

TUTORIAL: PCR ANALYSIS AND PRIMER DESIGN

Introduction

This chapter introduces you to tools for designing and analyzing PCR primers and procedures. At the end of this tutorial session, you will be able to:


- Design and analyze PCR primers using Vector NTI's primer design tool
- Add restriction enzymes to PCR primers
- Set parameters for optimum primer/target interactions and successful PCR
- Save primers and PCR results in the database
- Add primers to the Oligo List
- View and analyze PCR results
- Order Custom Primers from Invitrogen

Note: Press the F1 key to open Online Help for all of the dialog boxes in this tutorial.

1. Launch Vector NTI and Open a Molecule Display Window

- Launch Vector NTI using techniques described in previous tutorials (Select **Start > Programs > Invitrogen > Vector NTI Suite 10 > Vector NTI**).
- Using techniques learned in earlier tutorials, open a Molecule Display window containing ColE1.

2. Select the Region of ColE1

- Click the **Graphics Pane** button () on the Window Toolbar to activate the Graphics Pane.
- Select **Edit > Set Selection**. In the Set Selection dialog box, select the 5200–6400 region of ColE1, the target fragment for PCR.

3. Prepare for PCR Analysis

To perform PCR analysis on the selected fragment, select **Analyses > Primer Design > Find PCR Primers** on the menu bar. The Find PCR Primers dialog box appears (Figure 8.1):

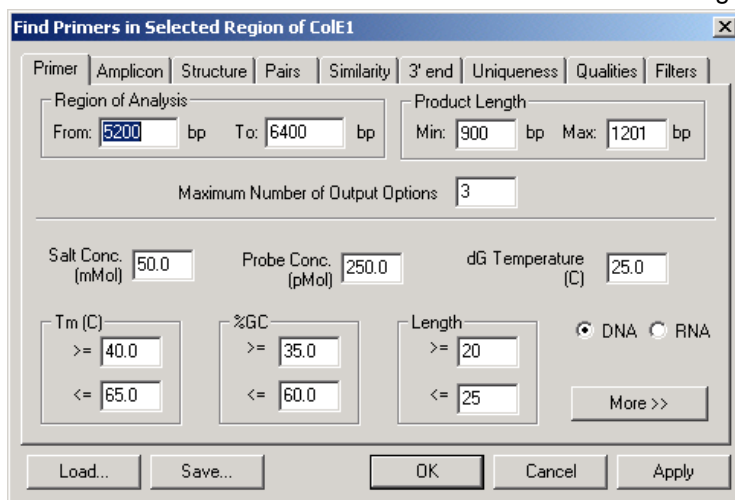


Figure 8.1 Find PCR Primers dialog box

With this dialog box, you can direct Vector NTI to search for convenient sense and antisense primers according to your specifications and to check primers' uniqueness.

Click the **More>>** button on the lower right to enable a complete view of the Find Primers dialog box (Figure 8.2):

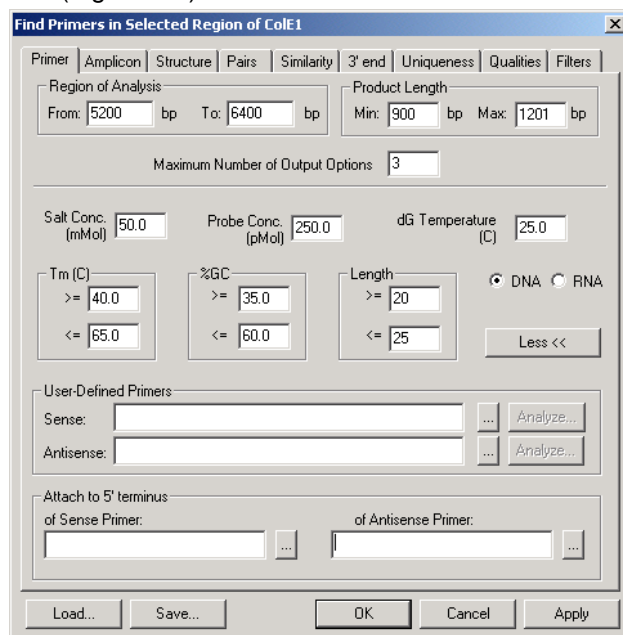


Figure 8.2 Find Primers dialog box

The **Primer** tab allows you to designate basic parameters for the PCR reaction and the primers that are generated. After clicking the **More>>** button, you can also define primers yourself and attach short sequences at the 5' and/or 3' ends of the product. (If you want the PCR target length to be the full extent of the selected target region, make sure that the Region of Analysis and Product Length sections of the dialog box reflect your selections.)

