MacVector 17

for Mac OS X

Using MacVector to simulate the Gateway®, TOPO®-TA and Zero-Blunt® cloning technologies from Invitrogen

MacVector, Inc.

Software for Scientists

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Introduction

Invitrogen market a number of kits and cloning vectors designed to simplify the cloning and sub-cloning of DNA fragments such as those generated by PCR amplification. Amongst the most popular are the Gateway and TOPO kits. For detailed information on the concepts, availability and pricing of these kits, please visit the Invitrogen web site at www.invitrogen.com.

MacVector includes support for simulating Gateway and TOPO cloning manipulations so that you can accurately document the sequences of the constructed molecules using a simple point and click interface.

Please note that this tutorial performs all ligations on the sequence. You can also do these ligations using the Cloning Clipboard. See the Cloning Clipboard Tutorial for more information on doing it this way.

Vector Sequences

There are a wide variety of vectors available both from Invitrogen and from other sources that can be used in TOPO and Gateway cloning. The sequences and maps of the Invitrogen vectors can be downloaded from their website. MacVector includes a nicely formatted selection of these vectors installed in the `/Applications/MacVector/Common Vectors/Invitrogen/` folder.

TOPO Cloning

This technique relies on the single-stranded DNA nicking and religation properties of Topoisomerase I from Vaccinia virus. This enzyme binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5ʹ-YCCTT on one strand. The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3ʹ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5ʹ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase. TOPO Cloning exploits this reaction to efficiently clone PCR products.

Invitrogen provide linearized vectors that have Topoisomerase covalently bound to each end – when mixed with PCR fragments, the fragments are joined to the vector with high efficiency, making

it a far more effective method for cloning PCR fragments than standard blunt ended DNA ligase approaches.

There are two main types of TOPO cloning;

- TOPO-TA Cloning
- Zero Blunt TOPO Cloning

There is a third approach that uses 4 residue single-stranded overhangs at one end of the vector to allow directional cloning of fragments. You can simulate this approach using MacVector, but it is not discussed in this tutorial. Please contact support@macvector.com if you need more information.

TOPO-TA Cloning

Taq polymerase has a non template-dependent activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. This reduces the efficiency of ligation into normal blunt-ended vectors. However, TOPO-TA vectors are supplied with single 3´ deoxythymidine (T) residues attached to the ends, providing a complementary overhang that increases the efficiency of ligation.
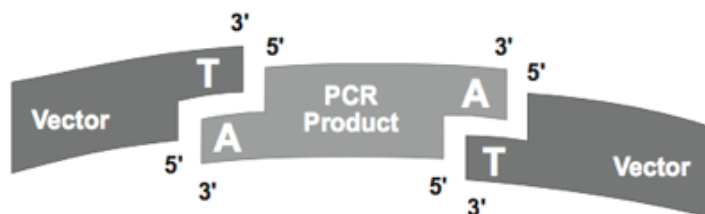

The basic steps required in MacVector to simulate a cloning like this are;

- Select the region in a source DNA molecule corresponding to the PCR fragment.

- Choose **Edit I Copy** to copy the blunt ended fragment to the clipboard.

- Open the target vector molecule and view the *TopoTA/BLNT* cloning site in the **Map** tab using the `Common Enzymes` set of sites.

- Select the *TopoTA/BLNT* site and choose **Edit I Ligate**.

- Click on the **Ligate** button to insert the fragment.

Example Tutorial

Select **File I Open** and navigate to the `/Applications/MacVector/Common Vectors/Invitrogen/` folder, choose the `pCR2.1 TOPO.nucl` file and click **Open**. Make sure you have the **Map** tab selected.
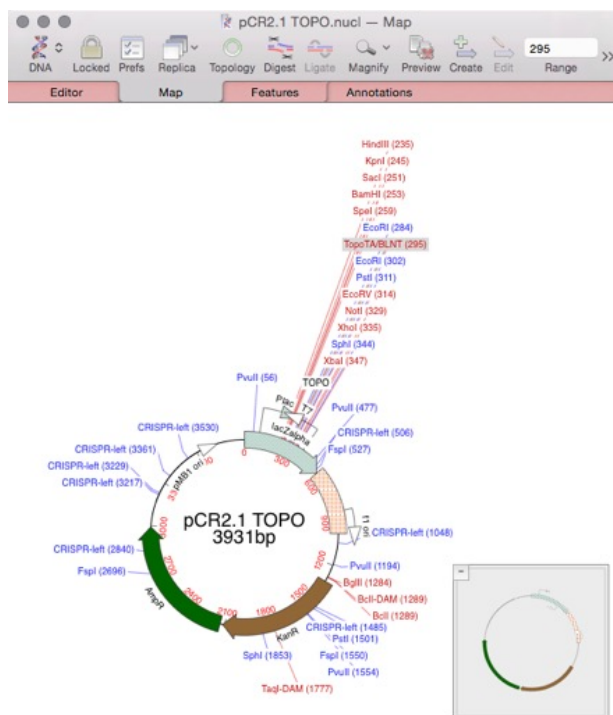
Note the ***TopoTA/BLNT*** site (selected in the screenshot for clarity) sandwiched between *Eco*RI sites in the *lac*Zalpha gene.

