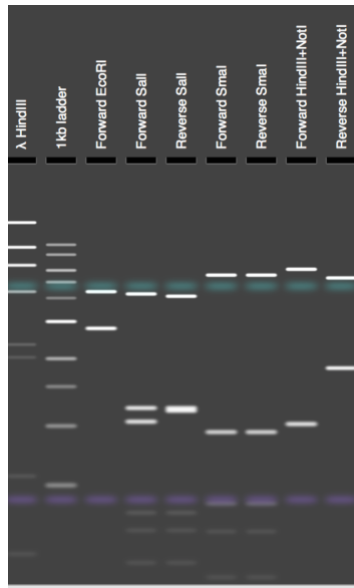


Repeat the drag and drop, but choosing first the *SalI* and then the *SmaI* sites in Forward. Finally click on the single *HindII* site, hold down the <shift> key, then click and hold on the single *NotI* site so that both sites highlight, then drag BOTH sites onto the agarose gel.



The gel updates with the predicted patterns produced by digesting **Forward** with each enzyme, or combination of enzymes. You can use the multiple-selection trick to digest a sequence with any number of restriction enzymes.

Bring **Reverse** to the front and repeat the drag and drop of *SalI*, *SmaI* and *HindIII* + *NotI* sites. If you are careful, you can drop each site slightly to the right of each of the **Forward** digests. Alternatively, you can click on a track and reposition it by simply dragging it to the left or right.



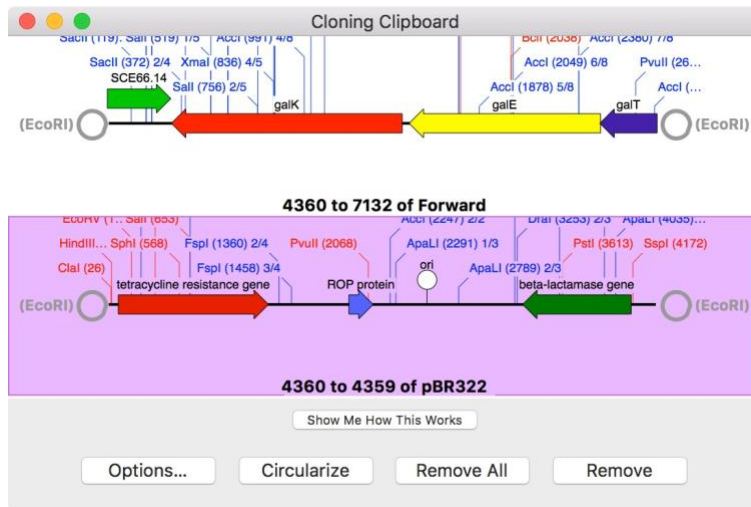
The gel updates to show the different digestion patterns. You can immediately see that the *SmaI* digests of the two constructs are identical, but the *SalI* and *HindIII*+*NotI* digests do give easily distinguishable patterns.

Removing A Restriction Site From A Vector

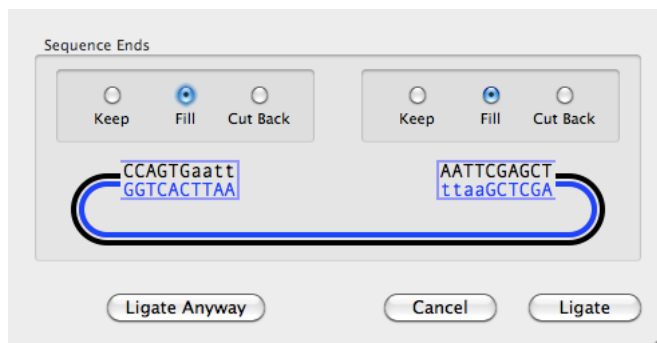
One common cloning operation that has historically been difficult to replicate in software is when you want to remove a restriction enzyme site from a vector by cleaving with an enzyme and the filling in or cutting back to remove the site and sometimes even creating a new RE site. This is easy to reproduce using the **Cloning Clipboard**;

Bring the pBR322 window to the front. Click on the *EcoRI* site at the 12 o'clock position and click on the **Digest** button

The **Cloning Clipboard** updates to show the linearized vector;

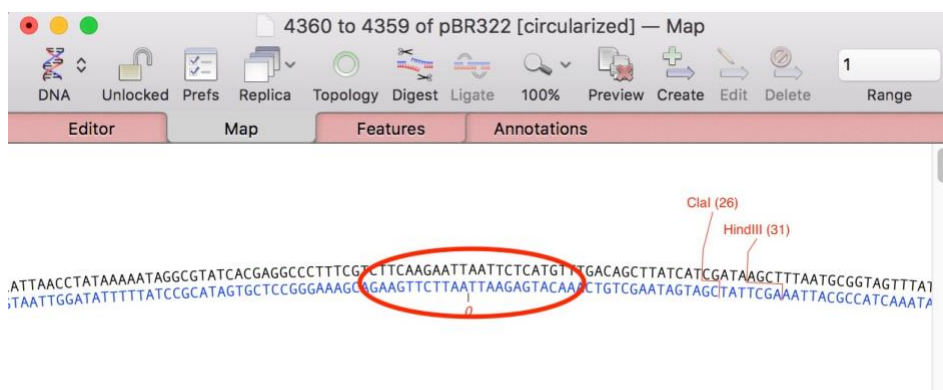


Click on the **Circularize** button then select the two **Fill** radio buttons to fill in the two ends of the fragment.