Note: The calculation for T_m is dependent on primer and salt concentrations; varying these concentrations can greatly affect the T_m for any given primer. Make sure to adjust these parameters according to your reaction conditions when performing your own PCR analysis to ensure that you obtain accurate T_m values.

For this tutorial, review the options on the **Primer** tab, but leave all the options unchanged.

Select the **Amplicon** tab (Figure 8.3). The options on this tab allow you to customize parameters relating to the resulting PCR product. %GC content for the product or a portion of the product next to the primer annealing site and allowed bases adjacent to the primer annealing site can be specified.

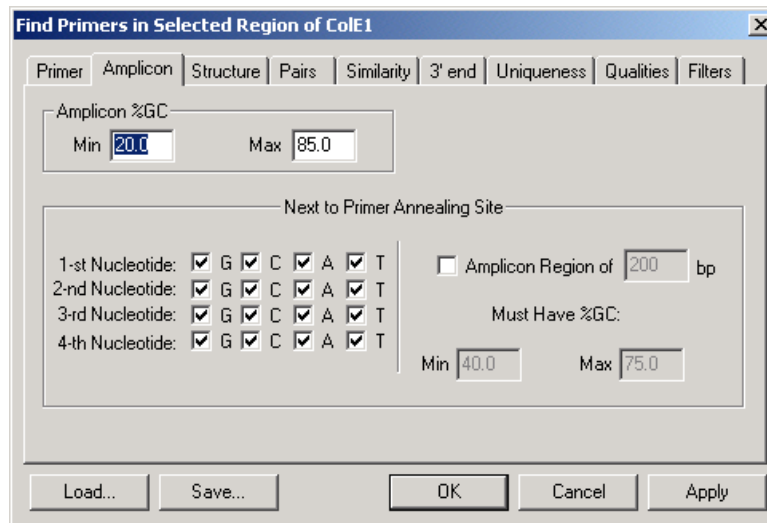


Figure 8.3 The Amplicon tab allows you to customize parameters relating to the resulting PCR product

Review the options, but leave all the options unchanged.

Select the **Structure** tab (Figure 8.4). The options on this tab set acceptable limits for nucleotide repeats, palindromes and hairpin loops for the primers. You can also check your primers/product for a selected group of restriction sites from this tab.

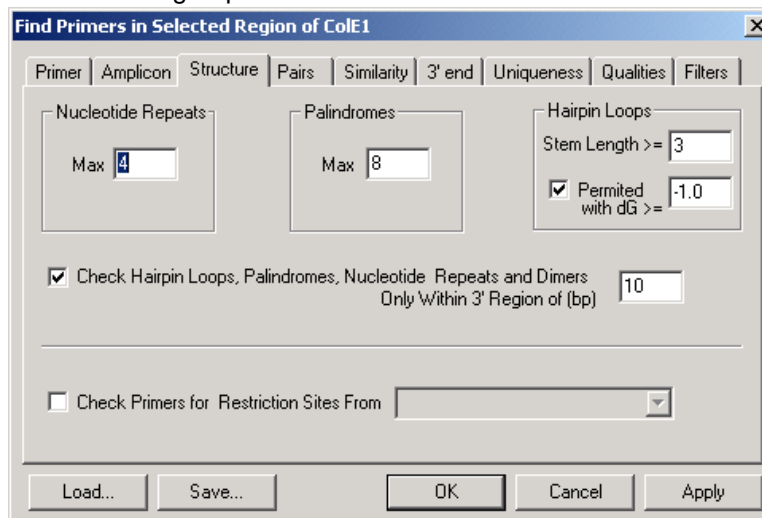


Figure 8.4 The Structure tab sets limits for nucleotide repeats, palindromes and hairpin loops in primers

Inspect all options, leaving them unchanged.

Select the **Pairs** tab (Figure 8.5). Options on this tab specify how closely parameters such as Tm and %GC, etc. must match between two primers in a generated primer set.