NB: If you do not see this site, you need to make sure you have the correct enzyme file set in the **Map** view preferences pane. To set this, click on the **Prefs** toolbar button to open the *Map View* preferences pane and switch to the *Scan DNA* tab;
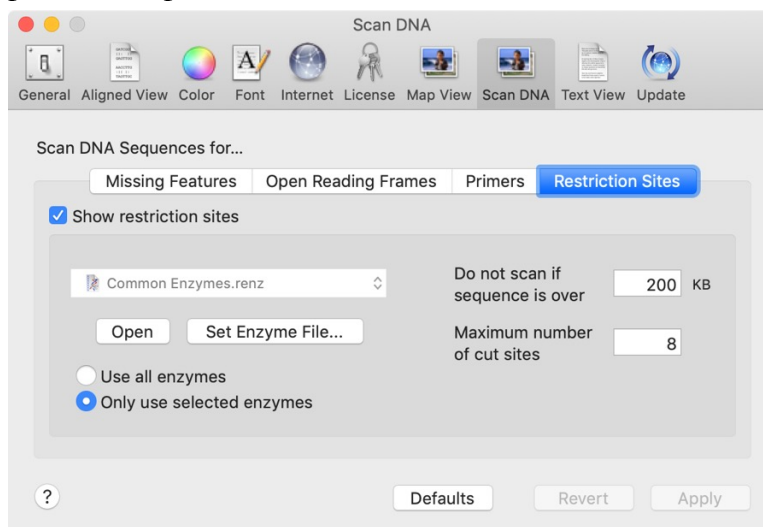

Click on the **Set Enzyme File…** button and choose the file `/Applications/MacVector /Restriction Enzymes/CommonEnzymes.`

MacVector 17.0 introduced a new *Restriction Enzyme Picker* floating window that lets you quickly turn on and off different enzymes. Make sure the **RE Picker** toolbar button is set to "on";
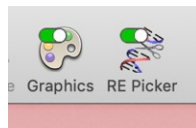

Then make sure the *TopoTA/BLNT* item is selected in the *RE Picker* list;
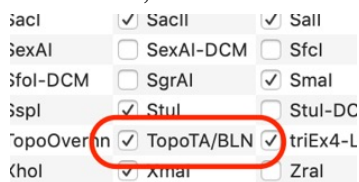
This should ensure that the ***TopoTA/BLNT*** "enzyme" is shown in the **Map** tab display.

Select **File I Open** and navigate to the
`/Applications/MacVector/Tutorial Files/Align To Reference/Sequence Confirmation/` folder, choose the `SequenceSample` file and click **Open**.

Make sure you have the **Map** tab selected. Click on the **ORF1** feature so that it highlights.
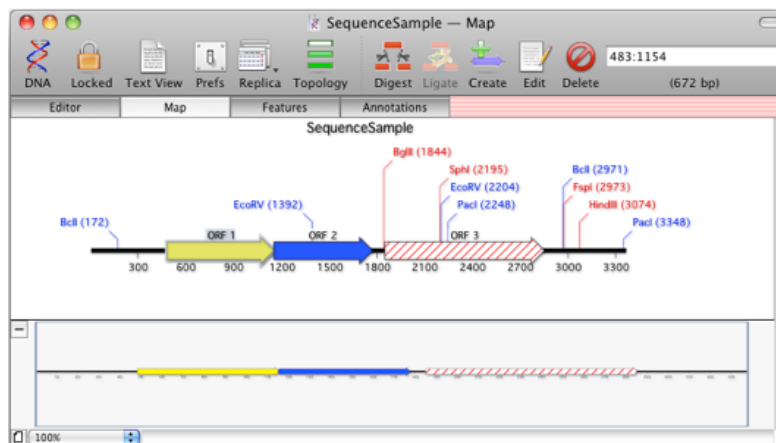
Next we will prepare a PCR fragment for cloning into the ***TopoTA*** site. This could actually be any blunt ended DNA fragment, but we will use a fragment generated by primers resulting from a **Primer3** analysis.

> Choose **Analyze | Primer Design/Test (pairs).** Make sure the
> settings match the following dialog and click on the **OK** button.

