

# MacVector 17.5

for Mac OS X

## Primer Design Tutorial

*MacVector, Inc.*  
Software for Scientists

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This version of the primer design tutorial was published in January 2020.

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## Introduction

MacVector provides a number of tools to help you design primers for use in Polymerase Chain Reaction (PCR) experiments. MacVector 12.6 introduced a new “Quicktest Primer” interface that simplifies the design of primers with mismatches and/or tails. This can be used in conjunction with the existing **Primer3** “Primer Design” functionality to design pairs of primers with mismatches and/or tails and to easily generate the predicted product of the reaction, complete with tails and/or mismatches. The Quicktest functionality was enhanced in MacVector 13 to display Restriction Sites around the primer location, including “one-out” sites and the effect of mutations on those sites on overlapping open reading frames. MacVector 14 introduced the concept of a “Primer Database” where you can store primers for use throughout MacVector.

This tutorial shows you how you can design a primer with a mismatch that changes a protein coding region to create a restriction enzyme site, add a tail containing a restriction enzyme site, find a matching primer to amplify a specific segment from a gene, add a restriction enzyme site tail to that primer, then finally generate a new DNA sequence representing the predicted product of the amplification.

## Sample Files

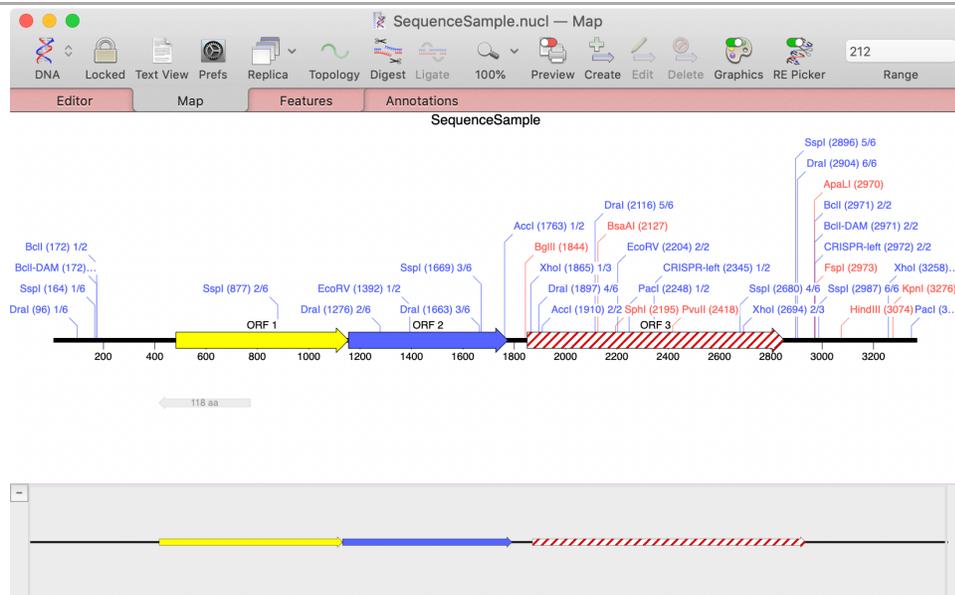
This tutorial uses a sample file that is installed with MacVector. You can find the file at this location;

```
/Applications/MacVector/Tutorial Files/Align To  
Reference/Sequence Confirmation/SequenceSample
```

## Tutorial

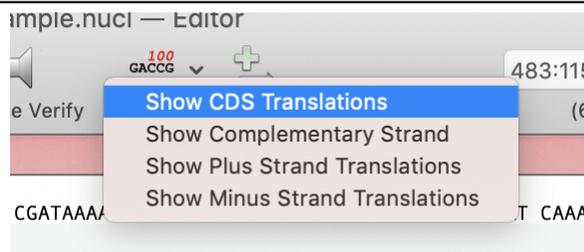
### Opening SequenceSample

Select **File | Open** and navigate to the `/MacVector/Tutorial Files/Align To Reference/Sequence Confirmation/` folder. Select `SequenceSample` and click **Open**. A window will open – click on the **Map** tab to view a graphical representation of the protein coding regions of the sequence.



Note there are three ORFs annotated on the sequence. You can see the translations of the ORFs in the **Editor** tab as well.

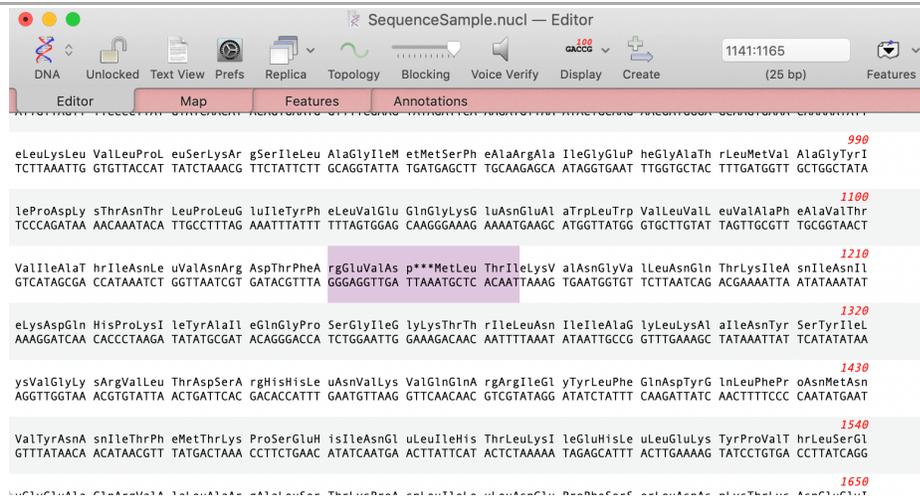
Click on the **Editor** tab. Then click and hold on the **Display** button. A popup menu will appear. Select **Show CDS Translations**.