Click on the **Ligate** button.

A new window appears containing the recircularized plasmid, but now missing the *EcoRI* site, here shown zoomed in to the residue level in the **Map** tab;



Multi-Fragment Gateway Cloning

The real power of the **Cloning Clipboard** becomes most apparent when you are trying to replicate 3-fragment cloning experiments. One common example of this type of approach is the MultiSite Gateway® Technology from Invitrogen. This uses *in vitro* recombination driven by a series of related phage *att* sites to quickly generate constructs made from multiple fragments with high efficiency. For more details, check out the information on the Invitrogen web site;

<http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning/MultiSite-Gateway-Technology.html>

For this example, we will see how simple it is to generate a construct consisting of three separate fragments joined end to end and inserted into a suitable target vector. Each fragment has been inserted into an “Entry” vector – the constructs can all be found in the /Applications/MacVector/Tutorial Files/Click Cloning/ folder.

vectorA.nucl – contains **fragA** flanked by *attL4* and *attR1* sites

vectorB.nucl – contains **fragB** flanked by *attL1* and *attL2* sites

vectorC.nucl – contains **fragC** flanked by *attR2* and *attL3* sites

pDESTR4R3.nucl – this is a “Destination” vector with *attR4* and *attR3* sites.

The strategy here is that we will join the fragments together to form a construct;

...*attL4-fragA-attR1/attL1-fragB-attL2/attR2-fragC-attL3*...

and then insert that into pDESTR4R3. For the purposes of manipulation of the *att* sites in MacVector, we can treat them just like restriction enzymes with long asymmetrical overhangs. While the experimental *in vitro* operation is a recombination rather than a ligation, the end results will be identical.

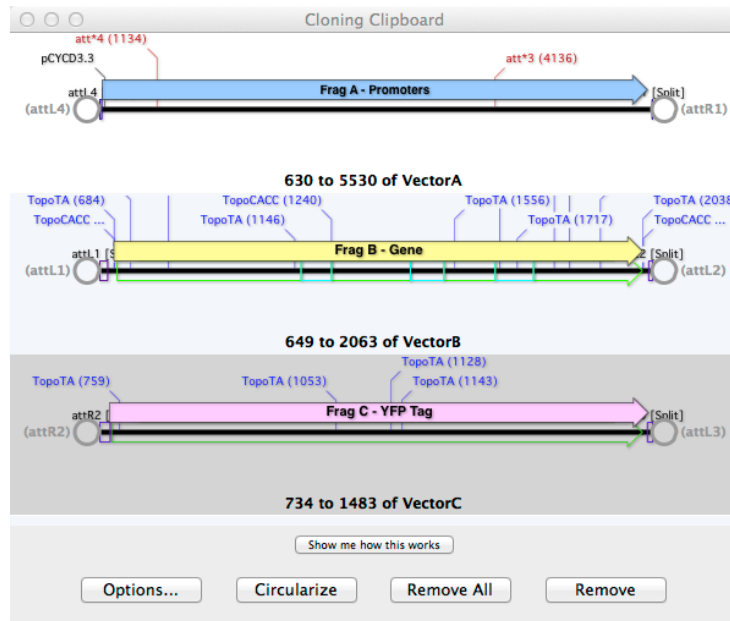
Choose **File | Open** and open each of the sequences vectorA.nucl, vectorB.nucl, vectorC.nucl and pDESTR4R3.nucl.

In the **Map** tab of vectorA.nucl, click on the *attL4* site at 629, hold down the <shift> key and click on the *attR1* site at 5530, then click on the **Digest** toolbar button.