> Make sure you have **Graphical map** selected in the result filter
> dialog, then click **OK**. In the **Map** window that opens, click on the
> first *PCR Product* object.
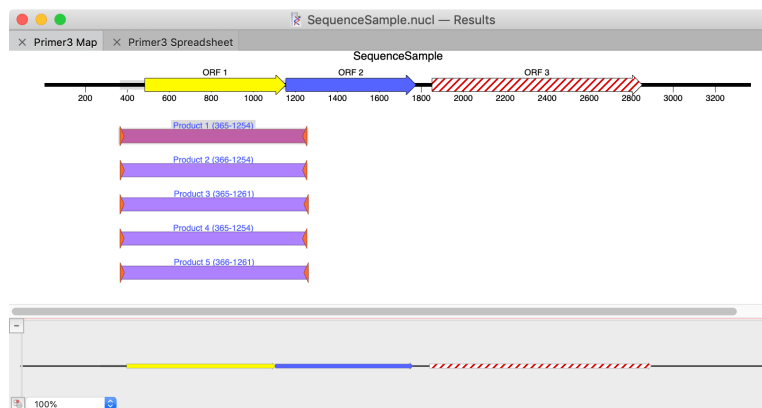
> Choose **Edit | Digest** to copy the selected PCR Product sequence
> to the clipboard. The fragment will appear in the *Cloning
> Clipboard* window
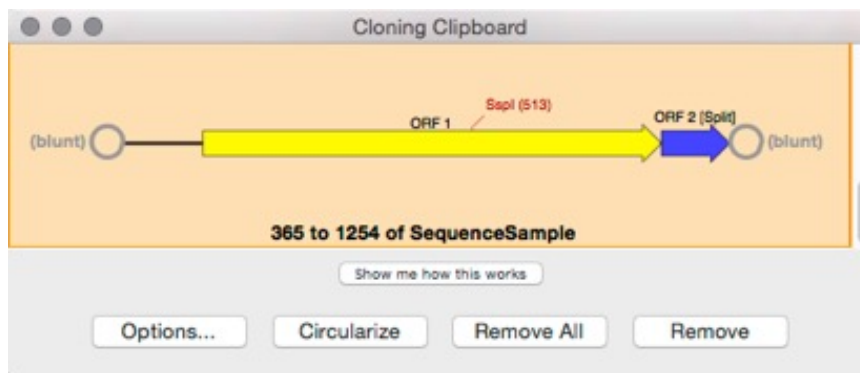
Switch to the **pCR2.1 TOPO** window **Map** tab, click on the
TopoTA/BLNT site and choose **Edit | Ligate**. The **Ligation**
dialog sheet should appear.
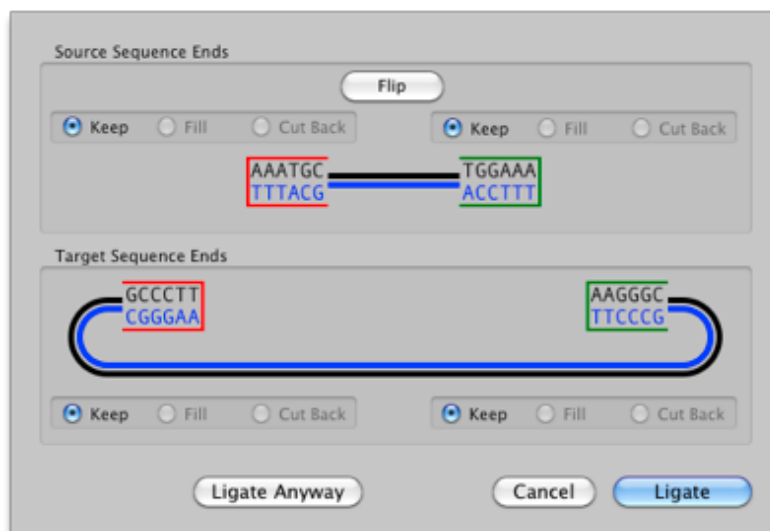
Note that there are some limitations in MacVector that affect the
VISUAL appearance of the cloning in the dialog, but will not
affect the final sequence of the construct. MacVector treats both
the source (the *PCR Product* fragment) and the destination
(*pCR2.1 TOPO*) as blunt-ended sequences;

```
...GCCCTT    AAATGCnnn...nnnTGGAAA    AAGGGC...
...CGGGAA    TTTACGnnn...nnnACCTTT    TTCCCG...
```

In reality, the vector has a 3'T overhang (i.e. is missing the 5'A at
each end) and the PCR fragment has an additional 3'A residue so
the cloning ligation should ideally be represented as;

```
...GCCCTT    AAATGCnnn...nnnTGGAAAA    AGGGC...
...CGGGA    ATTTACGnnn...nnnACCTTT    TTCCCG...
```

The end result of either ligation will be identical – when you press
the **Ligate** button the correct DNA construct will be created;

```
...GCCCTTAAATGCnnn...nnnTGGAAAAAGGGC...
...CGGGAATTTACGnnn...nnnACCTTTTTCCCG...
```