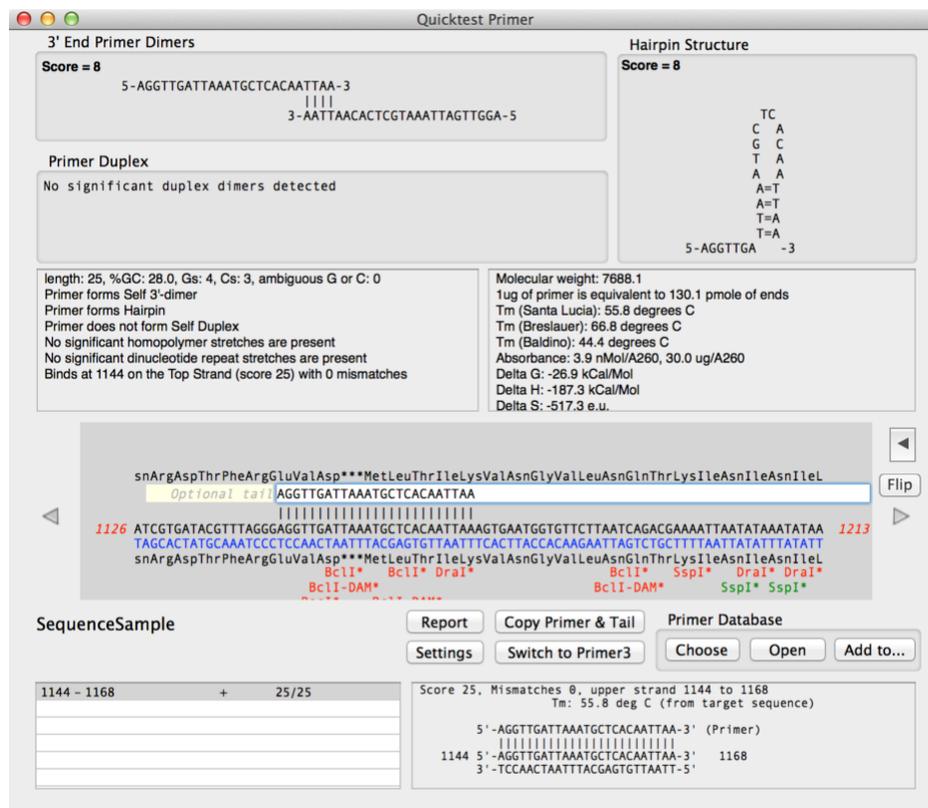
If you scroll through the sequence, you will see that the amino acid CDS translations are now displayed above the sequence.

## Invoking Quicktest Primer

Scroll through the sequence until you see the stop codon (\*\*\*) at the end of ORF 1 starting at residue 1152. Select approximately 20-25 residues across this codon.



From the main menu, choose **Analyze | Primers | Quicktest Primer (individual)**...



When **Quicktest Primer (individual)** is invoked with a short (200 residues or less) selection in the parental sequence window, the selected sequence is automatically copied and displayed in the central primer edit box (with the white background and the focus ring). The dialog is sensitive to the current active nucleic acid window so that if the primer in the edit box binds to that nucleic acid sequence, the matching template sequence is shown aligned

below the primer. The translations of any CDS features that overlap the binding region are shown below the sequence. Note that the plus and minus strands of the binding region are shown in the current selected colors (default of black for the plus strand and blue for the minus strand). If the primer binds preferentially to the minus strand, then the minus strand will be shown above the plus strand.

Finally, color-coded restriction enzyme sites are shown below the template sequence. Black sites are fully-matching restriction enzyme sites in the template sequence. The sites with an asterisk (e.g. BclI\*) are “one-out” sites (e.g. a 5 out of 6 match to the recognition sequence) where a single residue change could be made to create a complete recognition site. Green sites are those where changing the template sequence would NOT change the amino acid sequence of any overlapping protein coding sequence. These will also include sites where no coding sequence is annotated on the sequence. Red sites are those where introducing the residue change to match the complete site WOULD cause a change to the amino acid sequence of a protein coding open reading frame. For this feature to work as expected, your source sequence must be annotated with CDS features representing coding regions. All sequences downloaded from the GenBank database will be annotated appropriately.

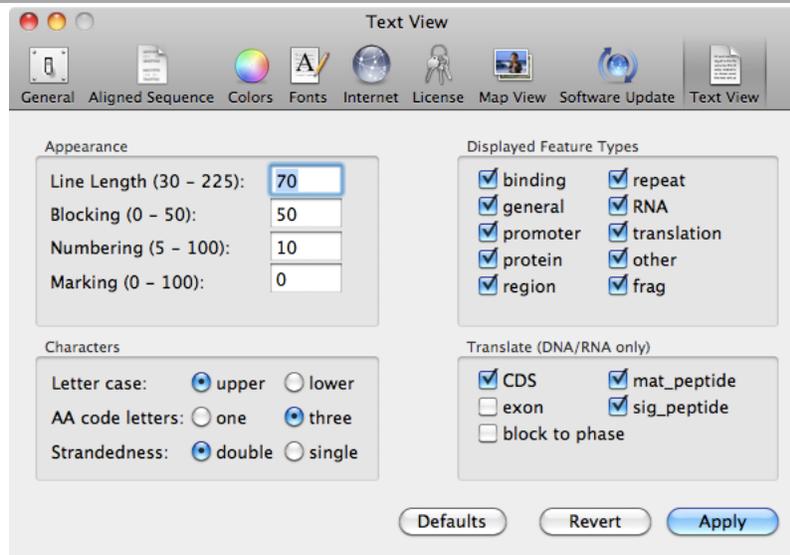
## Resizing the Dialog

There is a lot of information displayed in the **Quickest Primer** dialog and sometimes there will be far more restriction enzymes displayed below the template sequence than can be viewed in the existing box. However, if you have a reasonably large screen, you can resize the dialog and the central primer/template editing pane will increase in size;



## Changing the Amino Acid Display

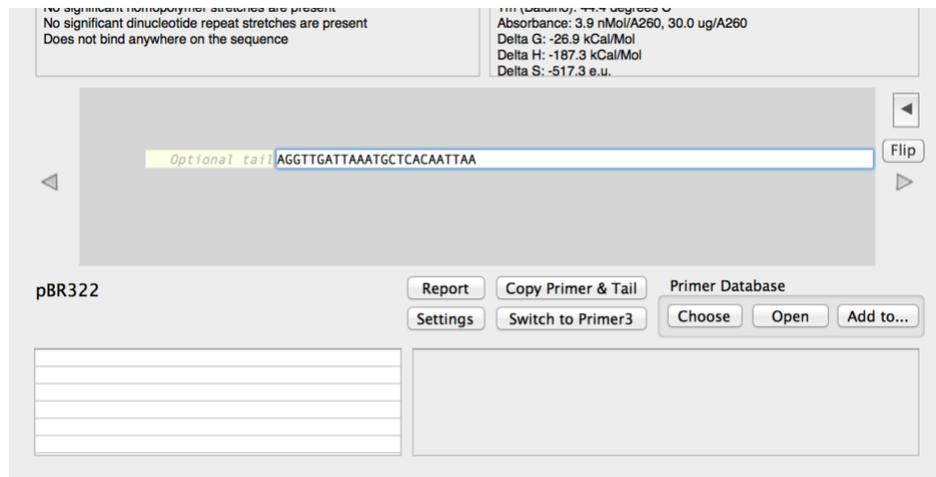
The default setting is for amino acids to display using the single letter codes rather than triplet codes (e.g. “A” rather than “Ala”). This tutorial uses the three letter codes as it is more obvious to discuss glutamine as “Gln” rather than “Q”. If you want to change your settings to match, choose the **MacVector | Preferences...** menu item and switch to the **Text View** tab;



Make sure you have **AA code letters** set to **three**, and then click **Apply**.