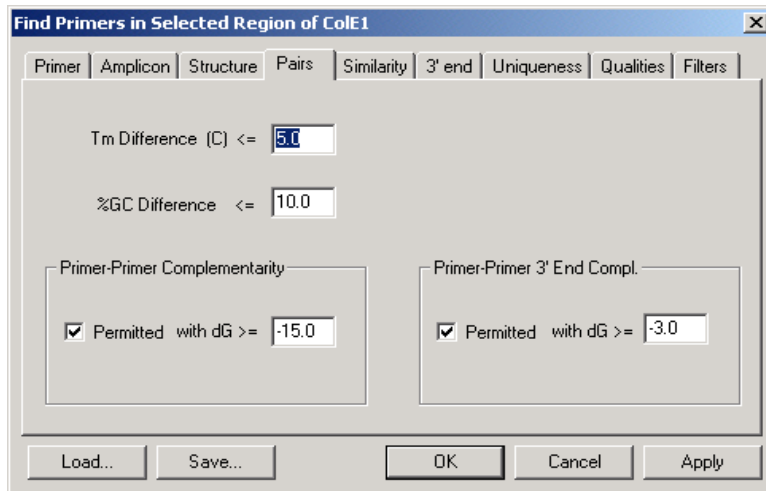


Figure 8.5 The Pairs tab specifies how closely parameters must match between two primers in a primer set

Inspect all options, leaving them unchanged.

Select the **Similarity** tab (Figure 8.6). The options on this tab determine the similarity relationship between the primers and the target sequence.

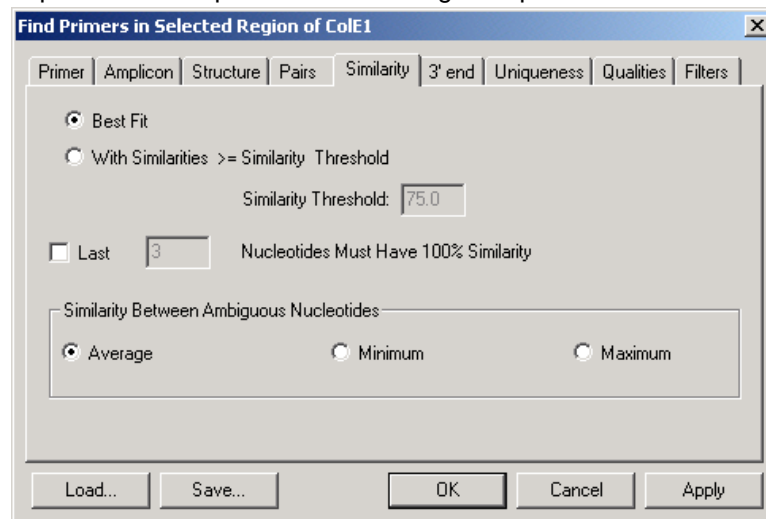


Figure 8.6 The Similarity tab determines the similarity relationship between the primers and the target sequence

Inspect all options, leaving them unchanged.

Select the **3' end** tab (Figure 8.7). The options on this tab allow you to set specifications for the 3' end of the primers generated by Vector NTI. Parameters such as dG and specific nucleotide content for the 3' end of both sense and antisense primers can be set here.

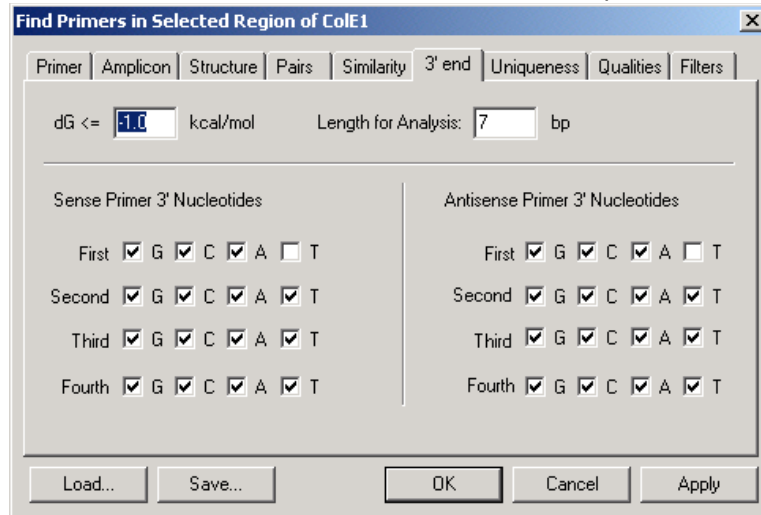


Figure 8.7 The 3' end tab allows you to set specifications for the 3' end of the primers generated by VNTI

Inspect all options, leaving them unchanged.

Select the **Uniqueness** tab (Figure 8.8). The options on this tab determine the uniqueness of the primers generated with respect to the PCR product. These parameters can be used to help ensure that generated primers bind to the desired template area with greater specificity than to the rest of the PCR product.