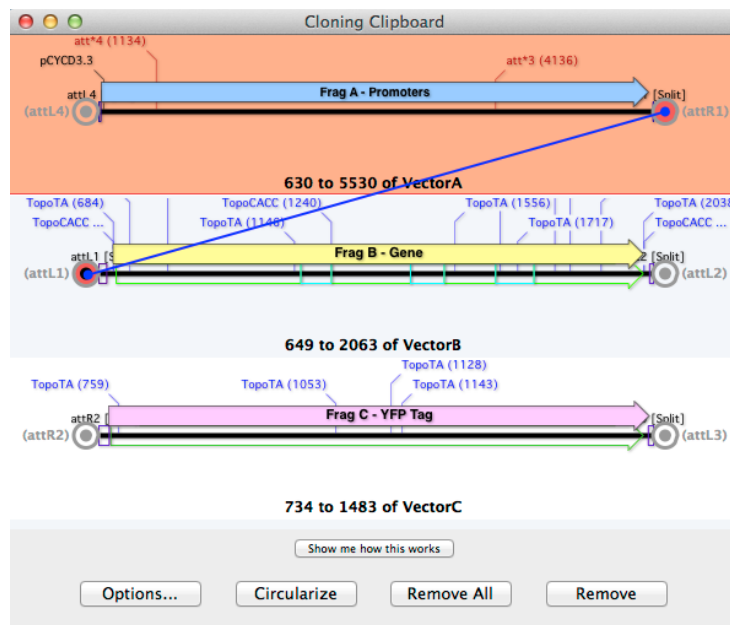
Switch to vectorB.nucl, select from the *attL1* site at 648 to the *attL2* site at 2063 and **Digest**.

Finally, switch to vectorC.nucl and digest the fragment from the *attR2* site at 733 to the *attL3* site at 1483.

The Cloning Clipboard should now look like this;

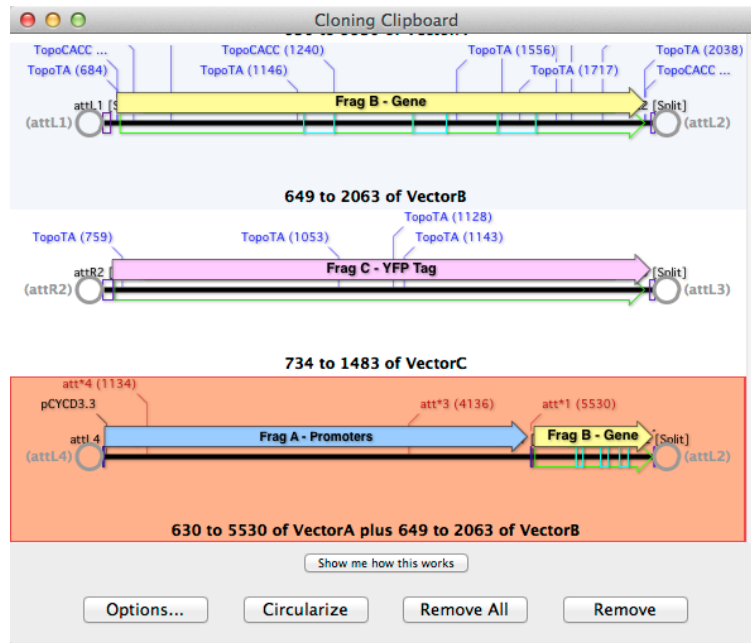


Click on the circle at the right end of **fragA** (marked as *attR1*), hold down the mouse and drag the line onto the *attL1* circle at the left end of **fragB**, then release the mouse.



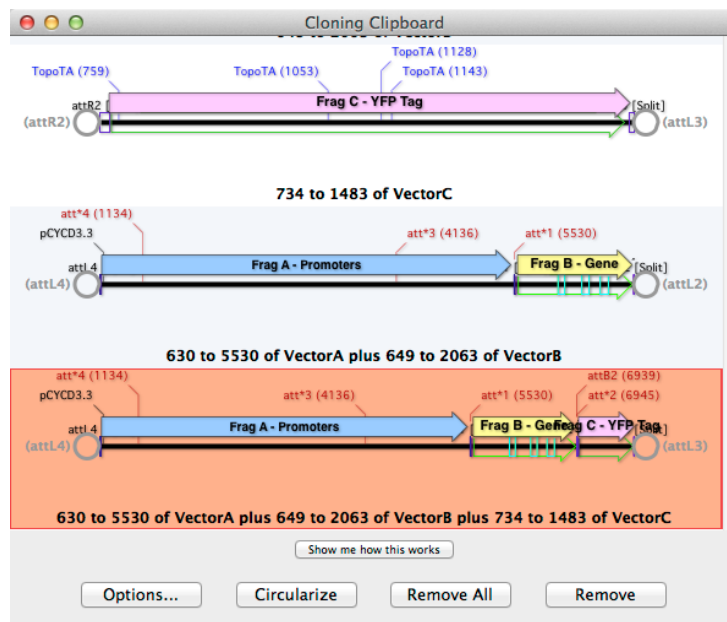
In the ligation sheet that drops down, click on the **Ligate** button.