Open an unrelated nucleic acid sequence – e.g. /MacVector/Sample Files/pBR322.

When the unrelated sequence window opens, the **Quicktest Primer** dialog refreshes and the binding information is removed (because the primer does not bind).



If you click back on the SequenceSample window, the binding information reappears. You can use this behavior to quickly see if/where any primer binds to any open DNA sequence.

## Nudging the Primer

When a primer binds to the target sequence, you can nudge it left and right to find the optimal location for the primer.

Make sure you have `SequenceSample` as the active window behind the **Quickest Primer** dialog. Click on the round left/right buttons and watch what happens to the display.

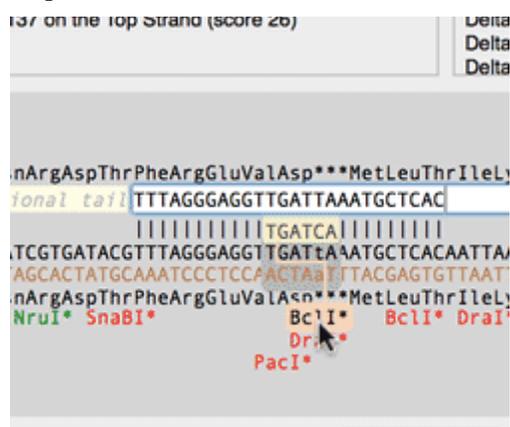
Each time you click on a button, the primer gets “nudged” along the sequence. Each of the display panes dynamically responds to the new primer location, so when you nudge you can see any hairpin loops or primer dimers graphically displayed in the upper panes. The scrollable text boxes are also updated to report the binding and secondary structural information and the primer statistics ( $T_m$ , thermodynamic properties etc). If possible, you want to avoid primers that might form hairpin loops or primer dimers. That may not always be possible, but at least this display alerts you to potential problems.

## Interacting with Restriction Sites

The restriction enzyme display is highly dynamic and interactive.

First, make sure the primer is nudged so that the \*\*\* termination codon is approximately centered in the primer. Move the mouse pointer over the *Bcl*I\* site that is below and slightly to the left of the \*\*\* codon.

While the pointer is hovering over the *Bcl*I\* site, the site highlights and the cut site and recognition sequence are temporarily displayed on the template sequence.



If you look carefully, you can see the mismatched residue is displayed in lower case. In this case, there is a “t” that would need to be changed to a “C” to create a functional *BclI* site.

Now click and hold the mouse button. This temporarily changes the sequence of the primer so that the “t” changes to a “C”.



The primer sequence changes and there is now a mismatch between the primer and the template, represented by the gap in the vertical match characters between the sequences. Also, a *BclI* site has now appeared above the primer sequence. Restriction sites that would be created by changes in the primer are displayed in black text above the primer. Conversely, restriction sites that would be destroyed by mismatches in the primer are displayed in grey strike-through text. Any changes to the amino acid sequence of overlapping open reading frames are shown in red text. In this case the \*\*\* termination codon has been replaced by a red Gln codon.

To make the change permanent, simply double-click on the *BclI*\* restriction site. In preparation for the next section of the tutorial, if you double-click, choose **Edit | Undo Edit** to revert the change.

## Introducing a Mismatch Manually

Nudge the primer so that the \*\*\* stop codon is approximately in the middle of the primer. Select the second “T” in the sequence ..GATTAAA.. and type a “C”.

The display updates to show that you have now introduced a mismatch and also shows the change in the amino acid sequence in red;

```

gAspThrPheArgGluValAspGlnMetLeuThrIleLysValAsnGly\
Optional tail GAGGTTGATCAAATGCTCAC
|||||
GTGATACGTTTAGGGAGGTTGAT_TAAATGCTCACAAATAAAGTGAATGGT
CACTATGCAAATCCCTCCAACCTAA_TTTACGAGTGTTAATTTCACTTACCAC
gAspThrPheArgGluValAsp**MetLeuThrIleLysValAsnGly\

```

The edit creates a new restriction enzyme site (*Bcl*I - TGATCA) as we saw above. You may also see that replacing the T with a C has introduced a potentially significant internal primer duplex;

```

Primer Duplex
Score = 18
      5-GAGGTTGATCAAATGCTCAC-3
      |||||
      3-CACTCGTAAACTAGTTGGAG-5

```

Continue to nudge the primer. You'll see that as you nudge the primer to the left, the primer information is updated in real time. Unfortunately, because *Bcl*I has a typical 6 base pair dyad symmetrical recognition site, we can't do anything about the internal primer duplex.

```

lAsnArgAspThrPheArgGluValAspGlnMetLeuThr
Optional tail GGGAGGTTGATCAAATGCTC
|||||
TAATCGTGATACGTTTAGGGAGGTTGAT_TAAATGCTCAC/
ATTAGCACTATGCAAATCCCTCCAACCTAA_TTTACGAGTG/
lAsnArgAspThrPheArgGluValAsp**MetLeuThr

```

## Adding a Tail Containing a Restriction Enzyme Site

For the next step we will add a tail containing a *Bam*HI restriction site to the primer.

Click in the "Optional tail" box and type the sequence "CGCGGATCC"

Note that we add 3 additional residues (CGC) before the *Bam*HI recognition sequence (GGATCC). Many restriction enzymes cannot cleave DNA if the recognition sequence lies too closely to the end of a double stranded molecule. Adding an additional 3 residues is usually sufficient to ensure that the enzyme will be able to cut the final PCR product. As you type, the restriction enzyme sites above the primer are automatically updated. You may find that adding the tail causes a potential hairpin loop to be created;

```

          BamHI      BclI
ValAsnArgAlaArgIleArgGluValAspGlnMetLeuThrIleLys
CGCGGATCCGGGAGGTTGATCAAATGCTCACAAAT
|||||
GTTAATCGTGATACGTTTAGGGAGGTTGAT_TAAATGCTCACAAATAA
CAATTAGCACTATGCAAATCCCTCCAACCTAA_TTTACGAGTGTTAATTT
ValAsnArgAspThrPheArgGluValAsp**MetLeuThrIleLys
          BclI*   BclI*   DraI*
          DraI*   FspI*
          PacI*

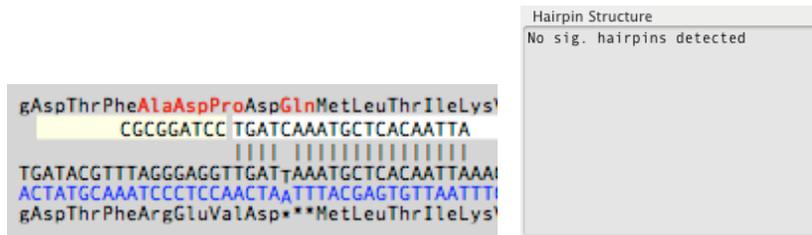
```

```

Hairpin Structure
Score = 10
      GGG
      G  G
      G  T
      C  T
      C=G
      T=A
      A=T
      G=C
5-CGCGG AAATGCTC-3

```

Nudge the primer to the right. Eventually you will find a location where the hairpin loop is reduced to just 3 residues and it falls below the significance threshold. Do not let the mismatch fall off the end of the primer or you will have to re-enter the T -> C change.