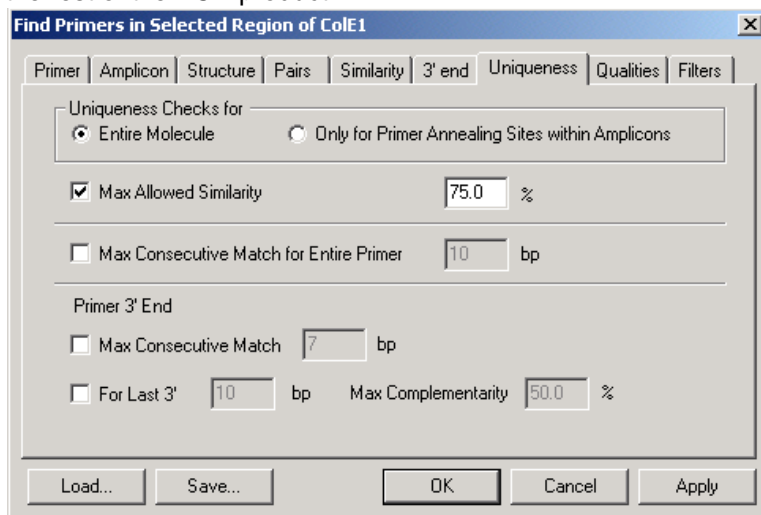


Figure 8.8 The Uniqueness tab determines the uniqueness of the primers generated with respect to the PCR product

Inspect all options, leaving them unchanged.

Select the **Qualities** tab (Figure 8.9). These parameters govern primer quality by determining how much weight should be assigned to parameters specified on the other tabs of the Find

Primers dialog box. These values affect scoring functions that evaluate the quality rating of the primer sets generated.

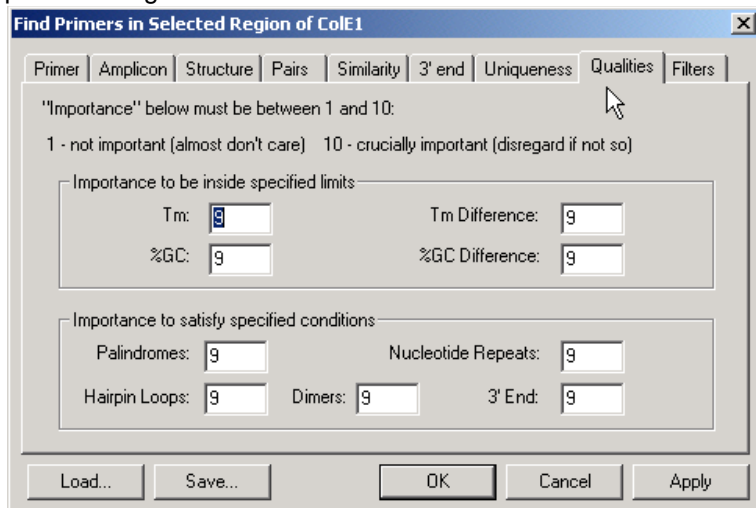


Figure 8.9 The *Qualities* tab governs primer quality by determining how much weight should be assigned to parameters specified on the tabs of the *Find Primers* dialog box

Inspect all options, leaving them unchanged.

Select the **Filters** tab (Figure 8.10). On this tab, you can select features that should either be excluded or included as regions to be considered for primer design. For example, if your sequence contains Repeat features, you may want to exclude them to obtain primers with greater specificity. Features can be added or removed by pressing the + and – buttons.

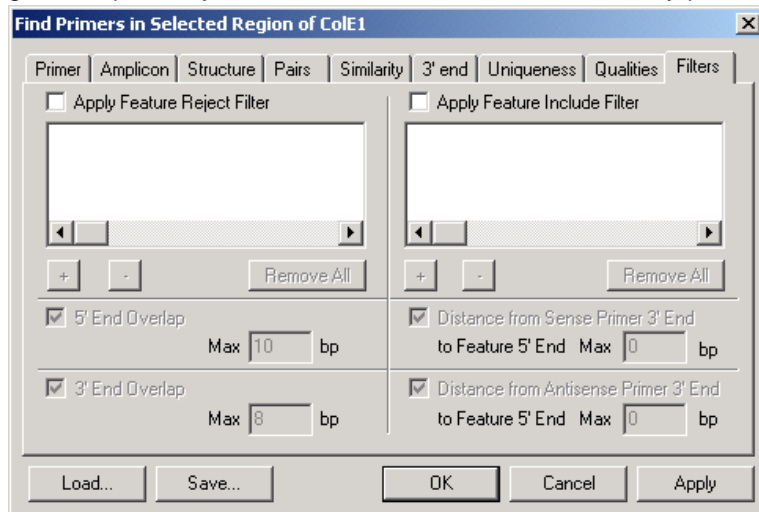


Figure 8.10 The *Filters* tab selects features that should either be excluded or included as regions to be considered for primer design

Leave all the options unchanged as you inspect them.