> Click on the **Ligate** button to join the two fragments
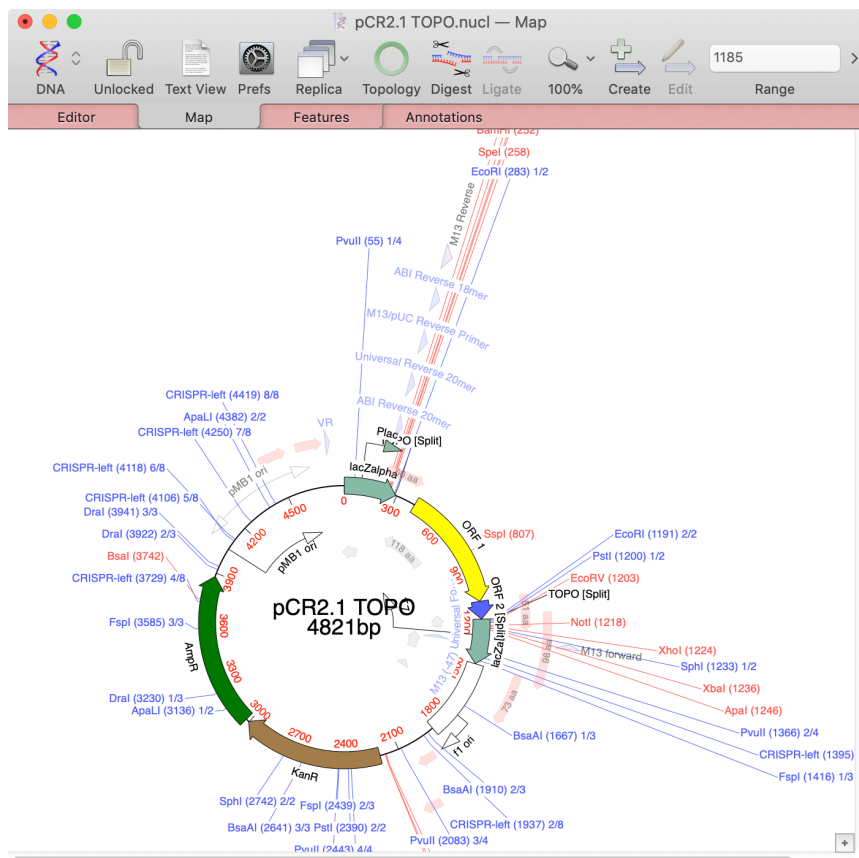
At this stage you would normally choose **File | Save As…** to save the construct under a different file name. Close the window without saving to make sure you don't overwrite the original vector.

Zero Blunt TOPO PCR Cloning

This technique is very similar to the TOPO-TA approach, except that the linear vector molecules are completely blunt ended and do not have 3'T overhangs. These vectors should be used whenever a "proof-reading" polymerase is used in the PCR reaction. These polymerases, like *Pfx* DNA Polymerase (also available from Invitrogen) do not leave 3'A overhanging residues but produce a completely flush end.

The strategy for Zero Blunt cloning simulation with MacVector is identical to the TOPO-TA approach. The Invitrogen vectors used for this (e.g. `pCR Blunt II-TOPO`, also in the `/Common`

`Vectors/Invitrogen/` folder) have the same ***TopoTA/BLNT*** site as the TA vectors. You can copy a PCR fragment, select the ***TopoTA/BLNT*** site in the **Map** tab of the vector and choose **Edit | Ligate** to paste the fragment into the vector.

Gateway Cloning

Gateway cloning is a technology that takes advantage of *in vitro* recombination using *att* sites derived from the bacteriophage lambda chromosomal integration system. Once a fragment of interest has been cloned into an "Entry" vector, flanked by *att* sites, it can be rapidly transferred into a wide variety of engineered "Destination" vectors by incubating the entry clone with destination vector and recombination proteins, then transforming a suitable host and selecting for the destination vector. Most Invitrogen Entry vectors use Kanamycin resistance as the primary selectable marker, whereas the Destination vectors use Ampicillin resistance. In addition, the Destination vectors typically contain a strain-specific lethal gene (usually ccdB) flanked by the att sites that gets replaced when the recombination event occurs, allowing direct selection for successful recombinant clones.

One final important feature of the system is that the recombination proteins have a slight flexibility in recognizing the sequence of the recombination sites, although the source and destination sites must be identical for recombination to occur. This means that by varying the sequence at the recombination site by a single residue, the system ensures that recombination always occurs in the desired orientation. There are 4 primary *att* sites used in Gateway cloning that are included in the Common Enzymes file;

*att*R1 CTTT **T**TTGTACAAA CTTG
*att*R2 CTTT **C**TTGTACAAA GTGG
*att*L1 CTTT **T**TTGTACAAA GTTG
*att*L2 CTTT **C**TTGTACAAA GTTG

*att*R1 and *att*L1 share the normal core sequence of the lambda *att* site, but *att*R2 and *att*L2 contain a C instead of a T within the core sequence. This difference ensures that *att*R1 can only recombine with *att*L1 and *att*R2 can only recombine with *att*L2. All the Invitrogen vectors contain matched flanking L1/L2 or R1/R2 *att* site pairs, so the recombination event always occurs in the same orientation, so you can exactly predict the product that will result from the recombination. There are other related L3/L4/L5 etc variations that can be used, but are far less common.

Creating an Entry Clone

There are a wide variety of Entry vectors available from Invitrogen and other sources, including many that use the TOPO-TA or Zero Blunt technologies described above. For this tutorial, we will use one of the simple Entry vectors with a typical restriction enzyme mediated cloning.