## Generating a Primer Report

Once we are happy with the primer and tail we have designed we may want to make a permanent record of the sequence of the primer and its characteristics. You can click on the **Copy Primer & Tail** button copy the full primer, ready to paste into other applications.

Click on the **Show Report** button.

This opens a separate report window summarizing the information displayed in the **Quicktest Primer** dialog, including the primer properties, potential primer dimer and hairpin loops, the presence of any additional binding sites on the target sequence and the details of the primary binding site, restriction enzyme sites and CDS translations. The report is designed to be a simple one page document that you can print or save as a PDF file to create a permanent record of the primer.

Quicktest Primer Report

5' C GCG GAT CCG GGA GGT TGA TCA AAT GCT CAC AAT 3'

Primer Properties		Analysis Parameters	
Length	34 residues	A-T score	2
GC	44.0 %	G-C score	3
Gs: 7, Cs: 4, ambiguous G or C: 0		Mismatch score	-5
Molecular weight	10492.0	3' end dimer score threshold	7
1ug of primer is equivalent to	129.4 pmole ends	Duplex score threshold	11
Primer Tm (Santa Lucia)	61.8 degrees C	Hairpin score threshold	8
Primer+Tail Tm (Santa Lucia)	72.8 degrees C	Hairpin minimum pairing	2 bases
Absorbance	3.0 nMol/A260	Hairpin minimum loop size	3 bases
Absorbance	31.7 ug/A260	Hairpin maximum loop size	10 bases
Delta G	-46.3 kCal/Mol	Primer concentration	50.0 nM
Delta H	-273.5 kCal/Mol	Monovalent cation (Na+) conc.	50.0 mM
Delta S	-732.5 e.u.	Divalent cation (Mg++) conc.	1.5 mM
3' End Delta G	-24.5 kCal/Mol	dNTP concentration	0.6 mM
No significant homopolymer repeat stretches are present			
No significant dinucleotide repeat stretches are present			

3' End Primer Dimers	Primer Hairpins
No significant 3' end dimers detected	<b>Score = 10</b>
	GGG G G G T C T C=G T=A A=T G=C 5-CGCG AAATGCTCACAAT-3

Primer Duplexes
<b>Score = 22</b>
5-CGCGGATCCGGAGGGTTGATCAAATGCTCACAAT-3       3-TAACACTCGTAAACTAGTTGGAGGCCCTAGGCGC-5

Primer Binding Details for SequenceSample

```

          BamHI      BclI
euValAsnArgAlaArg1leArg6luValAsp6InMetLeuThrIleLysValAsnGlyValLeuAsnGlnThrLysIleAsnIleAsnIleLysAspG
          CGCGGATCC GGGAGGTTGATCAAATGCTCACAAT
          |||||
1129 TGGTTAATCCTGATACGTTTAGGGAGGTTGAT*AAATGCTCACAAT*AAAGTGAATGGTGTCTTAATCAGACGAAATTAATATAATAAGGATC 1219
ACCAATTAGCACTATGCAATCCCTCCAAC*TTTACGAGTGTAAATTCACCTACCACAAGAAATAGTCTGCTTTAATTATATATATATTCCTAG
euValAsnArgAspThrPheArg6luValAsp**MetLeuThrIleLysValAsnGlyValLeuAsnGlnThrLysIleAsnIleAsnIleLysAspG
          BclI* BclI* Drai* BclI* SspI* Drai* Drai* BamHI*
          Drai* FspI* SspI* SspI* BclI*
          PacI*
  
```

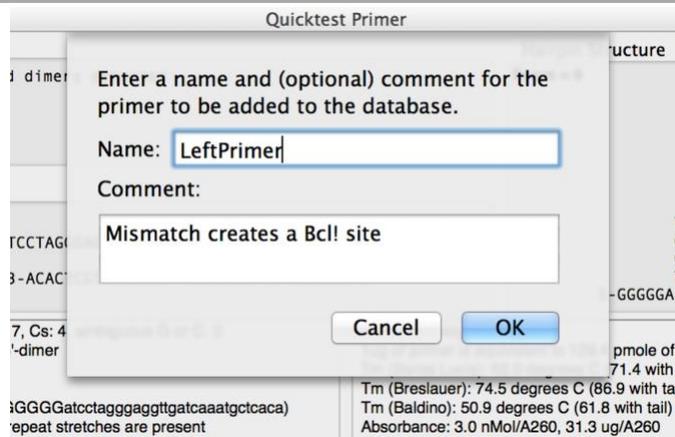
Alternate Binding Sites in SequenceSample

No alternate binding sites detected.

## Saving the Primer to the Primer Database

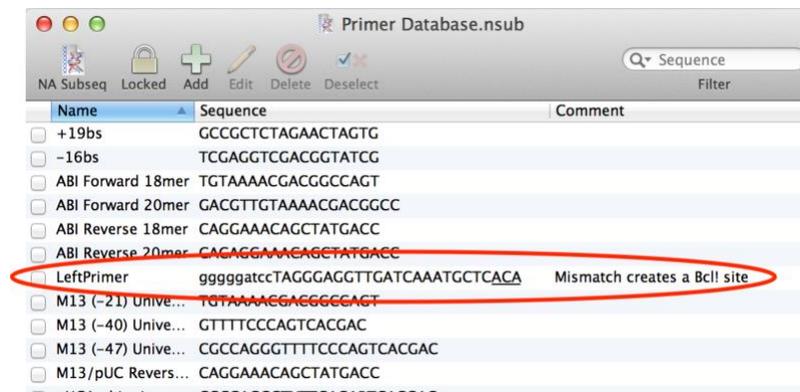
Once you have designed a primer, you may wish to add it to the “Primer Database”. The database is simply a file that exists somewhere on your hard drive. By default, MacVector uses the `Primer Database.nsub` file in the `/Applications/MacVector/Subsequences/` folder but you can set it to any file you wish by clicking on the **Primer Database -> Choose** button.

To save the current primer (with any tail), simply click on the **Add To...** button and a sheet will appear prompting for a name and comment



Enter a name and an optional comment, then click **OK** to save the primer. Now click on the **Primer Database -> Open** button

You should see the new primer in the list. Note how the tail is shown in lower case;



You can see that the last three residues of the primer sequence are underlined. This indicates to MacVector that the last three residues **MUST** perfectly match the target sequence before the primer can be considered a match.