Notice the **Load** and **Save** buttons on the lower left of each tab in the *Find Primers* dialog box. These buttons allow you to save your PCR settings to a file and load the settings file in for subsequent analyses. This precludes having to reset the various parameters manually for frequently used PCR condition settings. You need only save/load the settings in one of the tabs and the parameters for all the tabs in the *Find Primers* dialog box will be saved/loaded.


4. Perform PCR Analysis

In the Find Primers dialog box, press the **OK** button in the lower center, initiating the PCR analysis. When it is finished, a new folder is created, added and opened in the Text Pane of the ColE1 Display window.

5. Inspect the PCR Analysis Folder

Move the split bars as necessary to optimize viewing in the Text Pane. In the open PCR Analysis folder, inspect the subfolders.

- Each subfolder describes one possible set of primers for amplifying a fragment of the length within the maximum and minimum lengths specified in the Find Primers dialog box.
- The subfolders are listed in order of decreasing primer set quality rating which is calculated based on the importance factors assigned in the **Qualities** tab in the Find Primers dialog box. (Generally, maximum rating = 171.)
- Each subfolder contains useful information for the PCR reaction such as product melting temperature, optimum annealing temperature, sense and antisense primers with their melting temperatures, free energies, etc.

The sense and antisense primers are indicated with the symbol “” followed by their nucleotide sequences. (In Vector NTI, this same symbol is used to mark oligonucleotides created by many of the program's functions such as PCR analysis, molecule design, and hybridization. You can perform oligo analysis on any nucleotide sequence marked by this symbol in the Text Pane of a Molecule Display window.)

6. Perform Oligo Analysis on a PCR Sense Primer

To perform oligo analysis on the sense primer given in the first subfolder of the PCR Analysis folder, scroll through the Text Pane and verify that the folder labeled “**#1: Product of length 901**” is open. (Open it by double-clicking it, if necessary.)

Inside this subfolder, position the cursor on top of the sense primer, represented by the line **ATTACTCTTTTGAATGGTACTCCTG**. Open the corresponding shortcut menu by right clicking on the primer sequence. Choose the **Analyze** command, opening the Oligo Analysis dialog box (Figure 8.11):

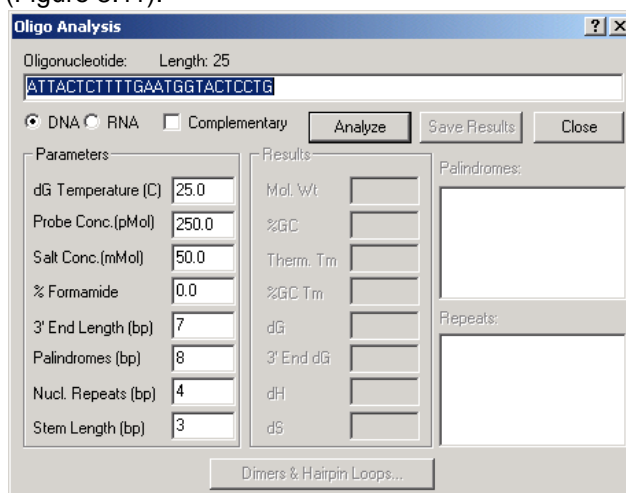


Figure 8.11 Oligo Analysis dialog box

In this dialog box you can set numerous parameters for oligonucleotide analysis. Leave the default parameters unchanged and press the **Analyze** button.

Note: The calculation for T_m is dependent on primer and salt concentrations; varying these concentrations can greatly affect the T_m for any given primer. Make sure to adjust these parameters according to your reaction conditions when performing your own oligo analysis to ensure that you obtain accurate T_m values.


The analysis results appear in the dialog box. Note the biochemical and structural parameters in the Results column. Press the **Dimers & Hairpin Loops** button to inspect the dimers and hairpin loops that may form if this primer is used.

Press the F1 key to view Vector NTI's on-line help information for this dialog box. Press the **Close** button.

7. Order Custom Primers

In Vector NTI Advance 10, you can order custom primers that you have generated and analyzed in the software. Custom primers are synthetic oligonucleotides made with your specified sequence for use in a variety of applications from PCR and sequencing to probes for gene detection. Custom modifications, such as standard deoxynucleotides, modified bases, 5' modified nucleotides (including fluorescent dyes, enzyme conjugates), and S-oligos for antisense studies can be designated. Specifications also include scale of synthesis, level of purity and 5' or 3' modifications.