A new ligated fragment should appear on the **Cloning Clipboard**;



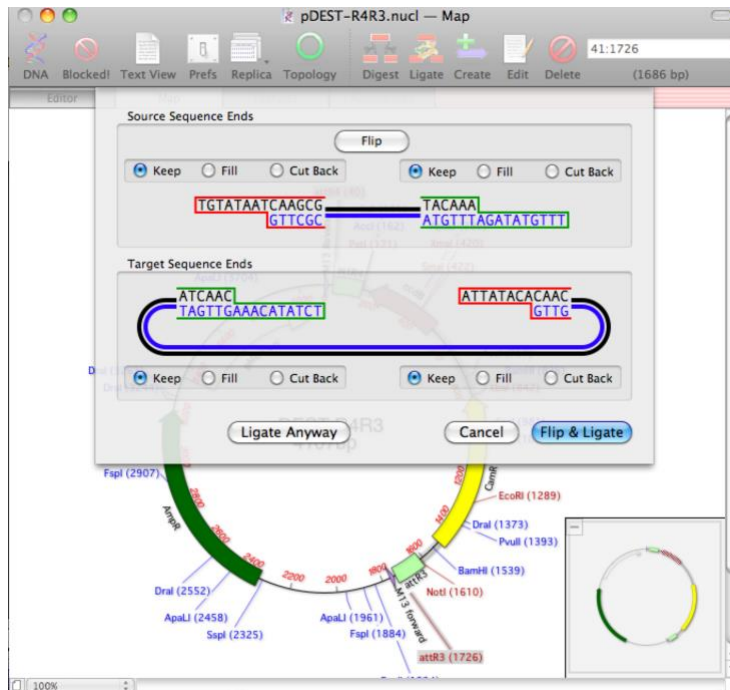
Now drag the *attR2* end of the **fragC** fragment to the *attL2* end of the new **fragA+fragB** fragment and **Ligate**.

Again, a new **fragA+fragB+fragC** fragment should appear in the **Cloning Clipboard** with an *attL3* site at one end and an *attL4* site at the other.

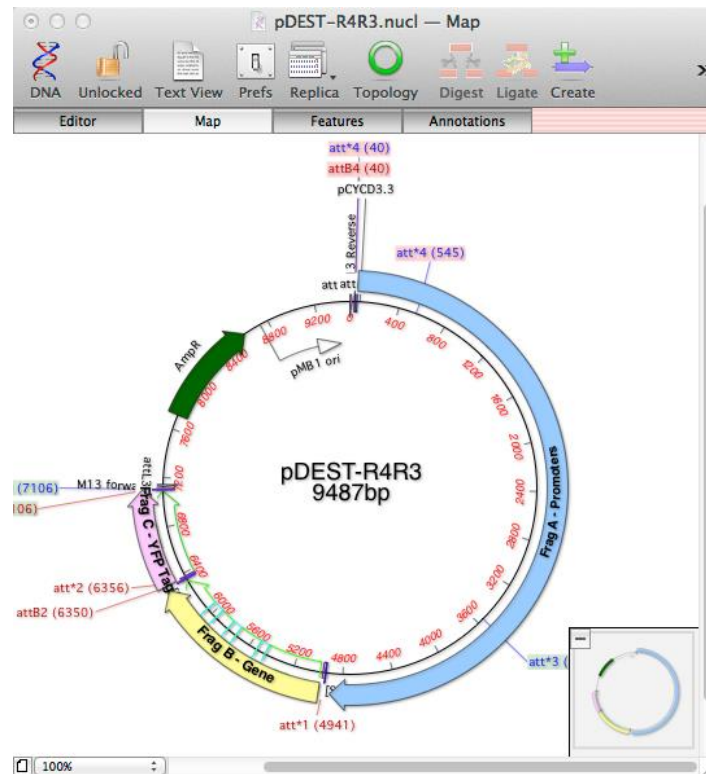


Leave the new fragment selected in the **Cloning Clipboard** and switch to the `pDEST-R4R3.nucl` window. Select the *attR4* site at 40 followed by the *attR3* site at 1726, then click on the **Ligate** button on the toolbar.

The **Ligate** toolbar button always tries to paste in the currently selected fragment on the **Cloning Clipboard**. Note that MacVector knows that the fragments needs to be flipped before it will “ligate” with the vector.



Click on the **Ligate** button.



The final construct has the three fragments inserted in the correct orientation and exactly duplicates results of an *in vitro* recombination experiment of a mix of all four starting plasmids.

These general approaches can be used to simulate any multi-fragment cloning, whether using regular restriction enzymes or Gateway *att* sites. The fragments on the **Cloning Clipboard** can be selected and deleted, or the entire clipboard cleared using the buttons at the bottom of the window. The fragments can also be re-ordered by dragging and dropping into a new location. Finally, you can **Edit | Copy** any selected fragment on the **Cloning Clipboard** to copy it to the regular shared system clipboard (also known as the “pasteboard”) if you want to paste the sequence into another application.