Double-click on the *LeftPrimer* item to open the **Primer Database Editor**.

Name:

Number of Parts:

Part #1 (Use lower case letters to indicate tails on primers)

Sequence:

Perfect match (X):

Allowed mismatch:  Offset:

Comments:

Problems

Nothing has changed.

Here you can see that the **Perfect match (X):** field has three X residues at the 3' end (padded with spaces to the length of the primer). The X residues must be present in the target sequence before a match will be reported. You can add X's at any point in this field to force a match in the target sequence.

Note also that **Allowed mismatch** is set to 3 in this case. The default for this is controlled by the **Max. Mismatches** field in the Quicktest Primer **Settings** dialog;

Self-complementarity thresholds

3' End Primer Dimers

Primer Duplex

Hairpin Structure

Primer Duplex Scoring

A•T base pairs

G•C base pairs

Mismatches

Reaction Conditions

Monovalent cation conc (mM)

Divalent cation conc (mM)

Nucleotide conc (mM)

Initial Primer conc (nM)

Template Binding

**Max. Mismatches**

Restriction Enzymes

Quicktest Primer uses the Restriction Enzyme file as configured for the single sequence Map tab. Use the Preferences Map View tab to select a different file

Use all enzymes

Only use selected enzymes

Include one-out sites

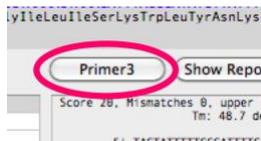
However, you can override the setting on an individual basis for any primer in the resulting Primer Database file, or override the settings when you actually run a **Primer Database Search** (see later).

You can also manually create new primers in the Primer Database file at any time. Simply click on the **Add** button and fill out the fields. Be sure to use lower case only for any 5' region that you wish to be treated as a non-binding tail.

## Finding a Suitable Matching Primer

Once the first primer has been designed, the next task is to find a suitable matching second primer for use in a PCR reaction. To do this we will make use of the integration between **Quicktest Primer** and the **Primer3** interface in MacVector.

Make sure you have the `SequenceSample` window as the front most window, then click on the **Primer3** button in the **Quicktest** dialog.



The Primer3 parameters sheet will drop down over the `SequenceSample` window and the **Quicktest Primer** dialog will close.

 A screenshot of the 'Primer Sequences' dialog box. It has several sections:
 

- Tail:** 5'-CGCGGATCC-3'. Below it is a text box containing 'TGATCAAATGCTCACAATTA' and a dropdown arrow.
- Two radio buttons: 'Use this primer' (selected) and 'Find 5' primer'. To the right, another set of radio buttons: 'Use this primer' and 'Find 3' primer' (selected). A 'Reverse & Complement' button is next to them.
- A checkbox for 'Hybridization Probe Sequence (Internal oligo for realtime PCR analysis)'. Below it is a text box and radio buttons for 'Use this primer' and 'Find Hybridization Probe'.
- A dropdown menu for 'Amplify Feature/Region'. Below it is a 'Region to Amplify:' section with two text boxes containing '1' and '3374', and a dropdown arrow.
- A checkbox for 'Advanced options'.
- Buttons for 'Defaults', 'Cancel', and 'OK' at the bottom right.

You can see that the main core of our primer is inserted into the 5' primer edit box and the tail is displayed over the top of that box. In addition, the **Use this Primer** radio button is selected for the 5' primer.

The trickiest part of using Primer3 to find a matching 3' primer is defining the region we want to be amplified. In this case, we know our designed primer overlaps the end of ORF1 and the start of ORF2. Lets set up the search to amplify from our primer position to somewhere beyond the end of ORF2.

Make sure the popup menu is set to Amplify Feature/Region, then click on the region button () and choose **1155 to 1775; CDS, ORF2** from the popup menu.

Because our primer overlaps the beginning of ORF2, MacVector will ensure that Primer3 accepts it as a valid primer. If the primer lay entirely within ORF2, we would need to adjust the **Region To Amplify** parameters so that the 3' end of the Region to Amplify would lie either within or to the right of the primer.

Click on the **Advanced Options** button.

You will see the advanced options area displayed. Note how the length, percent G+C, Tm and GC clamp options have all been pre-filled to match the characteristics of the core of the input primer. Normally you should not have to change these, as they will ensure that any 3' primers found will closely match your designed primer.

	Min.	Max.	Set	Opt.	
Length:	19	21	<input checked="" type="checkbox"/>	20	GC Clamp: 0
Percent G+C:	20	30	<input checked="" type="checkbox"/>	25	Maximum Poly-X: 5
Tm (°C):	46	50	<input checked="" type="checkbox"/>	48	Maximum difference in Tm: between primers (°C) 4

Click **OK** to run **Primer3**.

The Primer3 job will start and should complete within a few seconds. You should get a result sheet similar to this;

**Summary**

Primers...	Left	Right	Internal
Accepted:	0	144	0
Considered:	0	824	0

Primers discarded because of:

Would not amplify:	0	0	0
Too many Ns:	0	0	0
In region to be amplified:	0	0	0
Overlap excluded:	0	0	0
Bad GC% content:	0	574	0
No GC clamp:	0	0	0
Tm too low:	0	63	0
Tm too high:	0	29	0
High any complementation:	0	0	0
High 3' complementation:	0	0	0
High repeat:	0	0	0
High hairpin stability:	0	0	0
Long poly X:	0	14	0
Low quality:	0	0	0
High end stability:	0	0	0
Lowercase masking end:	0	0	0

Pairs...

Accepted:	10
Considered:	10

Reasons for rejection...

No target:	0
Product size:	0
Low product Tm:	0
High product Tm:	0
Tm diff too large:	0
High any complementation:	0
High 3' complementation:	0
No internal OLIGO:	0
High mispriming similarity:	0
High template mispriming:	0
Overlaps a better pair:	0

**Display Options**

Primer3 output

Spreadsheet

Graphical map

No left primers found

In particular, look at the **Pairs... Accepted** result. You should see that at least one pair has been accepted. If you don't see this then something went wrong – the most common problem is that you set up the region to amplify incorrectly, so that the left hand primer either did not bind within 200 residues of the region to amplify. If you click OK and examine the raw **Primer3 output** window it will usually have a message telling you what went wrong. If this is the case, simply choose **Analyze | Primers | Primer3...** and change the region to amplify settings.

Make sure the **Spreadsheet** and **Graphical map** checkboxes are selected, then click **OK**.