To initiate the ordering process, inside the PCR Analysis folder, scroll through the Text Pane and verify that the folder labeled "**#1: Product of length 901**" inside the subfolder is selected by positioning the cursor on top of the sense primer, represented by the line **ATTACTCTTTTGAATGGTACTCCTG**. Open the corresponding shortcut menu by right clicking on the primer sequence. Choose the **Add to Oligo List** command, opening the New Oligo dialog box, with the **General** tab activated. Enter **Sense primer - CoIE1** in the name box and click **OK**. Vector NTI verifies the oligo has been added.

Open the Oligo List dialog box by selecting **List > Oligo List**. Select the oligo named **Sense primer - CoIE1** and click the **Order** button ().

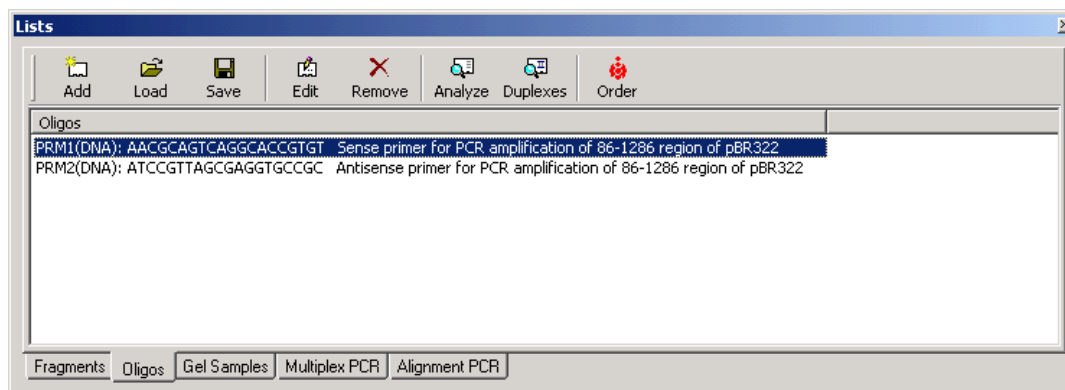


Figure 8.12 Oligo List dialog box

The Invitrogen webpage opens automatically, and your primer sequence is entered in the custom primer order form. If you in reality wanted to order the primers, at this point you would proceed to checkout. For this tutorial, however, simply inspect the Invitrogen Custom Order webpage, then exit and return to the Vector NTI workspace.

8. Save a PCR Primer to the Database

Now let's save the same primer to the database for future use. Right-click on the **ATTACTCTTTTGAATGGTACTCCTG** line in the Text Pane, to open the corresponding shortcut menu and select **Save To Database**. This opens the New Oligo dialog box, with the **General** tab activated (Figure 8.13).

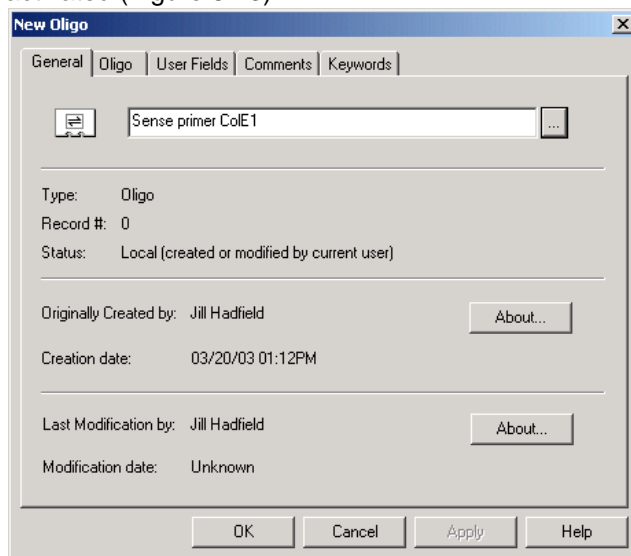


Figure 8.13 New Oligo dialog box with the General tab activated

Enter **Sense primer - ColE1** in the name box and click on the Oligo tab. The oligo sequence and description appear automatically in their respective text boxes.

Click on the **Keywords** tab. Click in the New Keyword entry box and enter **PCR-ColE1**. Press the **< Add** button to add your new keyword to the keyword list. Then select the existing **SENSE_PRIMER** keyword and add it to the keywords list, too.

Press the **OK** button. The primer is saved to the database under the name **Sense primer - ColE1**.