> Open the file `/Applications/MacVector/Common Vectors/Invitrogen/pENTR 1A` and make sure you have the **Map** tab selected.
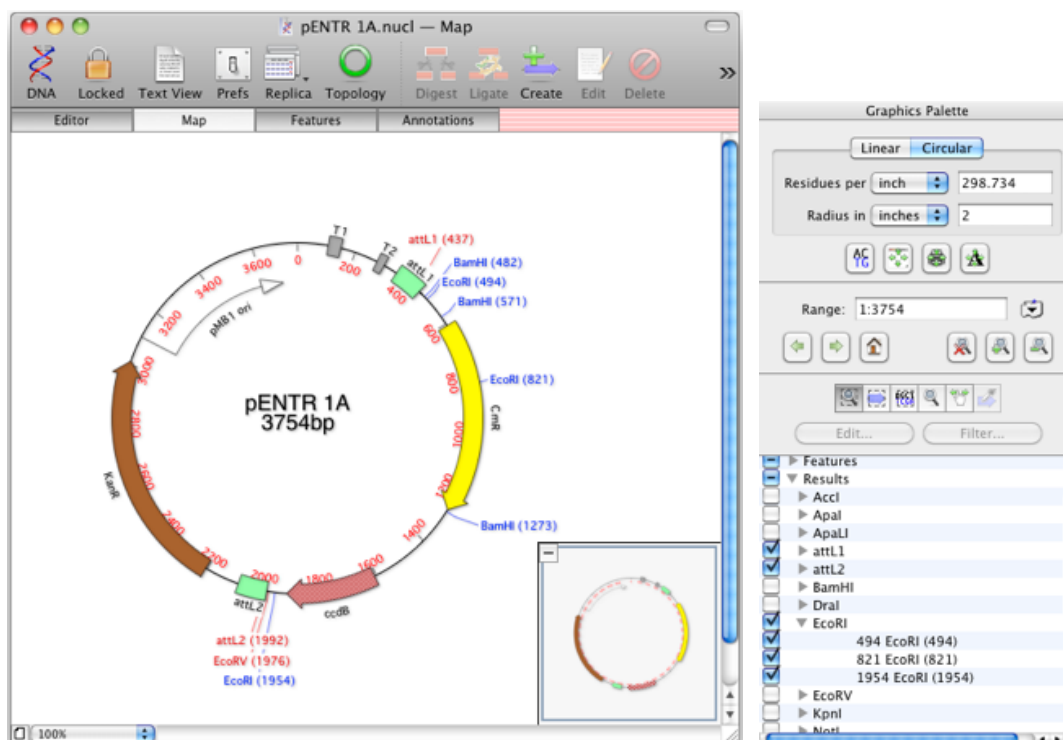

This is the vector we will use in the cloning experiment. Note that to clarify the image, we have turned off many of the restriction sites that would be displayed by default. If you want to do this, you can toggle the sites to be displayed using the floating **Graphics Palette** as shown above on the left. Alternatively, with MacVector 17.0 and later, you can use the new **RE Picker** to quickly filter and display sites of interest. In the `pENTR 1A` map, make a note of the *att*L1 (437) and *att*L2 (1992) sites, and also the pair of *Eco*RI sites at 494 and 1954 (there is a third *Eco*RI site at 821).

Open the file `/Applications/MacVector/Sample Files/GalCosmid` and make sure you have the **Map** tab selected.
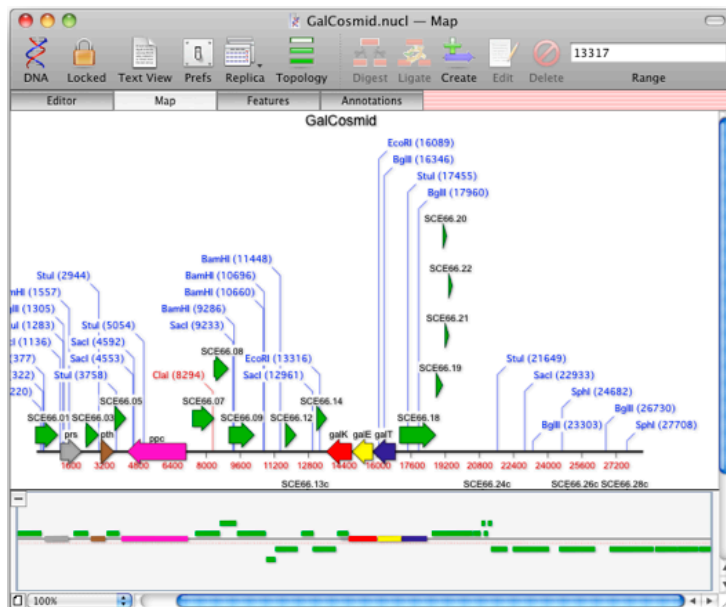

To clean up the display, again use the floating **Graphics Palette** or **RE Picker** to turn off all the restriction enzymes in the **Results** section except for *Eco*RI. Select the *Eco*RI site to the left of the *gal*K gene, hold down the **<shift>** key and then select the second *Eco*RI site on the right of the *gal*K gene.
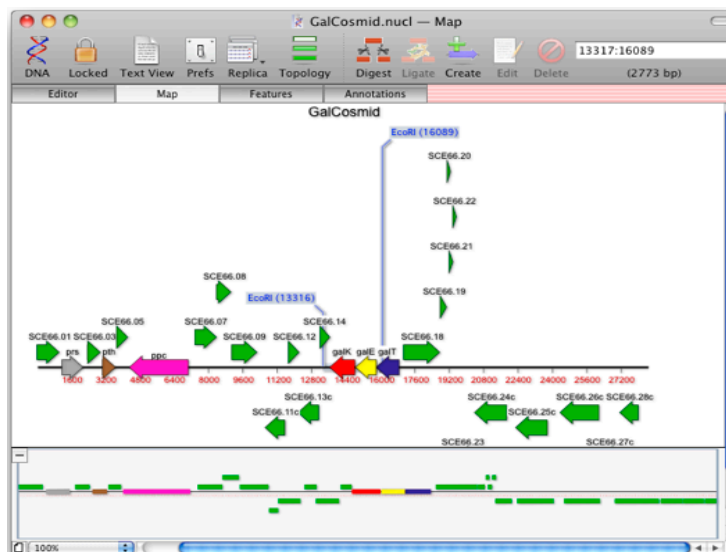

We are going to clone this *Eco*RI fragment into the corresponding sites in `pENTR 1A`.