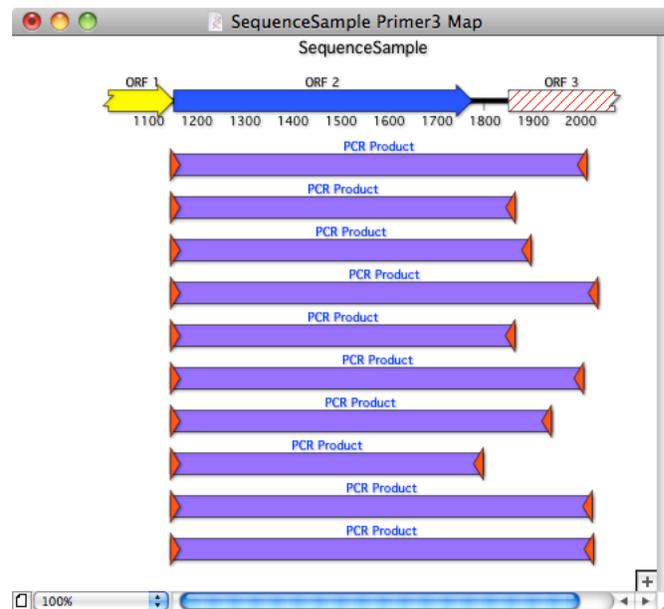
The SequenceSample **Primer3 Spreadsheet** tab displays a list of the pairs of primers found by Primer3.

Rank	L Start	L Len	L Tm	L GC%	L Sequence	R Start	R L	R Tm	R GC%	R Sequence	P Size	P Tm	Ta
1	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1887	20	52.1	40.0	CCTTAAACAACACTTGTCTC	743	71.8	51.0
2	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	2002	20	51.9	35.0	AACTATTATTATCCCGCTCT	858	72.3	51.3
3	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1876	20	52.1	40.0	ACTGTCTCGAGTATCTTCT	732	71.8	50.9
4	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1873	20	52.1	40.0	TGTCTCGAGTATCTTCTTG	729	71.7	50.9
5	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1949	20	52.2	40.0	ACCCATACC TACTATAAGCA	805	71.9	51.0
6	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1919	20	52.2	40.0	CTCAAGTAGACTTTGACCTT	775	71.8	51.0
7	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1875	20	51.8	40.0	CTGTCTCGAGTATCTTCT	731	71.8	50.9
8	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1921	20	52.2	40.0	TTCTCAAGTAGACTTTGACC	777	71.8	51.0
9	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1888	20	52.2	35.0	TCCTTAAACAACACTTGTCT	744	71.8	51.0
10	1145	20	52.0	35.0	cgcgatccGGTTGATCAAATGCTCACA	1869	20	52.3	35.0	TCGAGTATCTTCTTGCATT	725	71.7	50.9

You will see that all the left primers are the primer we designed using **Quicktest Primer**, complete with CGCGGATCC tail and the A->C mismatch. The Tm displayed (**L Tm**) is the Tm for the target site – i.e. the 20 residues where the tail-less core will bind. This does NOT take the

mismatch into account. The right hand primers are all suitable matching primers with similar  $T_m$ 's and length to our core starting primer. These are displayed as the “real” primer sequence i.e. the sequence you would send off for synthesis. They are the reverse complement of the plus strand of the target sequence. You can click in one of the primer cells and copy the primer to paste into an external application, or click elsewhere on a row to copy all of the data on the line so you have a record of the start, length and  $T_m$  data as well.

The `SequenceSample` **Primer3 Map** tab displays the results graphically (here shown zoomed in to the amplified region);

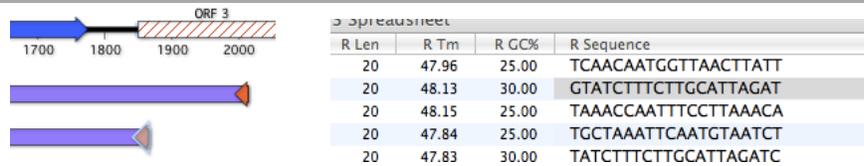


The **Map** is interactive. You can click on one of the small primer triangles, and then choose **Edit | Copy** and the sequence of the primer (including tail and/or mismatches) will be copied to the clipboard. Clicking on a purple PCR Product object followed by **Edit | Copy** will copy the predicted PCR product, again including mismatches and or tails in each of the primers.

## Adding a Tail to a Matching Primer

Once a suitable matching primer has been found, it is often useful to be able to add a tail to that primer as well. Again, we'd like to know if that might create significant secondary structure in the primer, so we need to use **Quicktest Primer** once more.

In the `SequenceSample` **Primer3 Map** results tab, click on the right hand primer of the second pair. Note how the same primer highlights in the spreadsheet and also in the parental `SequenceSample` **Editor** window.



Choose **Edit | Copy** to copy the primer sequence. Then choose **Analyze | Primers | Quicktest Primer** to open the **Quicktest Primer** dialog. If a primer is present in the main edit box, double-click in it to select the entire sequence, then choose **Edit | Paste** to replace it with the sequence you copied. Finally, if it is not already active, click on the original SequenceSample window to make sure **Quicktest** is reporting the binding results for the right sequence.

The **Quicktest Primer** dialog should appear and refresh with the new primer displayed in the primer edit box and with the template sequence “flipped” to show the correct binding between the new “minus strand” primer and the template. You should see that the upper strand of the template is now displayed in the “complementary strand” color ;

**Quicktest Primer**

**3' End Primer Dimers**  
**Score = 10**  
 5-GGGGATCCACTTGTCTCGAGTATCTTTCTTGCA-3  
 3-ACGTTCTTTCTATGAGCTCTGTTACACCTAGGGGG-5

**Primer Duplex**  
**Score = 16**  
 5-GGGGATCCACTTGTCTCGAGTATCTTTCTTGCA-3  
 3-ACGTTCTTTCTATGAGCTCTGTTACACCTAGGGGG-5

length: 25, %GC: 40.0, Gs: 4, Cs: 6, ambiguous G or C: 0  
 Primer forms Self 3'-dimer  
 Primer does not form Hairpin  
 Primer forms Self Duplex  
 Longest homopolymer: 5 (GGGGGatccacttgtctcgagtatctttcttgca)  
 No significant dinucleotide repeat stretches are present  
 Binds at 1852 on the Bottom Strand (score 25) with 0 mismatches

Molecular weight: 10430.9  
 1ug of primer is equivalent to 131.8 pmole of ends  
 Tm (Santa Lucia): 60.3 degrees C (70.1 with tail)  
 Tm (Breslauer): 69.4 degrees C (84.6 with tail)  
 Tm (Baldino): 49.3 degrees C (60.5 with tail)  
 Absorbance: 3.1 nMol/A260, 32.1 ug/A260  
 Delta G: -43.8 kCal/Mol  
 Delta H: -266.8 kCal/Mol  
 Delta S: -719.1 e.u.

lyIleGluProAspValGlnArgSerTyrArgGluGlnMet  
 GGGGATCC ACTTGTCTCGAGTATCTTTCTTGCA  
 1895 CCAATTCCTTAAACAACACTTGTCTCGAGTATCTTTCTTGATTAGATCTTAAACCTCTTTTATATTTTGAATACAAATTAT 1808  
 GGTTAAAGGAATTTGTTGTAACAGAGCTCATAGAAAGAACGTAATCTAGAGATTTGGAGAGAAATATAAAACTTATATGTTTAATA  
 lyIleGluLysPheLeuValGlnArgSerTyrArgGluGlnMet  
 DraI\* SpeI\* XhoI BglII DraI\* DraI\* AccI\* SspI\*  
 XbaI\*

SequenceSample  
 1852 - 1876 - 25/25

Report Copy Primer & Tail Primer Database  
 Settings Switch to Primer3 Choose Open Add to...