9. Add Restriction Enzyme Sites to the PCR Primers

You will load the sense primer you just saved to the database and then attach sequences to the 5' and 3' ends of the product to allow convenient cloning. Vector NTI will then find an optimum antisense primer.

Activate the Graphics Pane again and make sure the 5200–6400 region is still selected. (Check the status bar.) Open the Find Primers dialog box again by selecting **Analyses > Primer Design > Find PCR Primers** on the menu bar.

Locate the User-Defined Primers box in the Find Primers dialog box by pressing the **More>>** button on the **Primer** tab. (Any previously selected restriction sites will appear in the User-defined Primers Sense and Antisense text boxes by default. To continue with this tutorial, select each and press the **DELETE** button so each text box is blank.) Press the **Browse** button (**...**) to the right of the Sense Primer box. In the dialog box that appears, showing oligonucleotides saved in the database, select the "Sense primer - ColE1" oligo you just saved and click **OK**. The "Sense primer - ColE1" oligo is loaded into the Sense Primer text box (Figure 8.14). Note that you are not specifying the anti-sense primer, but leaving that up to Vector NTI.

To attach a *Bam*HI cloning site to the 5' end of your sense primer, locate the Attach to 5' Terminus of Sense Primer box and press its **Browse** (...) button. In the Choose Database Enzyme dialog box that opens, select the MAIN Enzymes subset. Scroll down to find and select *Bam*HI. Click **OK**. The *Bam*HI recognition site is entered into the Attach to 5' Terminus of Sense Primer box.

To attach a *Hind*III cloning site to the 5' end of the antisense primer, locate the Attach to 5' Terminus of Antisense Primer box and press its **Browse** button. In the dialog box, select the MAIN Enzyme subset and *Hind*III and click **OK**. The *Hind*III recognition site is entered into the Attach to 5' Terminus of Antisense Primer box.

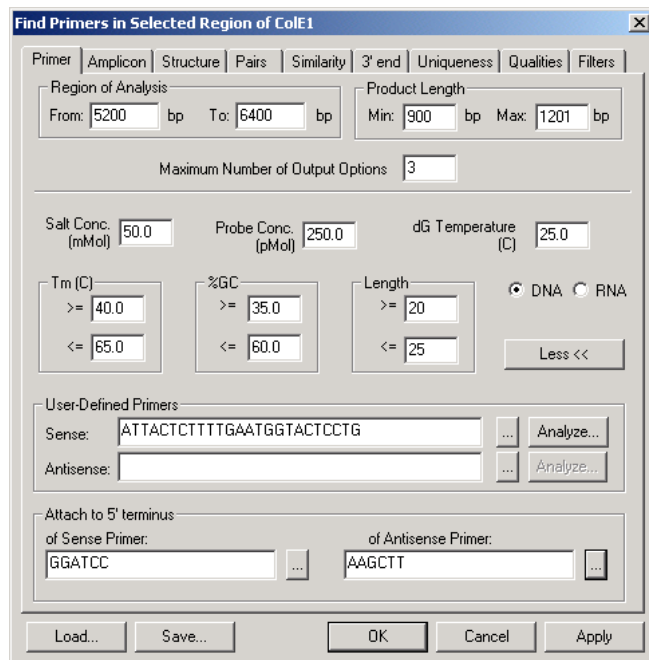


Figure 8.14 The “Sense primer - ColE1” oligo is loaded into the Sense Primer text box; restriction enzymes are designated for the sense primer terminus.

Locate and check the Check Primers for Restriction Sites From check box on the **Structure** tab, directing Vector NTI to search for certain restriction sites in the PCR product-plus-primer fragment you are creating. The restriction sites searched for are listed in the Restriction Endonuclease subset shown in the Check Primers for Restriction Sites From drop-down menu. Choose the **Palindromes/Non-Ambiguous** subset from the list (Figure 8.15).

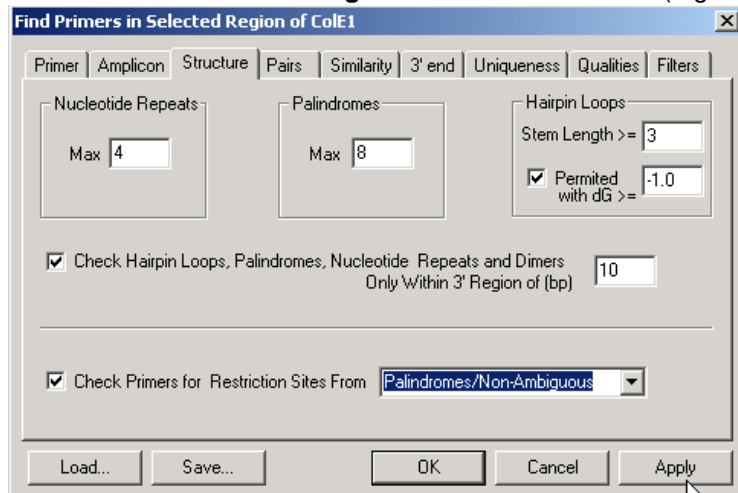


Figure 8.15 Searching for certain restriction sites in the PCR product-plus-primer fragment on the Structure tab

Now press **OK** at the lower center of the Find Primers dialog box. Press **OK** when Vector NTI shows the warning dialog box. Click **OK** to override the previous PCR analysis results.