> Select **Edit | Digest**. This will copy the selected fragment to the Cloning Clipboard, along with the *Eco*RI overhanging sticky end information.
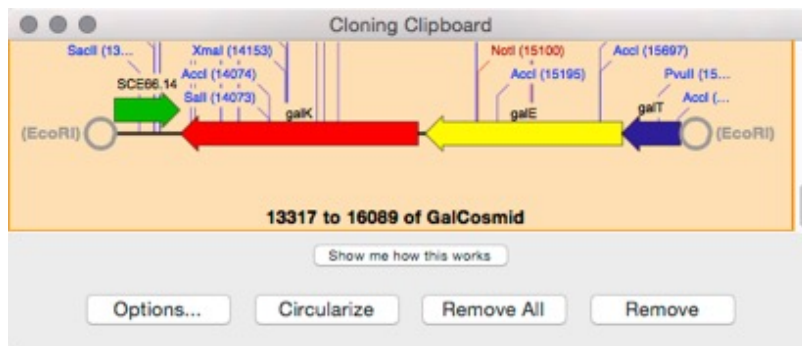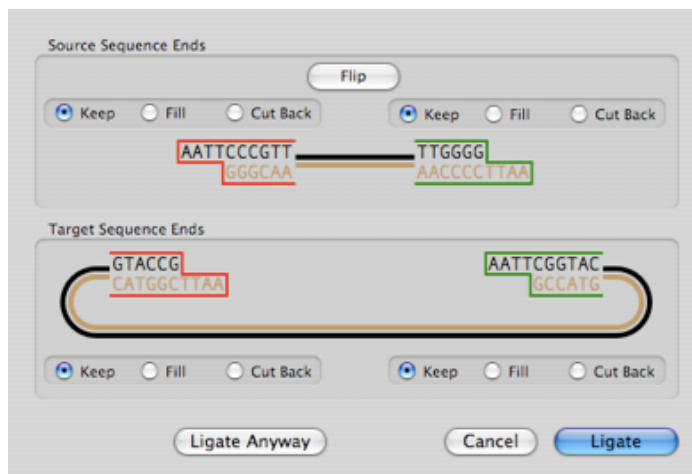

> Now switch to the `pENTR 1A` window and **<shift>**-select the two *Eco*RI sites there. Note that you must select the sites in a 5' to 3' direction (i.e. 494 followed by 1954).

Note that we are selecting the sequence fragment that we want to replace, in a similar way that you might replace a paragraph in a text document.
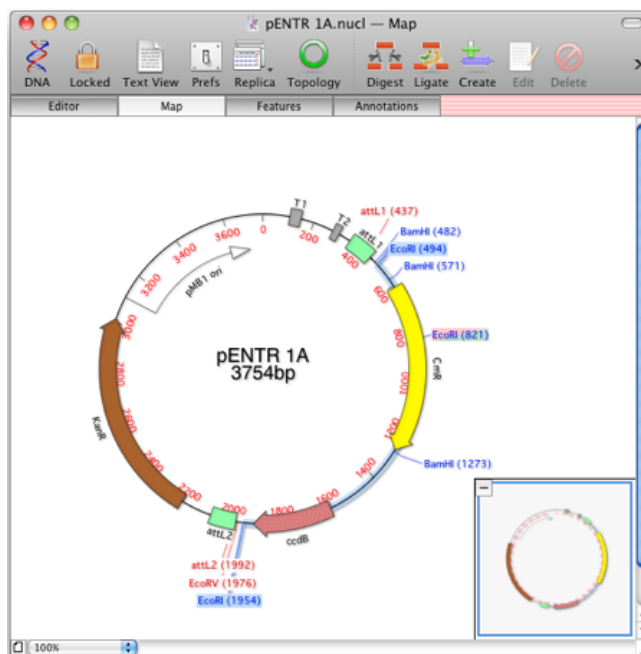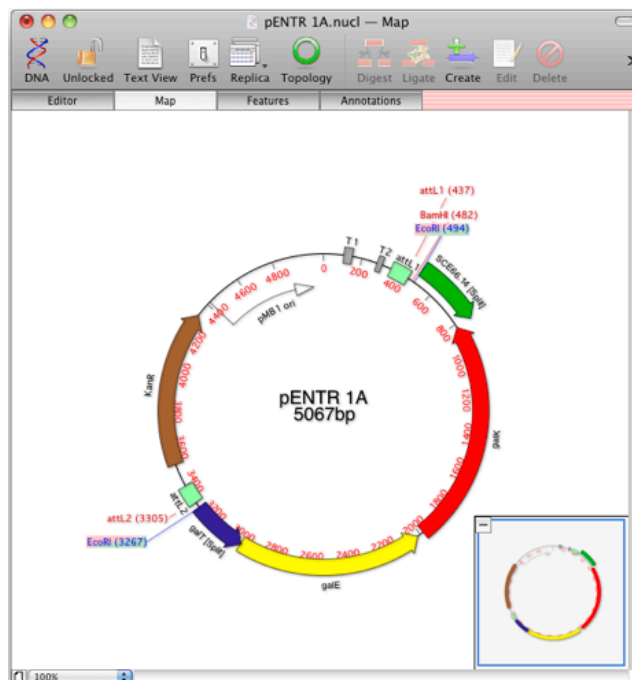