Score 25, Mismatches 0, lower strand 1852 to 1876  
 Tm: 60.3 deg C (from target sequence)  
 1852 5'-TGCAAGAAAGATACTCGAGACAAGT-3' 1876  
 3'-ACGTTCTTTCTATGAGCTCTGTTCA-5'  
 3'-ACGTTCTTTCTATGAGCTCTGTTCA-5' (Primer)

The “minus” strand is now shown above the black “plus” strand and the red numbering is larger at the left hand side than on the right hand side. This indicates that the primer is binding to the opposite strand, as you would

expect for the second primer in a PCR pair. This is also indicated in the list box at the lower left corner that displays the binding sites in the sequence.

Usually, the tail sequence will be remembered between invocations of the **Quicktest Primer** dialog. But if it is not;

Click in the “Optional tail” edit box and type in the same *Bam*HI restriction site tail we used previously (CGCGGATCC).

```

heLeuValAlaSerGlyTyrArgGluGlnMet
  CGCGGATCCGTATCTTTCTTGCATTAGAT
  |||
83 AACAACTTGTCTCGAGTATCTTTCTTGCATTAGATCT
TTGTTGTGAACAGAGCTCATAGAAAGAACGTAATCTAG
heLeuValGlnArgSerTyrArgGluGlnMet
  
```

Add the primer to the Primer Database, calling it “RightPrimer”. Finally, click on the **Primer3** button to reopen the **Primer3** sheet.

This time, because both primers have been defined (with tails), the Primer3 dialog will automatically switch into **Test Primer Pair** mode.

Note how the dialog remembers the last primer that we used for the 5’ end, including the mismatch we introduced and the tail we added. In this case, the new primer is for the 3’ end. MacVector always assumes that if you switch from **Quicktest** to **Primer3** with a primer that matches the minus strand (as we did here), then that should be treated as the 3’ primer. In Test Primer Pair mode, the analysis bypasses Primer3 completely and simply tests the primers for binding and reports the primer properties and the predicted amplification products.

Click **Test** to run the **Primer3** analysis

The analysis will proceed and you should be presented with a successful result dialog where just a single pair is considered and accepted.

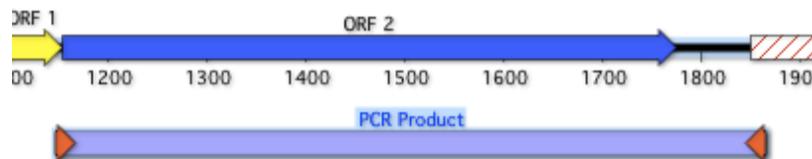
Click **OK** to display the result windows. <control>-click on the right hand primer in the **Primer3 Spreadsheet** result tab and select **Add Primer to Database** from the dropdown menu.

SequenceSample.nucl — Results

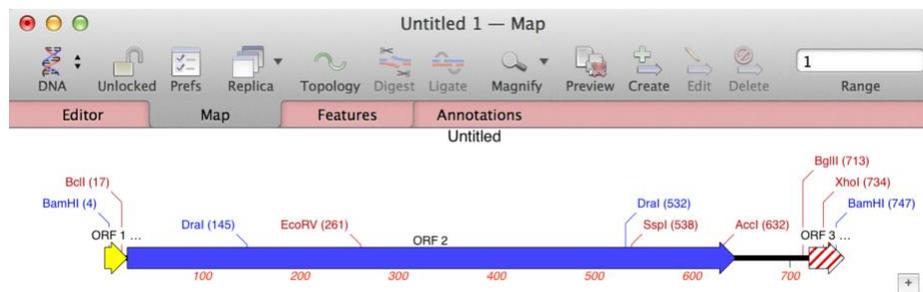
R Start	R Len	R Tm	R GC%	R Sequence	P Size	P Tm	Ta
1876	25	60.3	40.0	gggggatccACTTGTCTCGAG			

Copy  
Add Primer to Database

Click on the single PCR Product graphical object in the **Map** tab and choose **Edit | Copy**. This will copy the predicted PCR product, including the mismatch and tail from the 5' primer and the tail we assigned to the 3' primer



Finally, choose **File | New From Clipboard** and switch to the **Map** tab of the newly created *Untitled* sequence.



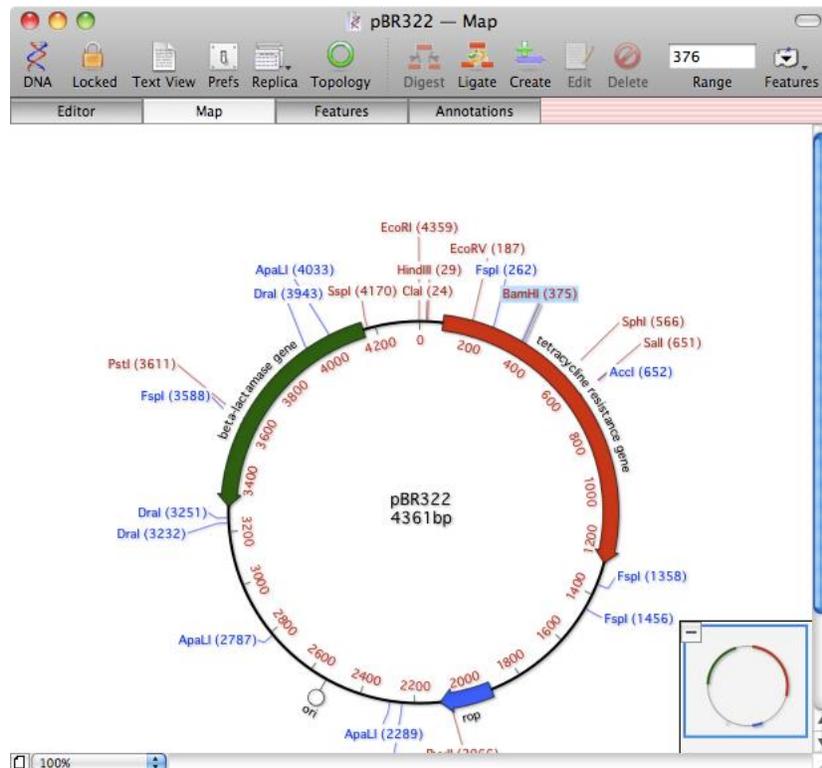
The new sequence retains all of the features from the source sequence i.e. ORF 2 and parts of ORF 1 and ORF 3. The sequence is now flanked by *Bam*HI sites from the tails we added to the primers. Finally, there is a *Bcl*I site near the left hand end – the mismatch we introduced into the 5' primer.