A new PCR analysis is performed using the additional information you have entered.

10. Inspect the New PCR Analysis Results

Inspect the subfolders of the new PCR Analysis results in the Text Pane.

Note that this time, all of the new options use the same sense primer, "Sense primer - ColE1", and that the REN sequences have been attached at the 5' ends of both primers. (They are separated from the primers by a space.) Only the antisense primers vary because they were not defined in the last dialog box.


The RENs appearing beneath each primer indicate those from the selected Palindromes/Non-Ambiguous subset whose recognition sequences are found within the generated fragment. The number of times that a REN recognition sequence is found within the PCR product is indicated. If no number is given after a REN's name, then that REN is found only in the primer or the attached sequence and not within the product.

11. Save the PCR Product to the Database and Open a Display Window


Place the mouse cursor on the folder labeled "#1: Product of length 919," and open the shortcut menu with a right click. Choose the **Save to Database and Create Window** command. In the dialog box that opens, in the Name field, enter **PCR Product 1**, and click **OK**. Choose the Main subset and click **OK**. The molecule is saved to the database, and a new Molecule Display Window is created containing the molecule.

12. Arrange the View of the Product



Arrange the new Molecule Display window to show your PCR product conveniently. Activate the

Text Pane, and click the **Link Panes** button () on the Window Toolbar. Most of the symbols in the Graphics Pane disappear because most of the folders in the Text Pane are closed.

Place the cursor on the Feature map folder and open the shortcut menu. Choose the **Expand**

Branch button () , opening the Feature map folder and all its subfolders. Now the signals are displayed in the Graphics Pane.

Locate the Restriction Map folder and open it with a double-click. Open the BamHI and HindIII folders by double-clicking them. These sites now appear in the Graphics Pane.

Activate the Graphics Pane and press the **Standard Arrangement** button. Arrange the graphics to fit the window so that the restriction sites and primers can be seen. (Use the **SHIFT + Zoom In** () and **Zoom Out** () buttons.) Use the **CTRL + Zoom** buttons to change only the horizon-

tal dimensions of the graphical map. (You can enhance your picture further using picture editing techniques described in Chapter 6.)

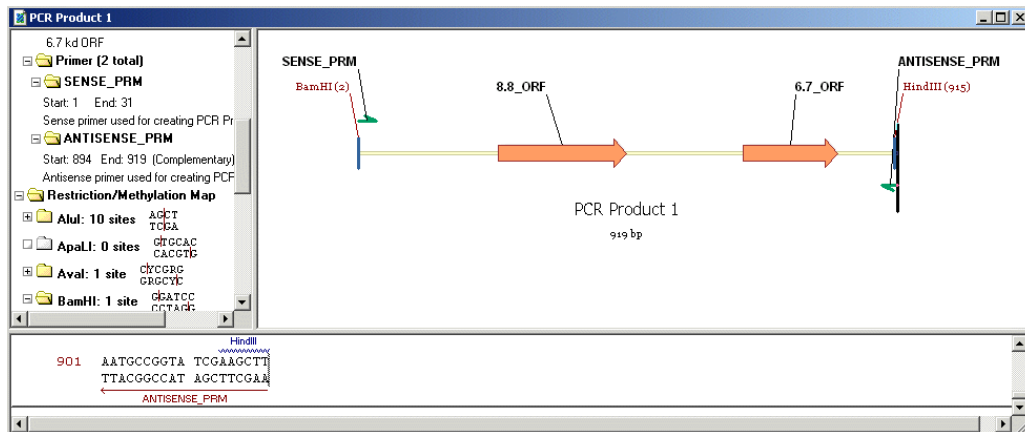


Figure 8.16 Standard Arrangement of restriction site and functional signal labels

13. Close Display Windows and Exit Vector NTI

This concludes the PCR analysis tutorial. Close all Molecule Display windows using the techniques described in previous tutorials: **File > Close**, then **File > Exit**.