> Select **Edit | Ligate...** The *Ligation* dialog sheet will appear.

Both ends have matching 5'-AATT-3' overhangs, so the dialog outlines the compatible ends in red and green to indicate what will happen when you click the **Ligate** button.

Click on the **Ligate** button. The copied fragment gets pasted into the vector.

Again, the image has been cleaned up by turning off all the sites except for *Eco*RI, *Bam*HI, *att*L1 and *att*L2. Note how the *att* sites now flank the cloned fragment.

> **<shift>**-select *att*LI then *att*L2 sites and choose **Edit I Digest** to place the fragment on the Cloning Clipboard.
> Open the file `/Applications/MacVector/Common Vectors/Invitrogen/pDEST 14`.

Again, you might want to turn off some sites to clean up the display. Note how the vector has attR1 and attR2 sites in a corresponding location to the *att*L1 and *att*L2 sites in `pENTR A1`. These sites will be highlighted in red and green to show that they are compatible with the digested fragment on the clipboard.

> **<shift>**-select the *att*RI and *att*R2 sites.
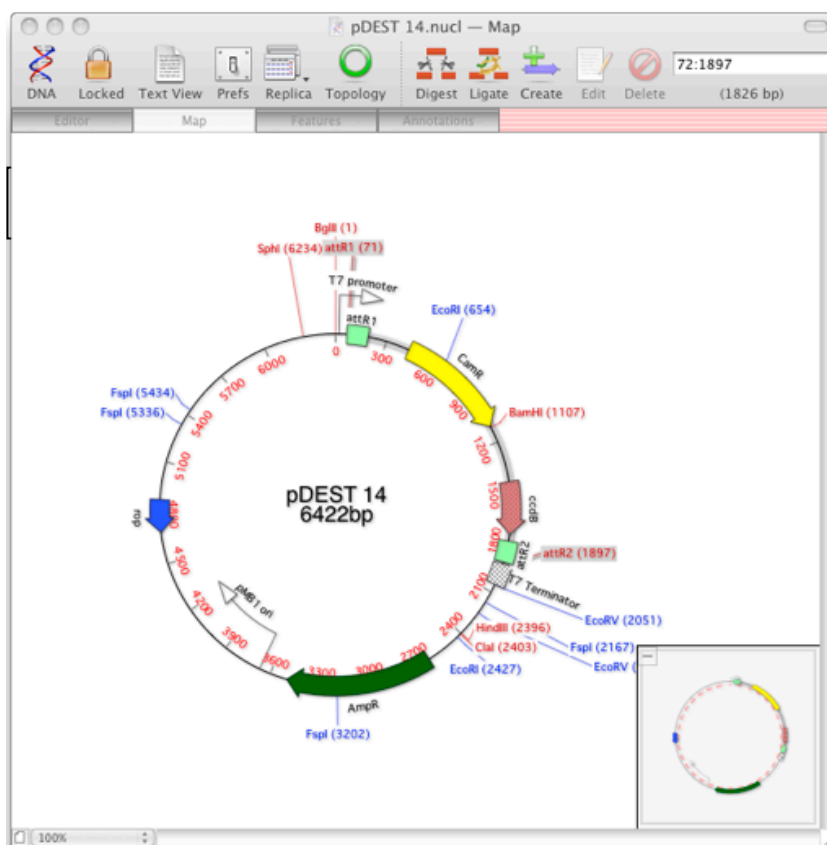
The **Edit I Ligate…** entry should become enabled when you select the sites. Note there is also a **Ligate** button on the toolbar.
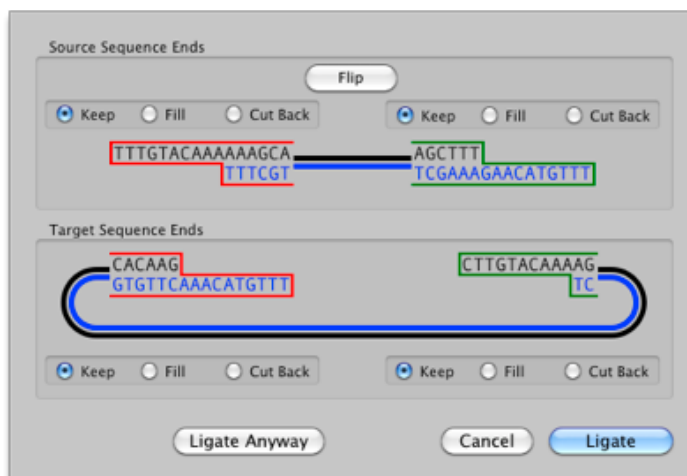
The **Ligation** sheet shows the "sticky ends" produced at the *att* sites. In this case, the overhangs are an artificial representation that serves to indicate compatibility using the usual approach MacVector uses for restriction enzymes. You can see how effective this is by trying to paste the fragment in the opposite orientation.