## Cloning the Amplified Fragment into a Vector

In this example, after completing the PCR reaction you would typically digest the amplified product with *Bam*HI and then clone it into a suitable vector. This is simple to emulate in MacVector. For this example we will clone the fragment into the *Bam*HI site of classic early cloning vector pBR322.

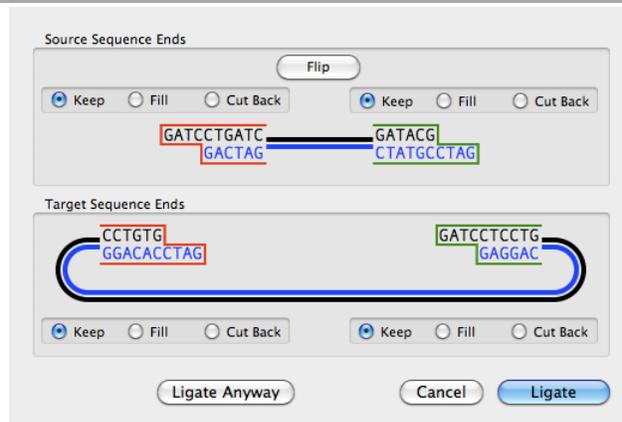
Click on the *Bam*HI site at the left side of the **Untitled 1 Map**. Hold down the <shift> key and click on the *Bam*HI site at the right side of the **Map**. Click on the **Digest** toolbar button.

Choose **File | Open** and open a suitable vector that contains a *Bam*HI cloning site. If you don't have a favorite vector, choose /Applications/MacVector/Sample Files/pBR322. Switch to the **Map** tab and click on the *Bam*HI site at position 375.



Click on the **Ligate** toolbar button.

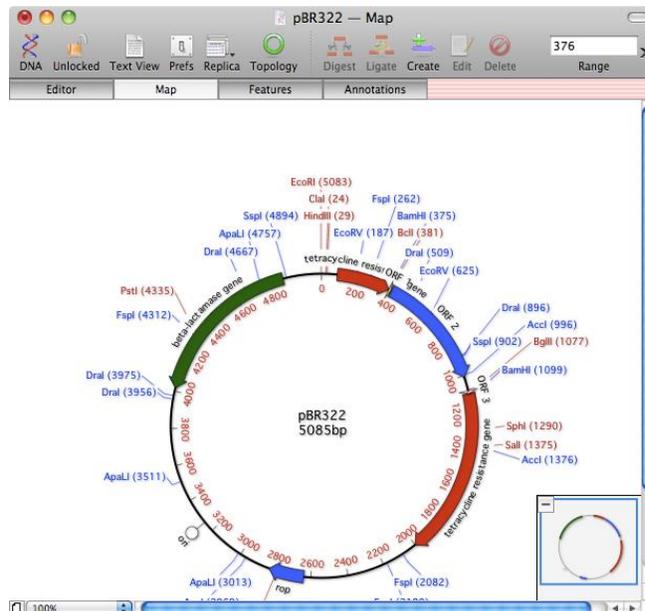
After unlocking the document, the ligation sheet will appear;



This gives you the opportunity to “flip” the incoming fragment so it is inserted in the opposite orientation, and also lets you fill or cut back sticky ends when emulating clonings that require manipulating overhangs. To learn more about “click cloning” with MacVector, check out [/MacVector/Documentation/Click Cloning Tutorial.pdf](#).

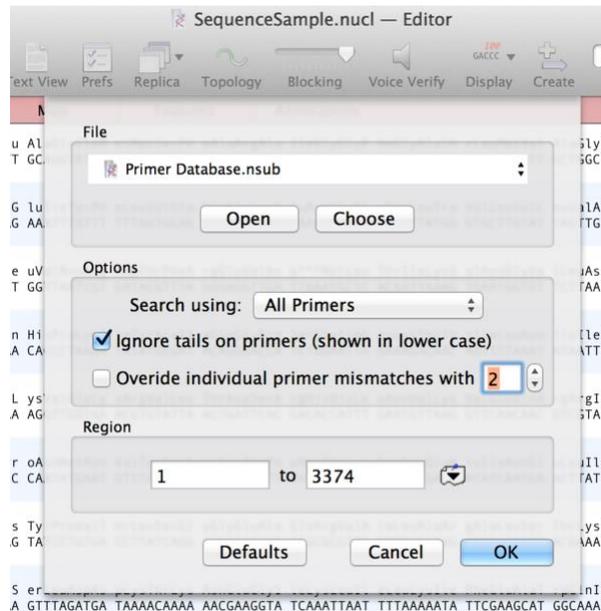
Click on the **Ligate** button.

The PCR fragment with *Bam*HI ends gets inserted into the target pBR322 document, creating a new molecule complete with all the features of the original target DNA sequence and including the new *Bc*II site we introduced with the mismatched primer.

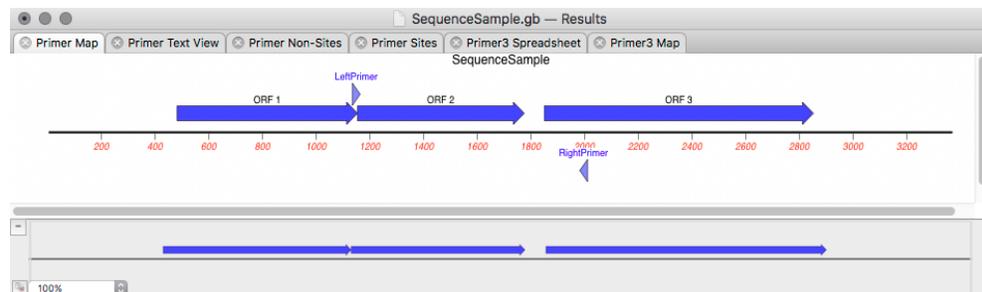


## Using the Primer Database Search Function

Bring the `SequenceSample` window to the front once more, then choose **Analyze | Primer Database Search**



This analysis is very similar to the **Nucleic Acid Subsequence** search function, except that it uses the `Primer Database.nsub` file by default and has a couple of extra parameters to handle how tails are handled and if you want to override the permitted mismatches parameter that is associated with each individual primer in the file.



## Additional Information

You can use the principles above to design any primer with or without tails or mismatches and document the final construct using MacVector's point and click interface. For more information on the "click cloning" interface, read the [Click Cloning Tutorial](#) in the Documentation folder. If you use T/A cloning to clone your PCR fragments, then you should read the [Gateway + TOPO Tutorial](#) document.