Click on the **Flip** button.
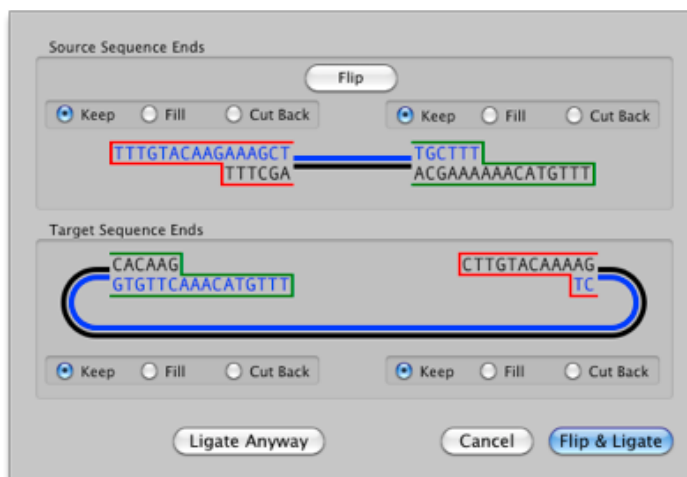
The source fragment gets flipped (reversed and complemented) – you can see that now the red and green outlines are crossed. If you examine the overhangs carefully you will see that the red outlined target end starts with a C that will only match the G on the very inside of the red source overhang. It will not match the green source overhang that has an A at that position. The default blue button changes text to **Flip & Ligate** to indicate that if you press it, MacVector will flip the fragment back again before pasting. MacVector only lets you paste the source sequence in a

biologically correct orientation unless you override the behavior by choosing the **Ligate Anyway** button.
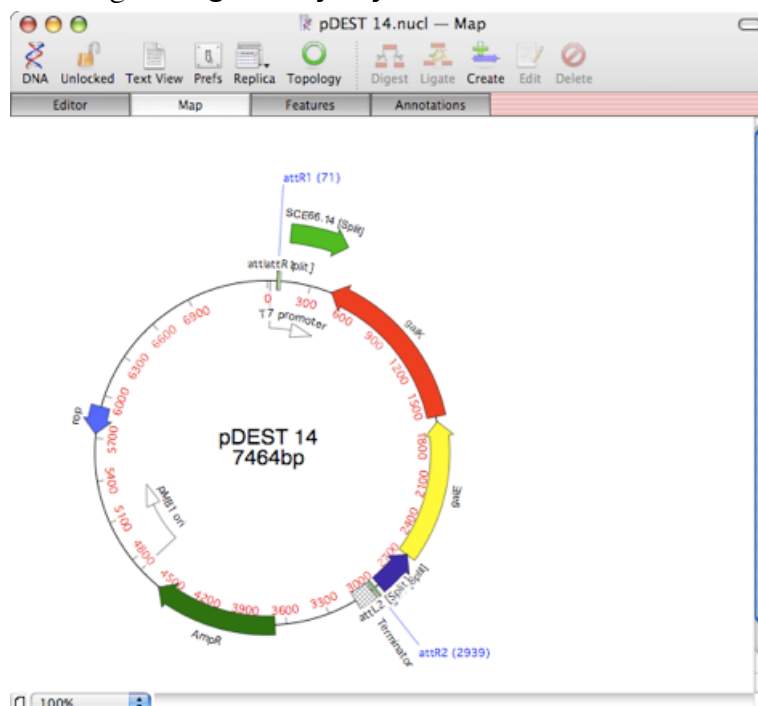
Additional Vectors

With the basic steps outlined above, you can simulate any Gateway, TOPO-TA or Zero Blunt TOPO cloning using MacVector. For additional details on the practical techniques involved in cloning using these technologies, or for the sequence of vectors not included in the MacVector `/Invitrogen/` folder, please see www.invitrogen.com.

Invitrogen tend to supply vectors as a plain sequence with a PDF map of the features. The easiest way to annotate the sequence with the common features shared by all Gateway and TOPO vectors is to use MacVector's Auto Annotation function. Paste the plain text sequence into a new MacVector Nucleic Acid document and then run **Database | Auto-Annotation**, selecting `/Common Vectors/Invitrogen/` as the sequence folder. That should annotate common features such as the *att* sites, replication origin, antibiotic resistance genes and any other features shared between the new vector and existing sequences in the `Invitrogen` folder. Then carefully examine the PDF map of the vector and add any new features that were missed by the auto annotation individually. To do that its easiest to type the location of the feature into the **Editor** tab **Range** control using a format like "123:456", then click the **Create** button to bring up the feature editor dialog.